Simultaneous Production of Ethanol and Hydrogen from Agricultural Waste and Forest Residues Available in North-East India

Thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

Soumya Sasmal

Department of Chemical Engineering

Indian Institute of Technology Guwahati

Guwahati- 781039, India

To my Parents for their love and

continuous

Encouragement

Department of Chemical Engineering
Indian Institute of Technology Guwahati
Guwahati-781039, Assam, India

..............................CERTIFICATE....................

This is to certify that the thesis entitled "*Simultaneous Production of Ethanol and Hydrogen from Agricultural Waste and Forest Residues Available in North-East India*" being submitted by **Soumya Sasmal** for the award of PhD degree has been carried out under our guidance and supervision. The work documented in this thesis has not been submitted to any other University or Institute for the award of any degree or diploma.

Dr. Kaustubha Mohanty

Associate Professor
Department of Chemical Engineering
Indian Institute of Technology Guwahati
Gwahati-781039, Assam, India

Dr. Vaibhav V Goud

Assistant Professor
Department of Chemical Engineering
Indian Institute of Technology Guwahati
Gwahati-781039, Assam, India

Acknowledgement

I have no qualms to admit that it was a great pleasure to work under the guidance of Dr. Kaustubha Mohanty, Associate Professor and Dr. Vaibhav V Goud, Assistant Professor of Department of Chemical Engineering, Indian Institute of Technology Guwahati. I am very pleased to acknowledge the continuous help and cooperation from Mr. Lukumani Borah , Mr. Prasun Bhattacharaya and Mr. Jayanta Kumar Mout.

It will never be enough to express in words the type and extent of help and cooperation that I had from Mr. S. Murugavelh, Mr. Krushna Prasad Shadangi and Mr. Venu Babu Borugadda.

Thanks are due to all the officials Of IIT Guwahati for providing me necessary helps needed for my Doctoral study.

I am also obliged to express my appreciation towards my beloved mother, father and family members for their enduring patience, and moral supports.

SOUMYA SASMAL

Abstract

Hydrogen and ethanol derived from lignocellulosic biomass are promising biofuels and of great potential to become alternatives to fossil fuels.The present researchfocuses on the carbohydrate platform for simultaneous production of ethanol and hydrogen in fluidized bed and stirred tank bioreactor. Areca nut (*Arecacatecheu*) husk, Moj (*Albizialucida*), and Bon bogori (*Ziziphusrugosus*) available in North-East India are chosen as the lignocellulosic biomass feed stocks for biofuel production. The cellulose concentration in Areca nut husk, Moj and Bonbogori was determined as 44.46%, 53.46% and 51.56%, respectively. The lignin concentration lies within the range 11-23%. Maximumcrystalinity was observed in Areca nut husk fiber (63.84%) followed by Moj (46.43%) and Bon bogori (42.46%). The calorific values of all the biomasses were found within the range of 17 $MJKg^{-1}$to 22MJ Kg^{-1}. To facilitatesaccharification process two types of pretreatment process were conducted viz. dilute acid pretreatment and ultrasound assisted lime pretreatment for allthe biomass samples. The Taguchi method of optimization was applied to optimize both the pretreatment processes.The ultrasound assisted lime pretreatment process was found better than dilute acid pretreatment process and it removed 68%, 65% and 64% of the lignin present in the naive lignocellulosic fibers of Bonbogori, Arecanut husk and Moj, respectively.The saccharification process was conducted at 50°C, 150 RPM for 96 hoursand the saccharification efficiency (ultrasound assisted lime pretreated) was highest in Bonbogori (82.5%) followed by Moj (80.1%) and Arecanut husk (79.5%).Among all the three biomass used in this study Bon bogori have showed the maximum saccharification efficiency therefore,Bonbogori was used for the fermentative production of both ethanol and hydrogen.Immobilizedwastewater sludge was used for simultaneous bio-ethanol and hydrogen production from the enzymatic hydrolysate of Bonbogori.The optimal factor combination for both ethanol and hydrogen production was achieved at 40°C temperature and 5.5 pH.Fluidized bed bioreactor performed better than the stirred tank reactor for production of both ethanol (0.21 ±.04 mol) and hydrogen (30.8± 0.5 mmol).

Index

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

List of Tables

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Appendix-A

List of Figures

Fig. No **Title** **Page No**

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Chapter VI

Appendix -A

Nomenclatures

ABBREVIATIONS

AA	Anaerobic Agar
AFEX	Ammonia Fiber Explosion
AGU	Anhydrous Glucose Unit
ANOVA	Analysis of Variance
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
BGL	Beta Glucosidase
BOD	Biological Oxygen Demand
BSS	Budesonide Submicron Suspension
CBU	Cello Biase Unit
CMC	Carboxy Methyl Cellulose
COD	Chemical Oxygen Demand
DOF	Degree of freedom
DTG	Differential Thermo Gravimetric
EC	Enzyme Commission
FAS	Ferrous Ammonium Sulphate
FBB	Fluidized Bed Bioreactor
FGB	First Generation Biofuel
FPU	Filter Paper Unit
FrGB	Fourth Generation Biofuel
FTIR	Fourier Transformation Infrared
GC	gas chromatography
GHG	Green House Gases
IEA	International Energy Agency
LHV	Lower Heating Value
MEA	Malt Extract Agar
MFC	microbial fuel cells
MNRE	Ministry of New and Renewable Energy
NREL	National Renewable Energy Laboratory
OECD	Organisation for Economic Co-operation and Development

OLR	Organic Loading Rate
OMC	Oil Marketing Companies
PCA	Plate Count Agar
PDA	Potato Dextran Agar
RON	Research Octane Number
S/N	Sound / Noise
SGB	Second Generation Biofuel
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
STR	Stirred Tank Reactor
SV	Sludge Volume
SVI	Sludge Volume Index
TCD	Thermal Conductivity Detector
TGA	Thermo Gravimetric Analysis
TGB	Third Generation Biofuel
TKN	Total Kjeldahl Nitrogen
XDH	xylitol dehydrogenase
XK	xylulose kinase
XR	xylose reductase
XRD	X Ray Diffraction

Summary

Fossil fuels currently supply most of the world's energy requirements. However, its future supply is limited, as oil is not an infinite resource. Scientists and policy makers must ensure to assess alternative sources of energy and determine what is scientifically possible, environmentally acceptable and technologically promising.Biofuels are an important form of renewable energy and making significant inroads toward a sustainable transport fuel. The ideal biofuel needs to be produced cheaply from nonfood feed stocks, to be readily available throughout the year, to serve as a drop-in replacement into existing infrastructure, and to be as energy dense as petrol or diesel. Lignocellulosic materials are promising alternative feed stocks for biofuel production such as bio-ethanol, bio-hydrogen etc. Ethanol has already attracted significant attention as an alternative fuel in the last two decades. In 1990, a Clean Air act amendment recommends ethanol as the additive oxygenates in automobile fuel. The bio-ethanol used in transportation sector is mainly prepared from maize, corn, sugarcane or sugar beet. Ethanol production from corn and sugarcane is a well-established process, while production from lignocellulosic materials has not yet been established. It is expected that, in near future wide variety of raw materials will be used for producing renewable fuels, including an array of recycled materials. Lignocellulosic materials are alternative, competent and cheaper sources for biofuel production because of their lower prices and no food vs. fuel issues. The prime aim in biofuel research is to develop an economical and sustainable translational technology for its production from the raw materials stated above. The total potential of producing energy irrespective of types of lignocellulosic biomass is approximately 20000 PJa^{-1}and among these the contribution of Asia is approximately 40% of the total potential of the world. Apart from all these facts foundation of the biofuel market needs the high level of public support that is usually justified by the expected energy, economic and environment benefits of biofuels. Despite the increasing use of biofuelssuch as biodiesel and sugar- or starch-based ethanol, evidence suggests that transportation fuels based on lignocellulosic biomass represent the most scalable alternative fuel source.

The growing demands and subsidies for ethanol and other biofuels have encouraged the entrepreneurs to set up bio-refineries. In that, lignocellulosic materials can be converted to bio-ethanol through the sugar-platform processes. Lignocellulosic biofuel production process involves collection of biomass, deconstruction of cell wall polymers into component sugars (pre-treatment and saccharification), and conversion of sugars to biofuels (i.e. fermentation). The preliminary step towards biofuel production is pretreatment. Pretreatment converts the recalcitrant lignocellulosic structure to reactive cellulosic intermediates before enzymatic hydrolysis. For a proficient enzymatic hydrolysis of cellulose into fermentable sugars, it is essential to modify the physical and chemical properties of the plant cell wall, which can be done through a pretreatment step. The complex processes for the conversion of lignocellulosic materials to ethanol make it very expensive compared to the sugar and starch platform for bio-ethanol production. Although, till date there is no commercial process available for bio-ethanol production from lignocellulosic materials, however the cellulosic platform has a great potential because of the availability of lignocellulosic materials. Apart from bio-ethanol another promising biofuel is bio-hydrogen. Hydrogen is presently produced from the reforming of fossil materials (90% of worldwide production, representing 45 billion tonnes) and generates high level of pollutant i.e., 10 tonnes CO_2 per tones H_2. The idea to produce hydrogen from microorganisms, such as bacteria and algae, via fermentation processes has re-gained tremendous interest recently. The production of hydrogen by different microorganisms is intimately linked to their respective energy metabolism.

In addition to hydrogen production, anaerobic fermentation also produces a significant amount of alcohols (such as ethanol). However, the hydrogen production is usually optimized by suppressing the ethanol production route. Therefore, it is a challenge to produce hydrogenand ethanol simultaneously, as the formation of alcohols would consume free electrons from NADH that is usually unfavorable for hydrogen production. Wastewater sludge is a proven source of microorganisms for the production of both hydrogen and ethanol. The challenge lies on improving its capability of hydrogen production by reduction of its methanogenic activity.

Thus based on the above brief discussion, it seems that there is enough scope to carry out research on simultaneous production of ethanol and hydrogen from lignocellulosic biomass materials. The PhD thesis with the title “**Simultaneous Production of Ethanol and Hydrogen from Agricultural Waste and Forest Residues Available in North-East India**”, was focused on the carbohydrate platform for production of aforesaid biofuels. Three unexploited biomass viz. Areca nut (*Areca catecheu*) husk, the most abandon agricultural residue in North-East India, Moj (*Albizialucida*), and Bonbogori (*Ziziphusrugosus*) which are well known among tribal people of North-East India as fuel woods, are chosen as the lignocellulosic biomass feed stocks for biofuel production.

Throughout the research project, the following objectives were established and accomplished:

- Physico-chemical characterization of lignocellulosic biomass
- To obtain lignin less pure fractions of cellulose and hemicellulose from efficient pretreatment of lignocellulosic biomass for optimum yield of hydrogen and ethanol
- Physico-chemical characterization of wastewater sludge
- Production and optimizationof both hydrogen and ethanol in a fluidized-bed reactor and stirred tank bioreactor

Thesis outline

The thesis is presented in the following seven chapters.

Chapter-I : Introduction
Chapter-II : Physico-chemical characterization of lignocellulosic biomass
Chapter-III : Pretreatment of lignocellulosic biomass
Chapter-IV : Saccharification of pretreated biomass
Chapter-V :Characterization of wastewater sludge
Chapter-VI : Simultaneous ethanol and hydrogen production through fermentation
Chapter-VII :Conclusions and future scope

A brief description of the content of each of the chapters is furnished below:

Chapter I: Introduction

In the beginning of this chapter a brief basic discussion on different sources of energy viz. definition, classification, advantages and disadvantages of different sources of energy and the need for bio-energy are discussed. The present international and national status of biofuels is also described vividly. Further, discussion on variousresearch works on biofuels and different opinions of several researchers, as well as the pathways of biofuel production are also described. Finally major research areas are compared with the current trend and work plan was organized from the review of literatures.

Chapter II: Physico-chemical Characteristics of Lignocellulosic Biomass

Biomass is a promising renewable energy resource, it can be defined as complex polymer of cellulose, hemicellulose and lignin; hence, it is also called lignocellulosic material. The various permutation and combination of those three components stated before provides unique characteristics to the biomass. These characteristics are different for each biomass. Beside biofuel production, the lignocellulosic biomasses may act as a raw material for production of value added chemicals. Therefore, characterization of varying composition of biomass and its analysis is essential for suitable application. The main emphasis of this chapter is to characterize the three chosen biomass samples i.e. Areca nut husk (*Areca catechu*), Moj (*Albizialucida*) and Bonbogori (*Ziziphusrugosa*) which are available in the region of North-East India, in details to find out the potentiality of these biomass as a feedstock for production of biofuel.Lignocellulose matrix provides basic structure of the plants; it is composed of three major components cellulose hemicellulose and lignin. The cellulose concentration in Arecanut husk was determined as 44.46%, Moj 53.46% and Bonbogori 51.56% respectively. The lignin concentration of three biomass samples was found to be between 11-23%. Various physical characterization (XRD, Calorific value, TGA) and chemical characterization processes (CHNSO, FTIR etc.) were applied in this study and their outcomes are also explained vividly. Maximum crystalinity was found in Arecanut husk fiber (63.84%) followed by Moj (46.43%) and Bonbogori

(42.46%). The calorific values of all the biomasses were in the range of 17 MJ $kg^{-1}$to 22 MJ kg^{-1}.

Chapter III: Pretreatment of LignocellulosicBiomass

The first step in biochemical conversion of lignocellulosic biomass is pretreatment. The purpose of this step is to enhance the rate of subsequent enzymatic hydrolysis and fermentation. Pretreatment is an unavoidable, but most energy-intensive step for biofuel production. The dilute sulfuric acid pretreatment process and ultrasound assisted lime pretreatment process was conducted and compared. Apart from mere comparative results, the chapter also includes the need of pretreatment and different pretreatment studies conducted by various researchers with different biomass materials. Based on the Taguchi method, an orthogonal array experiment (L9) was designed to determine the optimum conditions for both dilute acid and ultrasound assisted lime pretreatment method. The experiments were designed with three factors in three different levels viz. sulfuric acid concentration (vol%), solid loading (g mL^{-1}) and time of operation (min) for optimization of dilute acid pretreatment process and lime loading (weight of lime (g)/ weight of dry biomass (g)), biomass loading (weight of water (g) / weight of dry biomass (g)), duration of ultrasound irradiation (min) were chosen for ultrasound assisted lime pretreatment of the chosen biomasses respectively. The responses evaluated were the degree of cellulose crystalinity and delignification ratio for dilute acid and ultrasound assisted lime pretreatment process respectively.The recommended conditions for the dilute acid catalyzed pretreatment of all the biomass materials were acid concentration (2.5%), solid loading (1%) and time of operation (30 min) respectively and responses evaluated was degree of cellulosic crystalinity. The lime pretreatment process was conducted at 40°C for one week, with lime loading 0.75 g lime/ g dry biomass and water loading rate was maintained 20 g water/ g of dry biomass throughout the process. Ultrasound assisted lime pretreatment was conducted using the same parameters applied for lime pretreatment process but the time of operation was limited to eight hours. The responses evaluated for lime and ultrasound assisted lime pretreatment was the delignification (fraction of lignin removed from biomass) ratio of biomass.The ultrasound assisted lime pretreatment process removed 68% (Bonbogori), 65% (Arecanut husk) and 64% (Moj) of the lignin present in the naive lignocellulosic fiber respectively. The optimum conditions for both Moj and Bonbogri were observed at lime-loading ratio 0.75, biomass-loading ratio 20 and ultrasound irradiation duration 120 min, but for Arecanut husk, the ultrasound irradiation duration was 180 min. This chapter concludes with the recommendation of best pretreatment process (based on yield of sugar) i.e. ultrasound assisted lime pretreatment for the next downstream process i.e. saccharification.

Chapter IV: Saccharification of Biomass

The pretreatment step is followed by saccharification to convert the cellulosic sugars to fermentable sugars. The plant cell wall is a solid, composite lignocellulosic material; enzymes must work directly at the solid-liquid interface and engage in surface de-polymerization of individual cellulose chains to hydrolyze carbohydrate polymers. This surface ablation process results in a reaction rate several orders of magnitude slower than freely diffusing enzymatic reactions, because conversion is limited by substrate accessibility. Dilute sulfuric acid, lime and ultrasound assisted lime pretreatment processes were conducted to make the biomass more amenable for enzymatic saccharification process. The pretreated biomass samples were compared by means of enzymatic saccharification process using cellulase enzyme. The cellulolytic activity and cellobiase activity of the enzyme was found to be 45 ± 0.2 FPU mL^{-1} and 134 ± 0.03 CBU mL^{-1} respectively. The pretreated lignocellulosic biomass hydrolysis process was conducted at 50°C, 150 RPM for 96 hours at different enzyme loading (5, 10, 20 and 25 FPU g^{-1} of cellulose). The pH of the medium was maintained at 4.6. The ultrasound assisted pretreatment process yield more sugar than other two pretreatment methods. The saccharification efficiency of ultrasound assisted lime pretreated biomass was highest in Bonbogori (82.5%) followed by Moj (80.1%) and Arecanut husk (79.5%).

Chapter V: Characterization of Wastewater Sludge

In this part of thesis, several physical and chemical characteristics like proximate and ultimate analysis, thermo-gravimetric analysis, FTIR analysis of wastewater sludge, collected from the wastewater treatment plant of IIT Guwahatiwere analyzed and discussed elaborately. The chemical and physical features of wastewater sludge indicates that the sludge contains good amount of nitrogen (1.5% of TS), phosphorous (2.3% of TS) and other essential trace elements. These elements along with nitrogen and phosphorous facilitate microbial growth and reduces the need for addition of supplementary nutrient elements in the fermentation medium. The microbial cell number per gram of wet sludge was 6.14 $\times 10^7$ and 2.68 $\times 10^8$ for anaerobic (AA) and aerorobic condition (PDA) respectively.

Chapter VI: Simultaneous Ethanol and Hydrogen Production through Fermentation

This chapter describes the simultaneous production of hydrogen and ethanol using the saccharified ultrasound assisted lime pretreated Bonbogori. The experiments were conducted according to the taguchi methods of design of experiment in both stirred tank and fluidized bed bioreactor using wastewater sludge as a source of microorganisms. Four important parameters such as type of reactors, temperature, pH and organic loading rate were considered for the optimization process. The optimal factor combination for both ethanol and hydrogen production was achieved at temperature 40°C, OLR 50 kg L^{-1} and pH 5.5 respectively. Fluidized bed bioreactor showed better performance than the stirred tank bioreactor for simultaneous production of ethanol and hydrogen. In this designed conditions the optimum yield of ethanol and bio-hydrogen was found to be 0.21 mol, and 30.8± 0.5 mmol respectively.

Chapter VII Conclusions and future work

This chapter summarizes the inferences drawn from the present work and recommendations for future work. The overall aim of the thesis is to produce hydrogen and ethanol simultaneously from lignocellulosic biomass. The data shows that all the biomass studied here have the possibility to translate into bio-ethanol as well as bio- hydrogen. It can be concluded, that the research regarding obtaining the bio-ethanol and bio-hydrogen from lignocellulosic biomass can be optimized at pilot levels in near future.

Chapter I

Introduction

In this chapter, an outline of world energy scenario and future prospects of biofuel with respect to the global energy demand are discussed. Several biofuel types and their preparation methods, advantages, disadvantages and major research areas of biofuel production technologies are also described. From the discussion, state of art for the current work is derived and finally the organization of the total thesis has been presented.

1.1 Introduction

The word energy comes from the Greek word "*energeia*" and it appears for the first time in the *Nicomachean Ethics* of Aristotle during 4thcentury BC, which means "activity". Energy manifests the whole cosmos. Energy *per se* is the principle driving force to propagate the human socio economic development. It became the integral part of our livelihood since the existence of fire was discovered by friction of stones.Atpresent, the whole world depends on three different types of energy viz. (i) Fossil fuel energy, (ii) Nuclear energy and (iii) Non-fossil and non-nuclear energy [1]. Each sources of energy has their own advantages and limitations.

Nuclear energy is acquiredthrough either nuclear fission reaction, in which a nucleus of an element is broken into two or more nuclei and releases sufficient amount of energy, or nuclear fusion, where energy is released due to fusion of two or more atomic nuclei ofradioactive materials.The nuclear fission technology is widely used than the nuclear fusion technology but the initial high cost of making nuclear power stations, and management of life threatening high-level radioactive wastes makes it less popular.Furthermore, public recognition of nuclear plants is a serious issue and strongly depends on emotional factors: the incident of Chernobyl at Russia and fresh Fukushima accident in Japan in 2011 due to tsunami has reignited the debate about risks of running nuclear power stations. The usage of nuclear power is limited only up to 10.69% (Fig 1.1).Oil is still consideredas the most preferred source of energy (36.25%) followed by natural gas (24.46%) and coal (20.24%) whereas the world reserves of oil are only about 1258giga billion barrels [2]. The demand of oil throughout the world will increase to 14748 million barrel of oil equivalent until 2020 however, the world reserves of natural gas are about 1.41 m^3; gross production of gas is about 2.41 m^3 per year [3]. World coal reserves are about 9.11 tones and annual consumption is about 4.51 tones. The problems with fossil fuels for the near future are distribution and recovery, and these problems differ in detail for each of the types of fuel.

Utilization of fossil fuels such as petroleum oil and coal is well established but the fluctuation in oil prices and rising concerns aboutenvironment, an increasing amount of attention has been devoted for developing the cost-effective and environment friendly alternative energy resources [4].

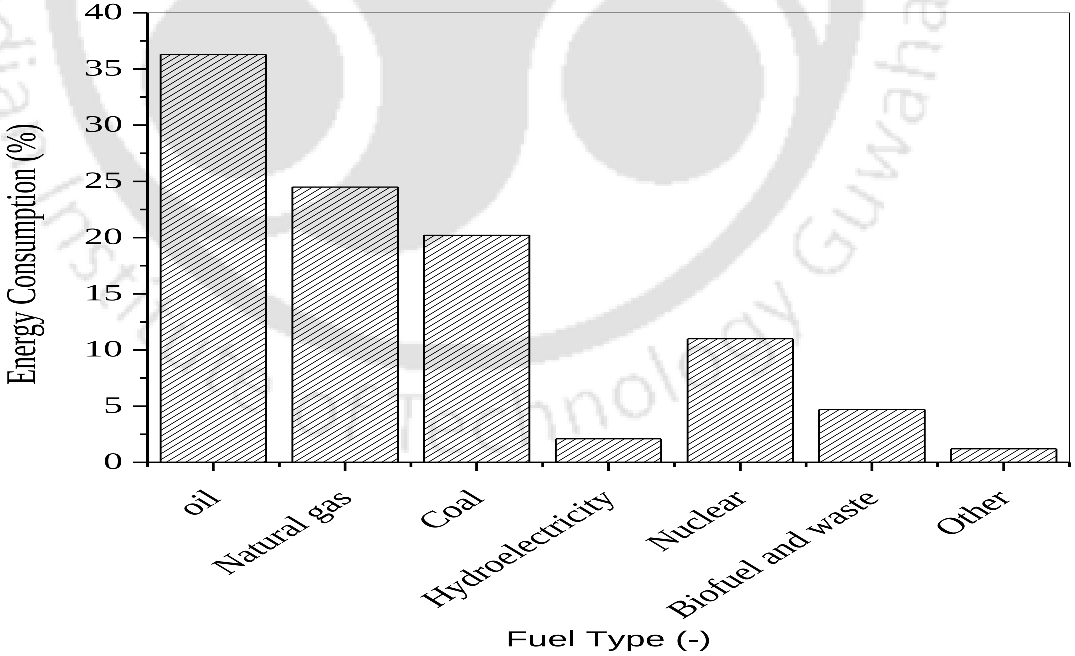

Fig 1.1 Energy consumption scenario of world

Source: Key world energy statics 2011, IEA report

Energy security is an indispensable feature of a country's independence and sustenance.The upshots from Fig 1.1 clearly show that 80% of the energy used by human being is not sustainable. Therefore,sustainable sources of energy should build up for each country to their own interests and growth. Thebiomass-based energy is one of suchsustainable sources of energy and is the third important energy source, after coal and oil.Energy from the sun is stored in plant biomass through photosynthesis therefore the biomass resource can be considered as organic matter, in which the energy of sunlight is stored in chemical bonds. When the bonds between adjacent carbon, hydrogen and oxygen molecules are broken by various chemical or physicochemical activities such as digestion, combustion, or decomposition, these substances release their stored chemical energy [5].

1.2 International Scenario of Biofuel

Renewable energy has been growing rapidly in most end-use sectors especially for power and transportation. Even in economic crisis, the European Union established many renewable energy facilities during 2011 unlike previous years. Further, for the same with the continuing fourth year, renewable energy accounted for more than half of the newer set-ups for the regional electric capacity and it was for more than 71% of total incorporations. The policies in support of the renewable energy have been the main motivation for inflating shares on renewable energy. About 118 countries, of which more than half are developing nations, currently have fixed renewable energy goals, and 109 nations have guidelines in support of renewable energy in the power sectors. The energy consumption pattern of OECD (Organisation for Economic Co-operation and Development) and non-OECD countries is illustrated in Fig 1.2. The pattern shows that the consumption of energy between OECD countries were more initially but after 2008 the scenario have changed and at present the non-OECD countries consumed 12.6 % more energy than OECD countries.

The scenario of global renewable energy (Table 1.1) reveales that the total consumption would increase up to 30 % by 2040 and among these; the biomass based energy consumption will play a major role.

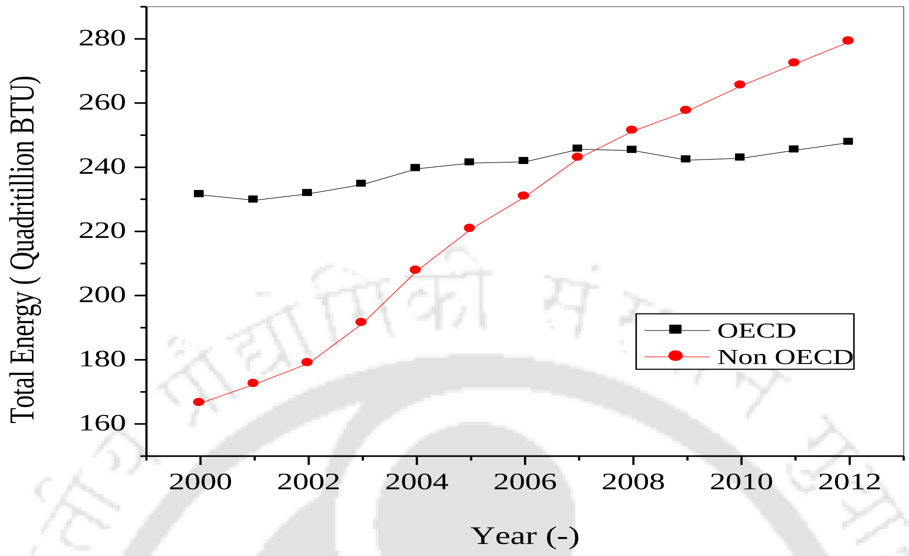

Fig1.2. Total energy consumption profile of OECD and Non-OECD countries.

Source: World Bank reports

Table 1.1 Scenario of usage renewable energy resources [6]

	2001	2010	2020	2030	2040
Total consumption (million tons oil equivalent)	10,038	10,549	11,425	12,352	13,310
Biomass	1080	1313	1791	2483	3271
Large hydro	22.7	266	309	341	358
Geothermal	43.2	86	186	333	493
Small hydro	9.5	19	49	106	189
Wind	4.7	44	266	542	688
Solar thermal	4.1	15	66	244	480
Photovoltaic	0.1	2	24	221	784
Solar thermal electricity	0.1	0.4	3	16	68

To keep this in mind more than thirty countries have already introduced programs for production of fuel ethanol from different lignocellulosic biomasses and among them countries like North and South America already initiated their own biofuel policy, at present Brazil producing more than 80% fuel ethanol [7].

Ethanol production from corn and sugarcane is a well-established process while production from lignocellulosic material is not yet a commercial route. The growing demands and subsidies for ethanol and other biofuels have encouraged the entrepreneurs to set up bio-refineries. A regional breakdown of the energy potential of different biomass materials (Table 1.2) proved that the potential of energy generation from biomass materials is quite evenly distributed among the various continents.

Table 1.2. Scenario of global bioenergy potentiality [Peta Joules per Annum (PJa^{-1})][8]

Energy Potential (Pj a^{-1})	**Asia**	**Africa**	**Europe**	**North America**
Wood	7700	5400	4000	12800
Stalk Plants	9900	900	1600	2200
Energy Plants	13900	13900	2600	4100
Dung	2700	1200	700	800

Kaltschmitt *et al*., 2003

The Asian countries can emphasis on planting energy plant based bio-refinery (13900 PJa^{-1}) whereas European countries have more potential on wood base bio-refinery process (4000 PJa^{-1}). The total potential of producing energy irrespective of types of biomass is approx 20000 PJa^{-1}and among these the contribution of Asia is approx 40% of the total potential of the world. Therefore, a significant potential exists in both developing and developed Asian countries for the production of biofuels from lignocellulosic biomass as well as the development of innovative technologies to efficiently convert biomass into fuels.

1.3 Indian Scenario of Biofuel

Energy is a critical input for socio-economic development and it plays a pivotal role in the growth and development of economy, particularly for a developing country like India, where per capita energy consumption is quite low, but increasing steadily.India has total reserves (proved & indicated) of 757 million metric tonnes of crude oil and1241 billion cubic meters of natural gas as on 1.4.2011.Gross Production of Natural Gas in the country at 52.22 billion cubic metres during 2010-11 is 9.95% higher than the production of 47.50 billion cubic meters during 2009-2010 [9]. There is no doubt that fossil fuels will continue to play a prevailing role in the energy scenario in our country in the next few decades. In contrast, renewable energy resources are abundant so it is virtually inexhaustible. India is rich in renewable energy resources whose potential has not been properly tapped. Therefore, their use should be encouraged in every possible way.The total potential for renewable power generation in the country as on 31st March 2010 was estimated to be 90,313 MWand this data shows that renewable energy no more remains a futuristic segment and is fast becoming a mainstay. The primary energy supply by fuel types in India have showed (Fig 1.3) that the main energy products consumed in India are oil, coal, biomass and gas [10].

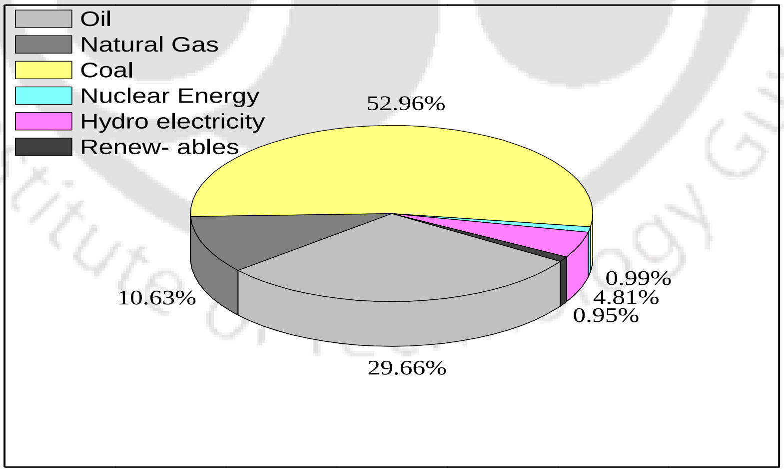

Fig 1.3 Primary energy consumption of fuel in India

Source: British petroleum statistical review of world energy 2011

The Ministry of New and Renewable Energy (MNRE) lays down the overall policy guidelines for renewable energy programmes and provides budgetary support for research and development and demonstration of technologies; facilitates institutional finance to institutions; and promotes private investments through fiscal incentives, tax holidays, depreciation allowance and remunerative returns for power fed into the grid. Indian government has mandated 10% blending of ethanol with gasoline, which amounted to about 1100 million liters of ethanol use. Further, India has a plan to expand biodiesel production to substitute 20% of diesel consumption by 2017. At present 5% blending of ethanol with gasoline has been taken up by the Oil Marketing Companies (OMCs) in 20 States and four Union Territories.The prescribed blending levels will be reviewed and moderated periodically as per the availability of bio-diesel and bio-ethanol. Beside this to avoid the fluctuations in the availability of biofuels, OMCs will be permitted to bank the surplus quantities left after blending of bio-diesel and bio-ethanol in a particular year, and to carry it forward to the subsequent year when there may be a shortfall in their availability to meet the prescribed levels. Moreover, the Bureau of Indian Standards (BIS) has also published IS: 2796: 2008 which covers specification for motor gasoline blended with 5% ethanol and motor gasoline blended with 10% ethanol [11].

Historically, biomass has been a major source of energy in each Indian household. Even in this century, several types of woods and cow dung are the holder of approximately 40% non-commercial energy sources of the total energy supply in India [12] but due to its highly inefficient conversion to useful energy, the use of biomass represents only 18% of total primary energy usage. Rural households in India predominantly use wood as fuel for cooking and water heating. Modern technologies facilitate derivation of energy from several organic materials and can be used in a variety of conversion processes to yield power, heat/steam, and fuel. In India, the uses of these technologies are focused on conversion of waste materials such as municipal, agricultural, or forest residues to biofuels. Production of biofuels in India can also be viewed as an option for generating rural employment and to promote rural development. Biofuel production will lead to creation of large number of jobs at multiple stages of the biofuel supply-chain, such as production (cultivation and harvesting practices), transportation, processing and marketing.

With the massive rate of urbanization, the demand for energy has grown assorted in the past few years and will continue to grow in future. The usages of energy in India (KT of oil equivalent) is shown in Fig 1.4.

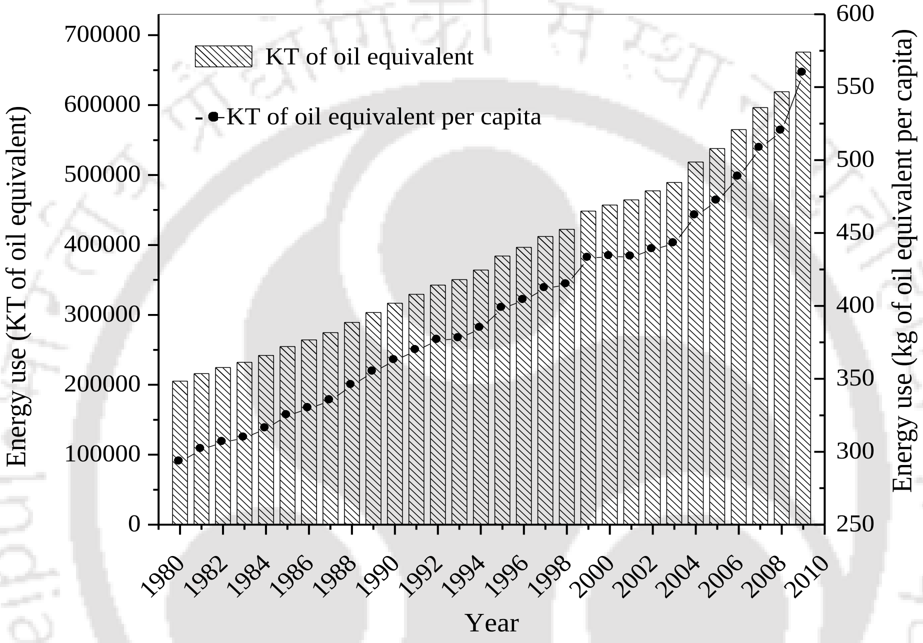

Fig 1.4. Usage of energy in India (1980-2010)

Source : International Energy Agency (IEA Statistics © OECD/IEA, http://www.iea.org/stats/index.asp)

Increasing rate of energy usage signifies the prosperity of the countries growth. Thus, there is an emerging energy supply-demand imbalance. Therefore, renewable energy has been an important component of India’s energy planning process since quite some time. Biofuels are one of the major divisions of renewable sources of energy. The production of biofuel in India since 2000 is tabulated in Table 1.3.

Table 1.3. Comparison of biofuel production (Thousand tonnes of oil equivalent)

Year	World	India	OECD	Non OECD
2000	9176	82	3841	5336
2001	10084	85	4350	5734
2002	11930	91	5406	6523
2003	14767	94	7045	7723
2004	16452	99	8549	7903
2005	19944	114	11013	8930
2006	25743	134	15054	10688
2007	34512	92	20494	14018
2008	46294	148	27728	18566
2009	52098	82	32569	19528
2010	59261	151	37130	22131

Source: British petroleum statistical review of world energy, 2011

From the available data it can be concluded (Table 1.3) that though the rate of biofuel production is quite high but the amount of biofuel production in India is not up to the mark compared to the rest of the world. The economy of India largely depends on agriculture which yields huge amount of agricultural residues and along with this good amount of forest residues can be harvested throughout the year which can be exploited for the production of biofuel. The major advantage of using biomass as a feedstock is its availability and there will be no upheaval for collection and formation of bio-refinery.

1.4 Overview of biofuel resources and environmental issues

Lignocellulosic biomass materials are the proven prodigiousreservoir from which liquid fuels and several important chemicals can be generated in a relatively simple, environment friendly and cost-effective manner. To avoid the dilemma between "foodsvs. fuel production", the focus is on utilization of agricultural waste products and forest residues rather than using food crops as the feedstock.

Biomass has always been a major source of energy for humanityand remains the dominant contributor for supplying energy to a large number of developing countries, such as India, where the dry lignocellulosic biomass materials are used to supply the household energy needs through traditional cooking stoves or open fires [13].

Among the various kinds of biofuels, thepopular substitute used for road transport vehicles is bioethanol. It has number of advantages over fossil fuelssuch as (1) it comes from a renewable resource, which contributes to sustainability, (2) biodegradable, (3) low toxicity, (4) readily available and (5) causes little environmental pollution [14-15]. Biofuels are commonly generated from the various activities of animals, plants and microorganisms. They can be solid like bio-char, liquid like biodiesel and bioethanol, or gaseous like biogas, biohydrogen. The heating value of biofuels are almost similar than its fossil counterparts, the lower heating value (LHV) of ethanol and butanol are 21.3 MJ/L and 27.8 MJ/L where as crude oil have 32.3 MJ/L respectively [16]. The biggest difference between biofuels and petroleum feed stocks is concentration of oxygen content [17]. Bioethanol is a high-octane fuel(RON value 108.6) and can be added into gasoline (RON value 95) as an octane enhancer [18-19]. It is used to partially replace gasoline to make gasoline-ethanol mixtures such as,E15 (15% ethanol and 85% gasoline) and E85 (85% ethanol and 15% gasoline). Production of biofuel in large scale can reduce the dependence on oil imports of the developing countries [20].

The inclusion of environmental externalities in the accounting of natural resources was already raised in the beginning of the last century.Energy scenarios have identified the need of immense CO_2 capture and storage deployment in industrial, fuel transformation and power generation sectors to limit the level of global CO_2 emissions. Presently all this energy comes primarily from non-renewable sources, mainly from fossil fuels.These Fossil fuels are responsible for many of the environmental problems that we are facing now-a-days. Change of climate, acid rain or the emissions of particulate matter are some of the impacts associated to the burning of fuels. Therefore, many countries put emphasison the use of clean fuel technologies. The latter implies the use of energy-demanding diminution technologies of pollutants such as SO_2, NO_x, particulated matter or CO_2, which in turn decreases the effective available energy from the fuels. Moreover, the energy of fossil fuels depends on the conditions of the environment. The anthropogenic greenhouse gases (GHG) emitted is increasing (0.06% annually) the CO_2 concentration in the environment and are provoking a global temperature increase.Consequently, the available energy of fossil fuels is being decreased. Therefore, strategies need to employ to stabilize and reduce concentrations of the green house gases from the atmosphere. Various sources of renewable energy are coming into existence, among all biofuels are gaining a lot of interest and are expected to play a crucial role in the global energy demand.

Biofuels can also be classified based on their feedstock, such as; first generation biofuels (FGB); second generation biofuels (SGB); third generation biofuels (TGB); and fourth generation biofuels (FrGB). FGB refer to biofuels made from primarily food crops such as sugar, starch, vegetable oils, or animal fats using conventional technologies, but FGBs are limited in their ability to achieve targets for oil-product substitution, climate change mitigation, and economic growth, because concerns exist about choosing the properfeedstock, usage of land and competition with food crops. The main drawback of first generation biofuels is the food-versus-fuel debate, one of the reasons for rising food prices is due to the increase in the production of these fuels [21-22]. Second-generation biofuels are produced from plant biomass or non-food biomasses e.g. lignocellulosic feedstock materials including by-products (i.e. cereal straw, sugar cane bagasse, forest residues), wastes (organic components of municipal solid wastes), and dedicated feedstocks (purpose-grown vegetative grasses, short rotation forestry and other energy crops) [23].Therefore, SGB refers largely to lignocellulosic materials, as this makes up the preponderance of the cheap and abundant nonfood materials available from plants [24]. The raw materials for production of TGB are algal biomasses (e.g.*Chlorococcum littorale*,*Eucheuma*sp,*)* whichhave the ability to produce high levels of carbohydrates instead of lipids as reserve polymers. These species are ideal candidates for the production of bioethanol as carbohydrates from algae can be extracted to produce fermentable sugars [25]. Fourth generation biofuels are based on conversion of vegetable oils and bio-diesel into bio-gasoline using algae by applying most advanced technologies [26].The TGB is about the processing of algae biomass for biofuel production, while the

FrGB is about metabolic engineering of algae for producing biofuels from oxygenic photosynthetic microorganisms [27].The most successful methods of producing biofuels from algal biomassas sole source of carbon are fermentation of microalgae to bioethanol and production of biodiesel *via in situ* transesterification of microalgal biomass. Several technical bottlenecks in algae biofuel researchand developmenti.e. third and fourth generation biofuel production, persistswhich can only be solved by the use of post-genome tools on these photosynthetic organisms.Annually, ~10^{11} tons of plant biomass, comprising mainly plant cell walls, is hydrolyzed by several kinds of microbes, and the energy released by this process equivalents to 640 billion barrels of crude oil [28]. Therefore, conversion of lignocellulosic biomass to fermentable sugars i.e.SGBmay stand for a viable alternative for the production of renewable fuels such as ethanol [29].

Carbohydrate is a major organic component of all biomass and, hence, a key substrate for producing biofuels. The main products of carbohydrate fermentation are acetic, propionic and butyric acids, along with ethanol [30]. Research around the world on production of ethanol from lignocellulosic biomass materials has been focused on developing cost-effective pretreatment technologies, improved cellulase enzyme activities, and co-fermentation of 6-C (e.g. glucose) and 5-C (e.g. xylose, arabinose) sugars to ethanol [31]. The preliminary step towards biofuel production is pretreatment to convert the recalcitrant lignocellulose structure to reactive cellulosic intermediates before enzymatic hydrolysis [32].For a proficient biological conversion of cellulose into fermentable sugars, it is essential to modify the physical and chemical properties of the plant cell wall, which can be done through a pretreatment step. The pretreatment process disintegrates the lignocellulosic matrix and removes some or all of the lignin, which causes increased porosity in the substrate and reduction in the size of the biomass particles [33-34]. Different pretreatments have varying mechanism of interacting with the plant cell wall components. In addition, there is an extensive variety of lignocellulosic materials, ranging from grasses, softwoods, and hardwoods, which also have different physical and chemical properties. For this reason, there is interdependence between pretreatment, the type of substrate, and the way it is eventually processed. Therefore,it is important to adopt appropriate pretreatment technologies based on the physico-chemical properties of each raw material (lignocellulosic biomass) because the concentration of building block constituents may vary in each year. Even the compositions of the biomasses which fall under same species may bemassivelychangeddue to varying seasons and climatic conditions. In addition, the choice of certain pretreatment has a large impact on all subsequent steps in the overall conversion scheme in terms of cellulose digestibility, generation of toxic compounds, energy demand in the downstream process etc. Therefore, the bioconversion of raw lignocellulosic biomass materials begins with characterization followed by pretreatment, saccharification and fermentation.

A wide range of physical, chemical, and biological based pretreatment processes exists. Various types of pretreatment processes include water-based, acidic, alkaline, and organic solvent pulping systemspainstaking by various researchers with different types of lignocellulosic biomass materials [35]. Some researchers have observed that same pretreatment processmay not be better suited to different types of lignocellulosic biomass materials [36].

Enzymatic hydrolysis or the saccharification process is succeeded by pretreatment, which breaks the cellulose and hemicellulose into monomers. Process is followed by fermentation,where various microorganisms are exploited to convert the monomers obtained after enzymatic hydrolysis process,to ethanol.Enzymatic saccharification has the potential to convert the carbohydrates of the lignocellulosic biomass to ethanol with high yields and low production costs [37]. Saccharification of lignocellulosics includes cellulase and xylanase enzymes, which are most commonly produced by soft-rot fungi such as *Trichodermasp*, *Penicillumsp*, *Aspergillus sp*andmany other genetically modified microorganisms [38]. Several researchers worked on the enzymatic hydrolysis process i.e. saccharification and their study revealed that a mixture of cellulases, including endo- and exo-cellulases and β-glucosidases, is required in order to break down the cellulosic micro fibril structure into its carbohydrate components in an efficient manner [39]. Challenges that remain in the bioconversion process includerecalcitrance of the lignocellulosic matrix.The series of enzymatic reactionsare observedduring thehydrolysis of both cellulose and hemicellulosic material to yield both five- and six-carbon sugars [40].

The yields of bioethanol using thermo-chemical platform tend to be lower. In terms of energy output, thermo-chemical production of bioethanol delivers about 2.6–

3.5 Giga J per tonne of dry biomass, compared to 6.9 Giga J per bone-dry tonne of wood for the Fischer–Tropsch process [41]. The production of ethanol through fermentative route begins after enzymatic hydrolysis or saccharification.Ethanol fermentation can be initiated by employingtwo commonly used microorganisms viz. yeasts *Saccharomycessp* and bacteria *Zymomonassp*[42].Unfortunately both of these two microorganisms cannot ferment pentose sugars, producedduring hydrolysis of hemicellulose stream of lignocellusic biomass. Yeasts *Candidasp*. which ferment cellobiose to ethanol does not prefer pentose as the source of metabolic activities. Enteric bacteria such as *Escherichia coli* can ferment both the pentose and hexose sugars derived from hemicellulose and cellulose. A soil bacterium *Klebsiella oxytoca*is capable of fermenting both hemicellulose-derived sugars and cellobiose and cellotriose as substrates [43].

Next to bioethanol another promising biofuel is biohydrogen. At present, the most economically competitive method for hydrogen production is steam reforming of natural gas, whichis also a kind of fossil fuel, and its usage fails to provide a solution to deal with the huge amount of carbon dioxide emissions during the reforming processes. Therefore, interest has grown to search for effective alternativetechnologies to produce renewable hydrogen cleanly and safely. The potential use of microorganisms for biological production of hydrogen as a future energy resource makes hydrogen metabolism an emerging field of research [44]. A large number of microbial species, including significantly different taxonomic and physiological types, can produce hydrogen, which are distributed across 10 out of 35 bacterial groups. These bacteria include strict anaerobes (*Clostridia*, *Methylotrophs*, methanogenic bacteria, Rumen bacteria, *archaea*), facultative anaerobes (*Escherichia coli* and *Enterobacter*), aerobes (*Alcaligenessp* and *Bacillussp*), photosynthetic bacteria, and *Cyanobacteria*[45-46]. In general, the isolated and identified mesophiles are mainly affiliated with two genera: facultative *Enterobactericeae*and strictly anaerobic *Clostridiaceae* [47-48]. In addition, aerobes such as *Bacillus*[49] and *Vibrio*sp [50] have been also cultivated or isolated for hydrogen production.

Both hydrogen and ethanol can be produced by dark fermentation of biomass using acidogenic bacteria (e.g. *Clostridialsp* or *Enterobactersp*). However, the metabolic pathway for hydrogen and ethanol production often conflicts with each other from the perspective of metabolic electron transfer thereby limiting the production to a single biofuel (either hydrogen or ethanol) [51]. Further in-depth study is required to optimize the metabolic pathway to produce both hydrogen and ethanol simultaneously.

1.5 Pathways of bioethanol and biohydrogen production

Biofuel can be produced by two routes (1) biochemical processing pathway and (2) thermal processing pathway i.e. gasification (Fig 1.5).

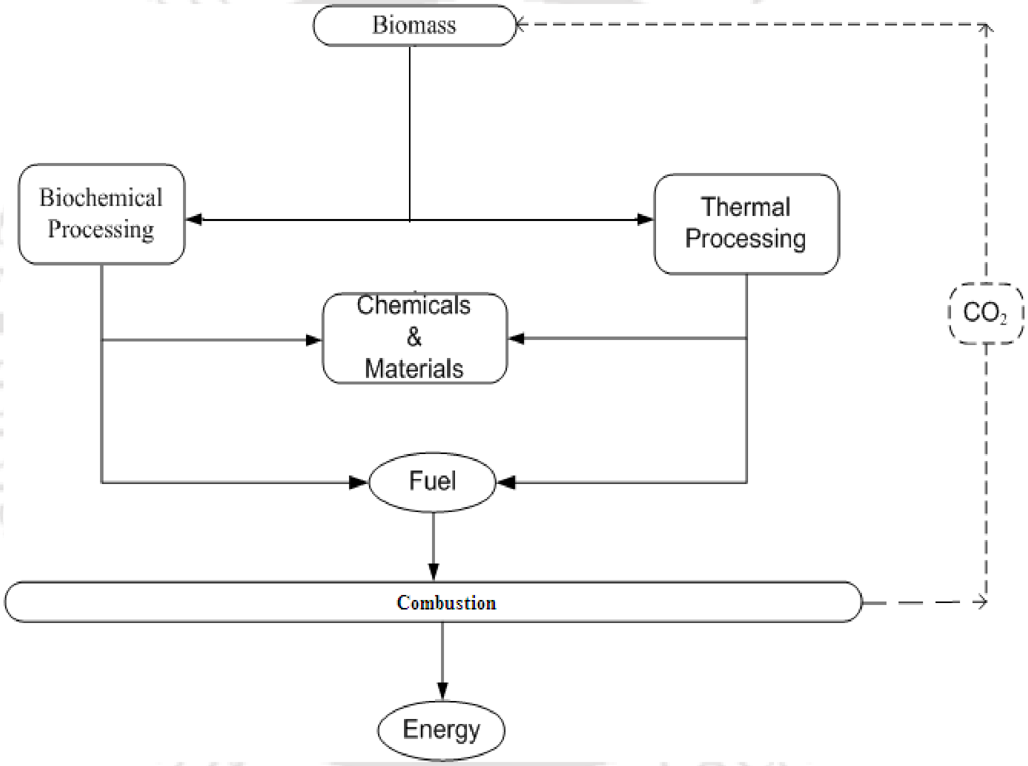

Fig 1.5 Schematic Diagram of biochemical and thermochemical processes that includes recycling of the energy.

The present study put focus on biochemical route of biofuel production. The carbohydrate platform involves the extraction of carbohydrates from biomass and the subsequent fermentation into ethanol. Bioconversion begins with a pretreatment stage to physically increase surface area along wood fibers. The glucose pathway is the simplest approach and involves extraction of readily fermentable six-carbon sugars present in biomass; this step is known as pretreatment of biomass followed by fermentation for the production of ethanol and hydrogen. Pretreated biomasses then hydrolyzed by means of cellulolytic enzymes, but the carbohydrates in lignocelluloses are not easily accessible for enzymatic hydrolysis. This recalcitrance is primarily due to the composition of lignocellulosic biomass and the way specific components interact with each other. Therefore, the first challenge is pretreatment of lignocellulosic biomass to reduce biomass recalcitrance, for improving the yield of fermentable sugars. Among pretreatments, being considered by various researchers includes water-based, acidic, alkaline, and organic solvent pulping systems. As with conventional pulping, these pretreatment methods tend to work best with a homogenous batch of wood chips. Some have observed that different pretreatments seem to be better suited to different types of lignocellulosic feedstocks [52]. The present study put emphasis on dilute acid pretreatment and ultrasound assisted lime pretreatment of lignocellulosic biomass materials. Pretreatment process is not sufficient to extract fermentable sugar from the lignocellulosic biomasses. The requirement of enzymatic hydrolysis followed by fermentation process is necessary to make the biofuels. During fermentation, both C5 and C6 sugars present in lignocellulose are fermented to ethanol under anaerobic/aerobic conditions. Historically, yeast (*Saccharomyces cerevisiae*) was used to ferment C6 sugars i.e. glucose. Similarly, other microorganism (e.g. *Zymomonas mobilis,Escherichhia coli*) has also been developed using genetic engineering tools, which can ferment both C6 and C5 sugars [53].

1.6 Scopes of current research and objectives

The conversion of the biomass into biofuels consists of several processing steps, such as pretreatment to remove lignin from the fiber matrix or hydrolysis of hemicelluloses to C5-sugar (pentose), hydrolysis of cellulose to produce C6-sugar (glucose), and fermentation to convert both sugars into biofuels. Yeast is conventionally used to convert only glucose, but recently some microorganisms are known to consume both C5- and C6-sugars, giving a higher bioethanol yield. Process configurations can be arranged as SHF (separate hydrolysis and fermentation), SSF (simultaneous saccharification and fermentation) or SSCF (simultaneous saccharification and co-fermentation).Both hydrogen and ethanol derived from lignocellulosic biomass arethe promising biofuels and have great potentials to become alternatives to fossil fuels. Hydrogenproductionviadark fermentation process by heterotrophic anaerobic microorganisms is known as more environmentally friendly and less energy intensive process. In addition,with hydrogen, anaerobic fermentation process also produces a significant amount of alcohols (e.g. ethanol). However, the hydrogen production usually optimized by suppressing the ethanol production route. Since ethanol considered as the most feasible alternative toliquid fossil fuels for the automobiles, there is a need to develop processes for simultaneous production of both hydrogen and ethanol. Current technologies mainly uses various types of reactors (such as stirred tank reactor, packed-bed, trickling biofilter etc.) for biofuel production. However, these reactors have their own limitations with respect to rate of mass transfer as well as maintaining growth conditions of the microorganisms. In contrast, fluidized-bed reactors are supposed to address these limitations with an aim of simultaneous production of both hydrogen and ethanol.

Pretreated lignocellulosic biomass results in two main streams: one rich in cellulose and other rich in hemicellulose. By choosing appropriate biocatalyst, carbohydrates obtained from both cellulose and hemicellulosecan be effectively converted to ethanol and hydrogen. Immobilized anaerobic sludge (mixed microflora) may prove to be a good biocatalyst for the dark fermentation process. It is anticipated that the fluidized-bed reactor with high mass transfer rate might optimize the yield of both hydrogen and ethanol.

The study embodied in this thesis isorganized into the following chapters containing the subsequent objectives.

- Physico-chemical characteristics of lignocellulosic biomass.

The lignocellulosicbiomass materials were initially analyzed for their physical (moisture content, crystallinity, elemental analysis, and ash) and chemical (composition) characteristics. Crystallinity is important to find out the *crystalline and amorphous cellulose in the biomass. Chemical composition such as cellulose, hemicellulose and lignin for each biomass were determined using NREL methods.*

- To obtain lignin less pure fractions of cellulose and hemicellulose from efficient pretreatment of lignocellulosic biomass for higher yield of hydrogen and ethanol.

Pre-hydrolysis of lignocellulosic biomass was performed using combination of physical (mechanical operation) and chemical (acid hydrolysis) techniques. The sizes of the particles were reduced using two-roller mill and the decrease in particle size facilitated to increase the cellulose reactivity. Two chemical pretreatment method viz-dilute acid pretreatment and ultrasound assisted lime pretreatment method were conducted on the screened biomass particles. The effects of various reaction parameters and optimization of reaction parameters such as temperature, acid concentration and reaction time on yield of fermentable sugars were extensively studied using Taguchi methodology.

- Physico-chemical characteristics of wastewater sludge

The seed sludge was collected from the final sedimentation tanks of municipal wastewater treatment plants located inside IIT Guwahati. The sludge was pretreated with HCl at pH 3.0 for 24 h to eliminate the methanogenic activity. The study includes several physical and chemical characterization like proximate and ultimate analysis, thermogravimetric analysis, FTIR etc.

- Production and optimization of both hydrogen and ethanol in a fluidized-bed reactor and stirred tank reactor

The pretreated biomass samples were saccharified using commercial cellulase enzymes.Production of both ethanol and hydrogen using this saccharified broth was conducted in both fluidized and stirred tank bioreactor. Taguchi method of *optimization process was applied to optimize as well as compare the entire process of biofuel production.*

The present study considered two forest residues namely bon bogori (*Ziziphus rugosa*) and moj (*Albizia lucida*) and one agricultural waste product areca nut husk (*Areca catechu*) as the lignocellulosic feedstock. These materials are plenty in North East region of India, the local people uses them as firewood (bon bogori and moj) to carry on their daily activities.

References

[1] ForseBerg CW. Sustainability by combining nuclear, fossil, and renewable energy sources. Progress in Nuclear Energy. 2009; 51(1): 192–200.

[2] Owen NA, Inderwildi OR, King DA. The status of conventional world oil reserves—Hype or cause for concern. Energy policy. 2010; 38(8): 4743-4749.

[3] Keshavarzian N, Anaraki SK, Zamani M, Erfanifard A. Projections of oil demand in road transportation sector on the basis of vehicle ownership projections, worldwide: 1972–2020. Economic Modelling. 2012; 29(5):1979–1985.

[4] Labban M. Oil in parallax: Scarcity, markets, and the financialization of accumulation. Geoforum . 2010; 41(4): 541–552.

[5] Lewis NS, Nocera DG. Powering the planet: Chemical challenges in solar energy utilization. PNAS, 2006; 103(43): 15729-15735.

[6] Kralova I, Sjöblom J. Biofuels-renewable energy sources: a review. Journal of Dispersion Science and Technology. 2010; 31:409–425

[7] Gallagher PW. A market and policy interpretation of recent developments in the world ethanol industry. Biofuels, Bioproducts and Biorefining,2007; 1: 103-118.

[8] Kaltschmitt M, Marten D, Frohlich N and Nill M, Energiegewinnung aus biomasse. Extermes Gutachten, WBGU-Materialien, berlin-Heidelberg, 2003.

[9] Basic Statistics on Indian Petroleum & Natural Gas 2010-2011. Ministry of Petroleum & Natural Gas Government of India.

[10] British petroleum statistical review of world energy 2011

[11] National Policy on Biofuels, 2009 Ministry of New and Renewable Energy. National Policy on Biofuels Government of India. http://www.mnre.gov.in/information/policies-2/

[12] Karandikar V, Rana A. Future of Energy Options for India in an Independent World. Reliance Industries Ltd.; p. 6. http://www.worldenergy.org/documents/p001145.pdf. Accessed September 2010.

[13] MacCarty N, Still D, Ogle D. Fuel use and emissions performance of fifty cooking stoves in the laboratory and related benchmarks of performance. Energy for Sustainable Development. 2010; 14 (3):161-171.

[14] Demirbas A. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy Conversion and Management. 2008; 49:2106–2116.

[15] Balat M. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management. 2011; 52: 858-875.

[16] Swanaa J, Yanga Y, Behnamb M, hompsonb R. An analysis of net energy production and feedstock availability for biobutanol and bioethanol. Bioresource Technology. 2011;102(2):2112–2117.

[17] Demirbas A. Political economic and environmental impacts of biofuels: a review. Applied Energy. 2009; 86: S108–S117.

[18] Eyidogan M, Ozsezen AN, Canakci M, Turkcan A. Impact of alcohol–gasoline fuel blends on the performance and combustion characteristics of an SI engine. Fuel . 2010;89(10): 2713–2720.

[19] Palmer FH. Vehicle performance of gasoline containing oxygenates. MI MechE 1986; C319/86: 33–46.

[20] Demirbas A, Demirbas I. Importance of rural bioenergy for developing countries. Energy Conversion and Management. 2007; 48: 2386–2398.

[21] Demirbas MF. Biorefineries for biofuel upgrading: A critical review. Applied energy. 2009;86,: S151–S161.

[22] Amani E,Chad H. Ethanol Expansion in the Food versus Fuel Debate: How Will Developing Countries Fare?. Journal of Agricultural & Food Industrial Organization. 2007; 5(2): 1-23.

[23] Naik SN, Goud VV, Rout PK, Dalai AK. Production of first and second generation biofuels: A comprehensive review. Renewable and Sustainable Energy Reviews. 2010; 14: 578–597.

[24] Eisberg N. Harvesting energy Chemistry and Industry.2006; 24–25.

[25] Goh CS, Lee KT. A visionary and conceptual macroalgae-based third-generation bioethanol (TGB) biorefinery in Sabah, Malaysia as an underlay for renewable and sustainable development. Renewable and Sustainable Energy Reviews. 2010; 14: 842–848.

[26] Demirbas AH. Inexpensive oil and fats feedstocks for production of biodiesel Energy Educ Sci Technol Part A, 2009; 23: 1–13.

[27] Jing L , Sheahan C, and Pengcheng F. Metabolic engineering of algae for fourth generation biofuels production. Energy Environ. Sci., 2011;4: 2451-2466

[28] Boudet AM, Kajita S, Pettenati J, Goffner D. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends in Plant Science. 2003; 8:576–581.

[29] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J. The path forward for biofuels and biomaterials. Science. 2006; 311:484–489.

[30] Ren N, Wang X, Xiang W, Lin M, Li J, and Guo W. Hydrogen production with high evolution rate and high yield by immobilized cells of hydrogen producing bacteria strain B49 in a column reactor. High Technol Lett. 2002; 8: 21–25.

[31] Cheng JC, Timilsina GR. Status and barriers of advanced biofuel technologies: A review. Renewable Energy.2011; 36: 3541–3549.

[32] Sánchez OJ, Cardona CA. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology. 2008; 99: 5270–5295.

[33] Mansfield SD, Mooney C, Saddler JN. Substrate and enzyme characteristics that limit cellulose hydrolysis Biotechnol Prog. 1999; 15: 804–816.

[34] Chundawat SP, Balan V, Dale BE. High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass Biotechnol Bioeng. 2008; 99 (6):1281–1294.

[35] Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies Bioresource Technology. 2005; 96:1959–1966.

[36] Mabee WE, Fraser ED, McFarlane PN, Saddler JN. Canadian biomass reserves for biorefining Applied Biochemistry and Biotechnology.2006; 129: 22–40.

[37] Han M, Kim Y, Kim Y, Chung B and Choi GW, Bioethanol production from optimized pretreatment of cassava stem. Korean Journal of Chemical. Engineering. 2011;28(1):119-125.

[38] Wilson DB. Cellulases and biofuels. Current Opinion in Biotechnology, 2009; 20(3): 295-299.

[39] Bansal P, Hall M, Realff MJ, Lee JH, Bommarius AS. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnology Advances. 2009; 27(6): 833-848.

[40] Robinson J, Keating JD, Boussaid A, Mansfield SD, Saddler JN. The influence of bark on the fermentation of Douglas-fir whitewood pre-hydrolysates. Applied Microbiology and Biotechnology. 2002; 59: 443–448.

[41] Sims R, Taylor M, Saddler JN, Mabee WE. From 1st- to 2nd-Generation Biofuel Technologies: An Overview of Current Industry and R&D Activities International Energy Agency, Paris, France .2008; 124.

[42] Amutha R, Gunasekaran P. Production of ethanol from liquefied cassava starch using co-immobilized cells of Zymomonas mobilis and Saccharomyces diastaticus. Journal of Bioscience and Bioengineering. 2001; 92 (6): 560-564.

[43] Cheng JJ, Timilsina GR. Status and barriers of advanced biofuel technologies: A review. Renewable Energy. 2011; 36(12): 3541–3549.

[44] Hallenbeck PC. Fermentative hydrogen production: Principles, progress, and prognosis International Journal of Hydrogen Energy. 2009; 34(17): 7379-7389.

[45] Lo YC, Chen WM, Hung CH, Chen SD, Chang JS . Dark H2 fermentation from sucrose and xylose using H2-producing indigenous bacteria: feasibility and kinetic studies. Water Research.2008;42: 827–842.

[46] Bisaillon A, Turcot J, Hallenbeck PC. The effect of nutrient limitation on hydrogen production by batch cultures of Escherichia coli. International Journal of Hydrogen Energy. 2006; 31: 1504–1508.

[47] Penfold DW, Forster CF, Macaskie LE. Increased hydrogen production by Escherichia coli strain HD701 in comparison with the wild-type parent strain MC4100 Enzyme and Microbial Technology.2003; 33:185–189.

[48] Collet C, Adler N, Schwitzguebel JP, Peringer P. Hydrogen production by Clostridium thermolacticum during continuous fermentation of lactose International Journal of Hydrogen Energy. 2004; 29:1479–1485.

[49] Patel SK, Sing M, Kalia VC. Hydrogen and Polyhydroxybutyrate Producing Abilities of Bacillus sp. From Glucose in Two Stage System. Indian Journal of Microbiology. 2011;51: 418-423.

[50] Redwood MD, Beedle MP, Macaskie LE. Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy. Reviews in Environmental Science and Biotechnology.2009; 8:149-185.

[51] Wu KJ, Lo YC, Chen SD, Chang JS. Journal of the Chinese Institute of Chemical Engineers. 2007;38: 205-213.

[52] Mabee WE, Fraser ED, McFarlane PN, Saddler JN. Canadian biomass reserves for biorefining Appl. Biochem. Biotechnol. 2006; 129: 22–40.

[53] Franden MA, Pienkos PT, Zhang,M. Development of a high-throughput method to evaluate the impact of inhibitory compounds from lignocellulosic hydrolysates on the growth of Zymomonas mobilis. Journal of Biotechnology. 2009; 144 (4): 259-267.

Chapter -II

Physico-chemical Characterization of Lignocellulosic Biomass

The lignocellulosic materials are cheap and readily available either in the form of agricultural waste or forest residues. These materials can be used as a source for energy generation either in the gaseous form (CO, H_2 etc) or in liquid form (ethanol, butanoletc) to meet the rising demand of energy. The reign of lignocellulosic materials for energy production is a proven fact in this era of energy research. The present study put focus on characterization of three biomass samples namely Areca nut husk (Arecancatechu), Moj (Albizialucida) and Bonbogori (Ziziphusrugosa), available in the region of North-East India. The study includes several physical characterization like proximate and ultimate analysis, thermogravimetric analysis, crystalinity and chemical characterization that embraced FTIR, Raman spectroscopy. The maximum crystalinity was observed in Areca nut husk fiber (63.84%) followed by Moj (46.43%) and Bonbogori (42.46%). All these properties combined together per se had shown that Areca nut husk, Bonbogori and Mojcould act as the impending potential candidates for biofuel production.

2.1 Introductionand review of literature

Biomass can be defined as complex polymer of cellulose, hemicellulose and lignin; hence, it is also called lignocellulosic material. The various permutation and combination of those three components stated before provides unique characteristics to the biomass. These characteristics are different for each biomass. Beside biofuel production, the lignocellulosic biomasses may act as a raw material for production of value added chemicals.

Depending on the geographical regions and environments or food habit of the local people the sources of biomasses are diverse; it may be agricultural waste products, forest residues, dedicated energy crops or combinations of thereof. The major advantage of energy production from agricultural waste or forest residues is negligible raw material cost. It is a win -win situation for both the energy producer and raw material harvesters. Therefore, research is going on to search for suitable agricultural wastes or forest residues, which can be utilized as renewable energy sources. The major lignocellulosic biomass species available in North -East region of India are bamboo, sugarcane, rice husk. All these biomass materials are the proven source of bio energy. Though these materials are plenty in nature, but to put more stress on these materials to generate energy is not beneficial for the dwellers in North East India, as all those crops require a systematic cultivation process which demands a huge dedicated cultivation lands apart from sound agricultural knowledge to cultivate. The present economic, social and geographical scenario of the North-east India may not cope up with such systematic cultivation system. The North-East India is well known throughout the world for its biological diversity, several forests and agricultural waste products exists here, fuel woods hold a consequential function in the societies abode at higher reaches of North East India, not only for cooking but also for heating and other day to day activities. There is a conspicuous lack of knowledge with regard to the biomass characteristics of indigenous tree and shrub species present in the forests and several provinces of North-East India, which retards to set up biomass based energy plants. In order to exploit the biomasses harvested from North-East India for its fermentable sugar and other valuable chemicals the chemistry of lignocellulose must be understood.

Cellulose consists of long chains of hydro-D-glucopyranose units (AGU) with each cellulose molecule having three hydroxyl groups per AGU, with the exception of the terminal ends[1]. The polymer contains three reactive hydroxyl groups at the *C*-2, *C*-3, and *C*-6 atoms, which are, in general, accessible to the typical conversions of primary and secondary alcoholic OH groups [2]. The un-branched β_{1-4} linkage results in linear chains that are stabilized by hydrogen bonds within the chain and between neighboring chains. A cellulose molecule may contain more than 104 glucose residues (mass 1–2 $\times 10^6$ Da) and can reach lengths of 6–8 μM.

Hemicelluloses are heterogeneous branched chain polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids, located beside cellulose as an amorphous mass. In contrast with cellulose, hemi-cellulose differs, in consisting primarily of xylose and other five-carbon monosaccharides. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans. The degree of polymerization and concentration of building block such as monosaccharide varies from plant to plant. The xylan of hemicellulose could be extracted quite well in an acid or alkaline environment, while glucomannan can hardly be extracted in an acid environment and needs a stronger alkaline environment than xylan for extraction[3]

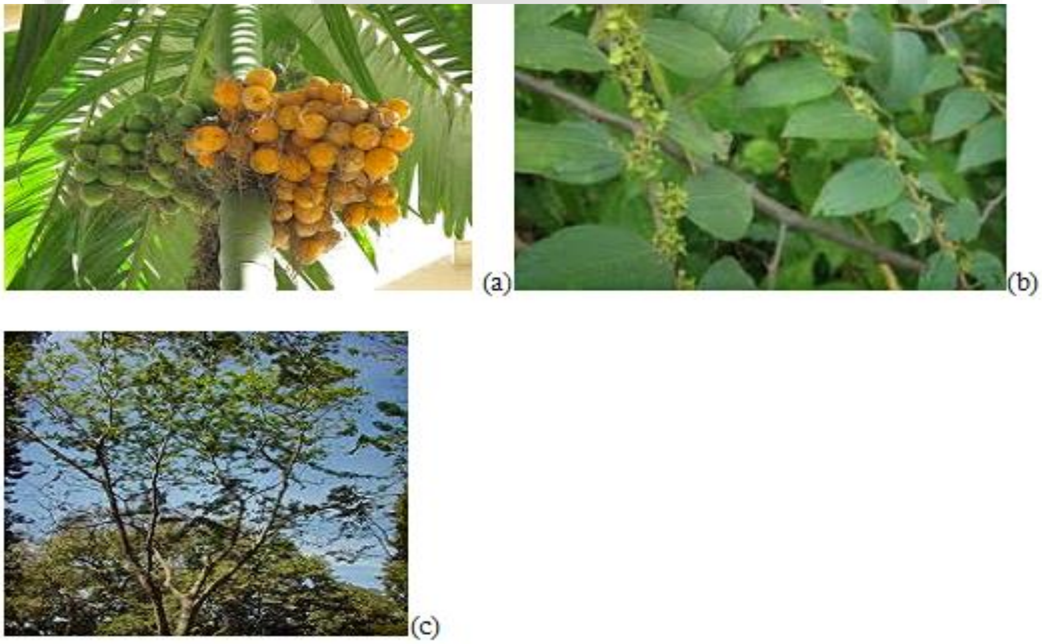

Fig. 2.1 Picture of selected lignocellulosic biomass (a) Areca nut (b) Bonbogori (c) Moj

Lignin is a complex high molecular weight polymer of coumaryl, conferyl and sinapyl alcohol. The concentration of these alcohols varies with plant to plant. This polymer intimately associated with cellulose and hemicellulose and imparts physical strength to the plant. The solubility of the lignin in acid, neutral or alkaline environments depends however on the precursor (*p*-coumaryl, coniferyl, sinapyl alcohol or combinations of them) of the lignin [4].

The present study focused on three biomass samples namely Arecanut husk,bon bogori and Moj. The Arecanut husk is well known as an agricultural waste residue in North-East India.Betel nut (*Arecan Catechu*), also known as Areca nut (Fig.2.1 (B)), has applications in human and animal medicine and is used in the paint and leather industries [5].*Areca* is a genus of about 50 species of single-stemmed palms in the family *Arecaceae*, found in humid tropical forests.The well-known member of the genus is *A. catechu*, the Areca nut palm. Several species of Areca nuts, known for their bitter and tangy taste, raw or dried are routinely used for chewing. These nuts are widely used as a popular masticator in India and Pakistan. India is the largest producer of Areca nut in the world. Karnataka tops the list of Areca nut producing states with 40 per cent of the country’s total output. India is also the largest consumer of it in the world at 3.2 lakh tones a year. The total area under the crop is estimated at 2.7 lakh hectares and the annual production is estimated at around 3.3 lakh tones, with Karnataka and Kerala accounting for nearly 72 per cent of total output. Over six million people are engaged in Areca nut cultivation, processing and trade. More than 85 percent of the area under cultivation is made up of small and marginal holdings [6]. The Areca nut husk is considered as an agricultural waste product. The nut has commercial importance and is processed by boiling the whole nut. The husk of the fruit is removed and it has no other traditional use. Husks are left in piles to dry; it is often a nuisance to the processor. The large amount of solid husk, formed during separation of kernel from the fruit, disposed without any treatment, though this lingocellulosic material contains a good amount of fermentable sugar (21%) [7]. Therefore, a number of wide opportunities exist to use this so-called agricultural waste material as a feedstock for bio-energy production.

Bon bogori and Mojare the rapid growing trees found in subtropical and tropical regions of world. Both these plants are common flora in Assam and other states of North-East India. Local tribal people and villagers traditionally preferred it as fuel wood.The major advantage of using such kind of biomass is its availability and there will be no upheaval for collection and formation of bio-refinery.

The present study deals with few important characteristics of lignocellulosic biomasses which are significant for biofuel production, e.g. crystalinity, thermogravimetric analysis (TGA) and Fourier transformation infrared (FTIR) study etc.

2.2 Materials and methods

The biomass samples used in this study were collected from Kamrup district of Assam. The detail nomenclatures of biomass are illustrated in Table 2.1. The air-dried samples of biomass were chopped and sieved (mesh size - BSS 30) to get homogeneous powder (diameter of particles lower than 5mm). The powdered biomass samples were used as a starting material for characterization. The characterization scheme of biomass samples is shown in Fig. 2.2.

Table 2.1. Botanical nomenclature of biomass samples

		Botanical Nomenclature		
Common name	Class	Family	Genus	Scientific name
Arecanut husk	*Liliopsida*	*Arecaceae*	*Areca*	*Areca catechu*
Moj	*Magnoliopsida*	*Leguminosae*	*Albizia*	*Albizialucida*
Bonbogori	*Magnoliopsida*	*Rhamnaceae*	*Ziziphus*	*Ziziphusrugosus*

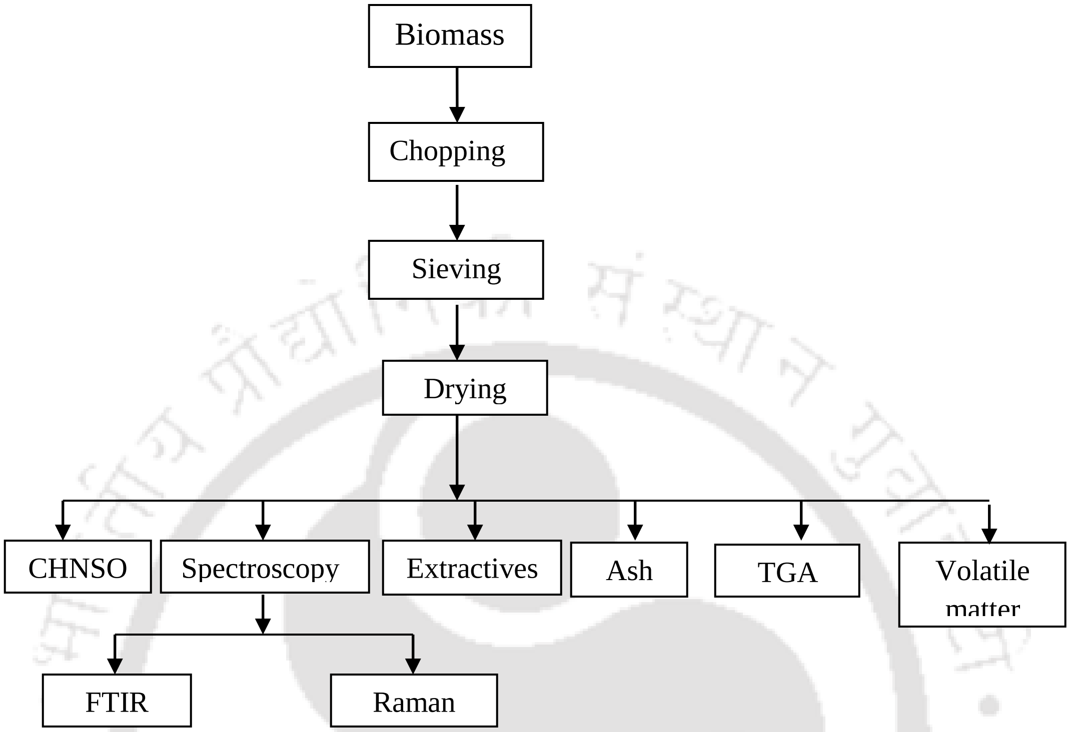

Fig. 2.2. Scheme for characterization of biomass samples

2.2.1 Physical and chemical characterization of biomass

2.2.1.1 CHNSO analysis

The basic elements of any biomass are carbon, hydrogen, nitrogen, sulphur and oxygen. The sample (1.0 mg) was used in a tin boat assortment for percentage composition of carbon, hydrogen, sulphur and nitrogen analysis and the percentage oxygen was determined by means of difference.

2.2.1.2 Ash content

The inorganic materials present in the biomass material are labeled under the inclusive term “Ash”. The ash materials can be classified into three broad groups, water soluble(free ionic form), organic solvent soluble and precipitated as relatively pure compounds i.e. crystalline or amorphous forms [8]. The inorganic materials loosely adhered with the biomass are known as extractable ash content. These are easily detached from the biomass by simple extraction or washing techniques. Extractable ash is the result of soil remaining in the biomass. Structural ash materials are adhered with biomass strongly**.** The cations (K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+}) present in the ash has a positive inhibition effect on enzyme, which helps to hydrolyze the cellulosic substrates into glucose and other readily fermentable sugars [9]. Therefore, the estimation of ash present in the biomass is necessary before processing the biomass for biofuel production. The procedure to estimate the amount of ash present in biomass was adopted from the NREL protocol [10]. 2 (±0.2) gm of biomass was put into an oven dried moisture free crucibles, and heated up to 575 ± 25 °C in a muffle furnace for three hours. The dry weight of ash content in biomass samples were calculated using the following Eq.s (2.1) and (2.2).

$$Dry\ weight\ of\ the\ sample = \frac{Weight\ (air\ dried\ sample) \times Total\ solid\ (\%)}{100} \quad (2.1)$$

$$Ash\ (\%) = \frac{weight\ (ash+crucible) - weight\ (crucible)}{dry\ weight\ of\ the\ sample} \times 100 \qquad (2.2)$$

2.2.1.3 Moisture content

The moisture content in biomass material is the amount of water present in the lignocellulosic biomass fiber. Quantification of moisture present in biomass is necessary because biomass materials are hydrophilic in nature, adsorption of moisture leads to lower the energy density present in it [11]. The moisture content of the biomass was determined using the method described elsewhere [12]. The moisture content was calculated by using Eq. (2.3).

$$\text{Moisture (\%)} = 100 - \left(\frac{Weight_{dry\ sample}}{Weight_{\ sample\ as\ received}} \times 100\right) \qquad (2.3)$$

2.2.1.4 Determination of calorific value

The calorific values of all the biomass sampleswere determined in a static bomb calorimeter; sealed Parr 1108, using the procedure described in ASTM manual [13].

Each biomass sample was mixed thoroughly in the sample bottle and converted into pellet, taking care that theheaviesandlights (fluff)weredistributed well in the sample and then send for analysis. Approximately 1.0 g of sample in the form of pellet of each species were measured out and put into pre-weighted crucibles. A cotton thread was attached to a platinum ignition wire and placed in contact with the pellet. Approximately 1mLof distilled waterwas poured into the bomb and then filled with oxygen to a consistent pressure between 20 and 30 atm (2.03 and 3.04MPa). The calorimeter was placed in an isothermal jacket with an air gap separation of10 mm between all surfaces. The electrical energy for ignition, discharged through a platinum wire of ~40 V, was calculated using the change in potential across a 1256 or 2900 mF capacitor. Thebombcalorimeterwassubmergedinacalorimeter canefilled with distilled water. The calorimeter jacket was maintained at constant temperature by circulating water at 25°C.

2.2.1.5 X-ray diffraction analysis

The enzymatic hydrolysis of lignocellulose is affected by many factors. The limiting factors have been traditionally divided into two groups: those related to lignocellulose structural features and those related to the mechanism and interactions of the cellulolytic enzymes. However, the heterogeneous nature of lignocellulose and the multiplicity of enzymes make it difficult to fully understand the interactions between enzyme and lignocellulose; furthermore, the interactions change as hydrolysis proceeds. Therefore, it is apparent that the rate and extent of biomass hydrolysis are inextricably linked to biomass structural features. Generally, structural features can be categorized into two groups: physical and chemical. Physical structural features include cellulose crystallinity, degree of cellulose polymerization, pore volume, accessible surface area, and particle size. It is broadly accepted that highly crystalline cellulose is less accessible to cellulase attack than amorphous cellulose; therefore, crystallinity affects the efficiency of enzyme contact with cellulose. Cellulose crystallinity are key factors that determine biomass digestibility.

The strong crystalline arrangement of cellulose retards enzyme hydrolysis of polymeric sugar molecules, which leads to lower yield of fermentable sugar as well as ethanol [14]. The crystalline structure of cellulose was first established by Carl Von Nägeliin 1858 [15]. Crystalline regions are formed due to the hydrogen bonds between cellulose chains and Van- der Waals forces between the glucose molecules in cellulose and hemicellulose[16]. Therefore, a decrease in cellulose crystallinity influences the initial rate of cellulose hydrolysis by cellulase enzyme[17]. Cellulose crystalinity was determined by wide angle X-ray diffractometer (Brucker D8 Advanced X-ray diffraction measurement system). Lignocellulose samples were transferred to the glass sample holder and analyzed under plateau conditions. The radiation was generated with an accelerated voltage of 40 KV and a current of 40 mA. The scan scope was between 7° to 40° with a step size of 0.02° with determination time 1sec / 0.02°. Crystalinity index along with the intensity of the main crystalline plane (002) and the amorphous fraction was calculated using the intensity at ~18°of 2θ for amorphous fraction and maximum intensity of (002) plane at ~22.8° of 2θ for crystalline fraction [18]. The crystalinity of the biomass samples were determined using Eq.2.4 as stated below,

$$CRI = \frac{(I_{002} - I_{amor})}{I_{002}} \times 100 \qquad (2.4)$$

where,

CRI = Crystalinity Index

I_{002} = Intensity at 22.8°

I_{amor} = Intensity at 18°

2.2.1.6 FTIR analysis

Fourier transform infra-red spectroscopy (FTIR) is capable of providing accurate predictions of the chemical composition of biomass. An FTIR spectrum contains information on the fundamental vibrations and has more separation between similar signals, e.g., hydroxyls for carbohydrate isomers [19].FTIR study of the subjected biomass samples were carried out by dispersing the dried (50 °C for 24 h) powdered samples (2 mg) of the fibers of biomass on IR grade potassium bromide (200 mg). Shimadzu, IR Affinity 1 was used to obtain the FTIR spectra with a resolution of 1 cm^{-1}.The reproducibility of the spectra of all the samples was verified on two sample preparations; from 64 to 100 scans with a resolution of 4 cm^{-1}. Baseline and other necessary corrections for penetration depth and frequency variations were applied using Shimadzu IR solution 1.5 software supplied with the equipment.

2.2.1.7 Thermogravimetric analysis

Thermogravimetric analysis provides a rapid method for determining the temperature assisted decomposition profile of a material and the kinetics of its thermal decomposition. The principle composition of biomass materials includes cellulose, hemicellulose and lignin. Several researchers reported that thermal decomposition of biomass samples follow first order reaction kinetics and therefore the decomposition rate of biomass is directly proportional to the concentration of its individual components [20, 21]. It was also observed that, TGA study helps to estimate approximate weight percentage of the cellulose, hemicellulose and lignin in biomass samples, as explained in the previous section.

The prepared biomass samples were stored in air tight plastic containers until they were needed for the TG analysis. The procedure used for TG analysis is the same as described by Ergudenler and Ghaly (1991)[22]. The sample sizes of all the three biomass samples were kept almost same 10 ± 0.5 mg. TG analysis of all the three biomasses was carried out in METTLER TOLEDO, Switzerland (Model no TGA 851e/LF/1100) at the heating rate of 10 °C min^{-1} under nitrogen atmosphere. The continuous records of weight loss vs. temperature data was obtained and analyzed through the software (STAR) provided by the manufacturer of the TGA.

The intensity of degradation at higher temperature is an important character of lignocellulosic biomass. Presence of more sugar than lignin in fibres of biomass materials, leads rapid degradation and formation of gases such as CO_2 and CO etc.

Therefore the researchers' works on biomass gasification accentuate more on value of T_{50} (temperature at level of 50% weight loss) and R_{50} (rate of weight loss at T_{50})because higher the value of R_{50} indicates rapid consumption of biomass in gasifiers. The study presents these two parameters for each biomass separately.

2.2.1.7Determination of extractives

Extractives present in biomass comprised of non-structural aromatic compounds, which possess one or more phenolic hydroxyl groups [23]. These components are different types of fatty acids, resins, tannins etc; all these materials have a prominent effect on analysis of structural carbohydrates and lignins. The knowledge of the amount of extractives helps to estimate exact amount of fermentable sugars present in biomass. For estimation of extractives in the biomass samples NREL protocol was used [24].The biomass samples (5 gm) were extracted using soxhlet extraction apparatus through extraction thimbles (single thickness, cotton cellulose thimble with external length 94 mm and ID 33 mm). Three stages of extraction; n-hexane, followed by ethanol (95%) and distilled water was performed and each extraction operation was executed for 6 h. In first step, the extraction of n-hexane soluble compounds was carried out to separate the class of compounds such as non-polar lipids, hydrocarbon compounds and terpenoids, etc. The raffinated biomass of the first step was extracted with ethanol to separate compounds such as chlorophyll, polar waxes, sterol and other minor constituents. The final raffinate biomass was extracted with distilled water to separate inorganic materials and non-structural carbohydrates [25]. The solvent in each extract was removed in rotary evaporator under reduced pressure.

2.2.1.8 Lignin, cellulose and hemicellulose content

The lignin, cellulose and hemicellulose content in the biomass samples were determined using thermogravimetric analysis (TGA). The biomass samples were heated under nitrogen atmosphere at temperature range between 30 °C to 700 °C with heating rate of 10 $°Cmin^{-1}$. The data generated during the experiment were analyzed through STAR software and a profile of weight loss vs. temperature was obtained for each biomass sample.

The hemicellulose and cellulose part of biomass usually start to decompose at around 206 °C and 315 °C and decomposition of lignin start decomposing above 330 °C respectively [26].The thermal decomposition study conducted on rapeseed (*Brassica napus L),*sponge ground fibers, sugarcane bagasse banana fibers by several researchers reveals that the thermal decomposition of hemicelluloses occurred at temperatures ranging from 150 °C to 350 °C, cellulose was decomposed at temperature range of 275 °C - 350 °C, and lignin was featured by gradual decomposition for the temperatures between 250 °C and 500 °C [27]. The present study follow the same approach to estimate lignin, hemicelluloses and cellulose content of the three biomass samples.

2.3 Results and discussion

The utility and significance of the three biomasses were delineated in previous sections. The detail physical and chemical properties with respect to biofuel production are discussed as follows.

The proximate and ultimate analyses of all the biomass samples were elicited in Table 2.2. The volatile matter present in Areca nut husk is more but it had less amount of fixed carbon content than other two biomass materials. The volatile matter comprises of hydrogen, oxygen and carbon whereas fixed carbon comprises only carbon. The results of ultimate analysis revealed that the oxygen content in Areca nut husk is more than Bonbogori and Moj. The higher amount of oxygen in Arecanut husk is responsible for the higher volatile matter content (Table 2.2) but lowers the calorific value. The calorific values of Areca nut husk (17.83±0.5 MJ kg^{-1}), Bonbogori (21.24±0.5 $MJkg^{-1}$) and Moj (20.35±0.2 $MJkg^{-1}$) was determined using the method described in previous sections. All the biomass studied here have lower sulphur content, which implies less amount of hydrogen sulfide gas production or SO_x emission during gasification process; which paves the way to use this biomass as a feedstock for gasification purpose too [28].

Table 2.2 Proximate and ultimate analysis of biomass samples

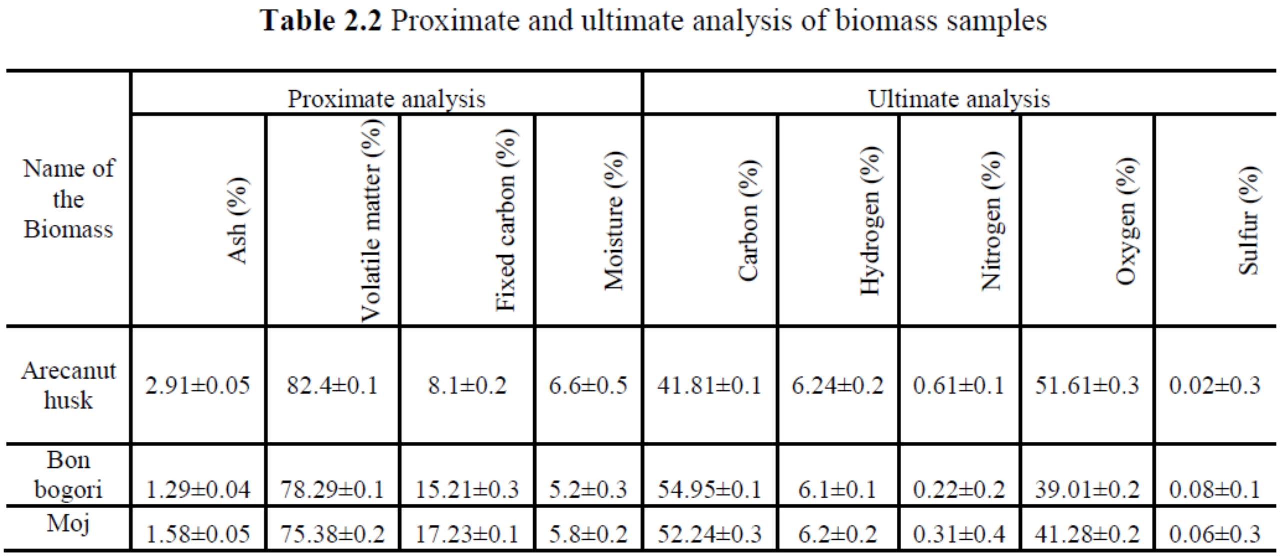

Table 2.2 Proximate and ultimate analysis of biomass samples

Name of the Biomass	Proximate analysis				Ultimate analysis				
	Ash (%)	Volatile matter (%)	Fixed carbon (%)	Moisture (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Oxygen (%)	Sulfur (%)
Arecanut husk	2.91±0.05	82.4±0.1	8.1±0.2	6.6±0.5	41.81±0.1	6.24±0.2	0.61±0.1	51.61±0.3	0.02±0.3
Bon bogori	1.29±0.04	78.29±0.1	15.21±0.3	5.2±0.3	54.95±0.1	6.1±0.1	0.22±0.2	39.01±0.2	0.08±0.1
Moj	1.58±0.05	75.38±0.2	17.23±0.1	5.8±0.2	52.24±0.3	6.2±0.2	0.31±0.4	41.28±0.2	0.06±0.3

The results showed that Areca nut husk has higher percentage of ash content compared to other two biomasses under study. The ash content retards the enzymatic hydrolysis i.e. saccharification of biomass samples. Therefore the Areca nut husk as a lignocellulosic stock material for biofuel production demands thorough washing to remove water soluble and loosely adhered inorganic materials along with a customized pretreatment method to reduce ash content.

The removal of moisture from all the biomasses started at around 70 °C (due to evaporation of water) and maximum moisture removal was taken place at 105 °C. It was very difficult to remove all the moisture associated with the fibers of the biomass due to the hydrophilic nature of lignocellulose; therefore, a minor amount of moisture can always be detected.

Extractives derived from lignocellulosic materials include resins, terpene acids, waxes etc, which protect plants from termites and other microorganisms. These materials are chemically polar and non-polar in nature and can be used as green chemicals so have a value other than just for biofuel production. Therefore this compound needs to be extracted before processing the lignocellulosic biomass as a biofuel feedstock. To remove non-polar elements hexane and ethanol extraction process was performed. It was observed that Bonbogori contained more amounts of non-polar materials than Areca nut husk and Moj. The final raffinate after solvent extraction processes was extracted with hot water. This step facilitated to extract polar materials present in the residual biomass sample. The water extractives were more in Moj than Areca nut husk and Bonbogori. The total amount of extractive content was more in Bonbogori followed by Moj and Areca nut husk (Table 2.3).

Table 2.3. Extractive (g) from 100 g of biomass samples

Name	Hexane extract	Ethanol Extract	Water Extract	Cellulose	Hemi-cellulose	Lignin
Areca nut husk	4.77±0.2	3.05±0.2	6.25±0.4	44.46±0.3	28.08±0.2	11.03±0.1
Bon bogori	8.11±0.1	7.75±0.3	2.98±0.2	51.56±0.3	8.73±0.1	20.51±0.1
Moj	5.21±0.1	3.35±0.1	7.61±0.1	53.46±0.1	6.37±0.2	22.97±0.1

The thermal decomposition study of all the biomass samples was evaluated using constant isothermal ramp program in TGA. The detail thermal decomposition behaviors of the three-biomass samples are illustrated in Fig. 2.3.

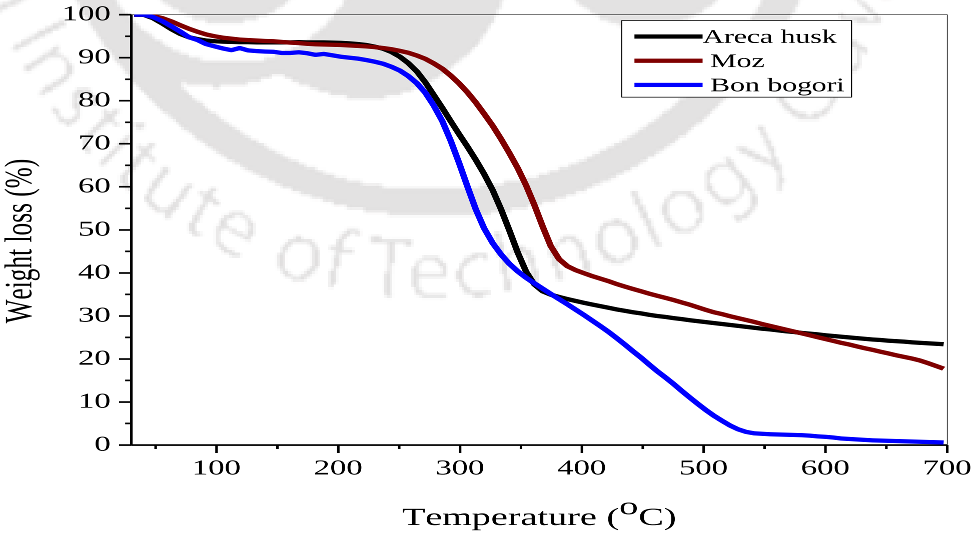

Fig. 2.3. Thermogravimetric analysis of biomass samples

The major degradation of Areca nut husk, Moj and Bonbogori observed at around 280 °C, 355 °C and 305 °C respectively (Fig. 2.3), which signified the degradation of structural sugar components. Differential thermogravimetric study (DTG) showed two major grooves for all the biomass samples (Fig. 2.4). The first one was observed within 300 °C – 400 °C range and the second one was at around 500 °C. The value of weight loss at the region between 250 °C – 350 °C signifies the amount of hemicellulose and cellulose respectively, the lignin degradation started at around 500 °C as described in previous section (Fig.2.4). The results of present study agrees well with the degradation pattern of other cellulosic fibers like banana, sugarcane bagasse and sponge gourd fibers, *Acacia mangium* and *Acaciahybrid* wood species reported by several researchers[29,30]. Cellulose, hemicellulose and lignin content of the biomass sample such as Moj estimated by thermogravimetric method is comparable with the values reported by Kakati and Konwer (2001) [31].

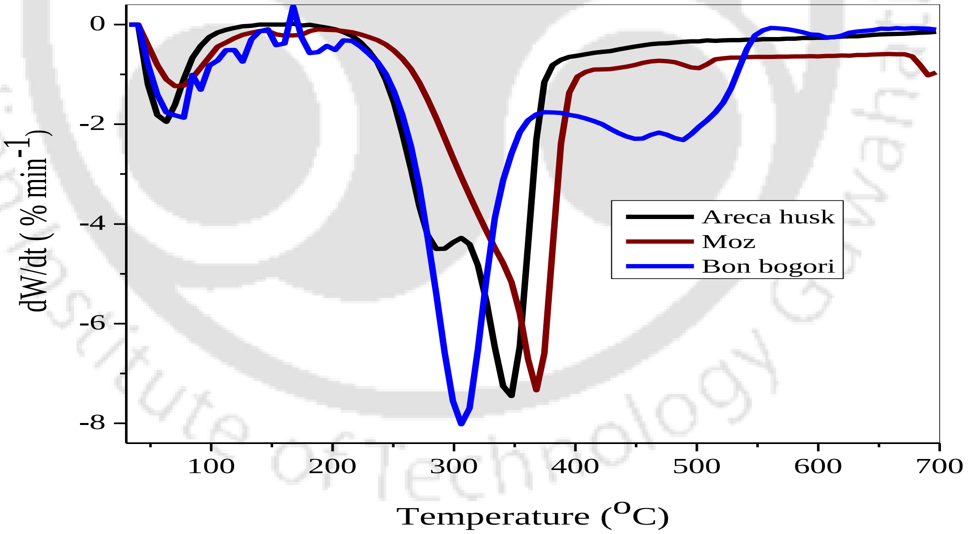

Fig. 2.4. Differential Thermogravimetric analysis of biomass samples

The comparative analysis of the value of T_{50} and $R_{50}$was shown in Fig. 2.5.Higher value of R_{50} indicates that the biomass was relatively active at its T_{50} value. It was observed that all the biomass had almost similar value of R_{50} (~ 7.2-7.4). The highest T_{50} value was observed in Moj (356 °C) followed by Areca nut husk (340 °C) and Bonbogori (320 °C). The reported value of T_{50} and R_{50} for oil palm shell, oil palm frond, rice husk, paddy straw found to be in between 363 °C - 410 °C and 2.9 -3.19 respectively [32].

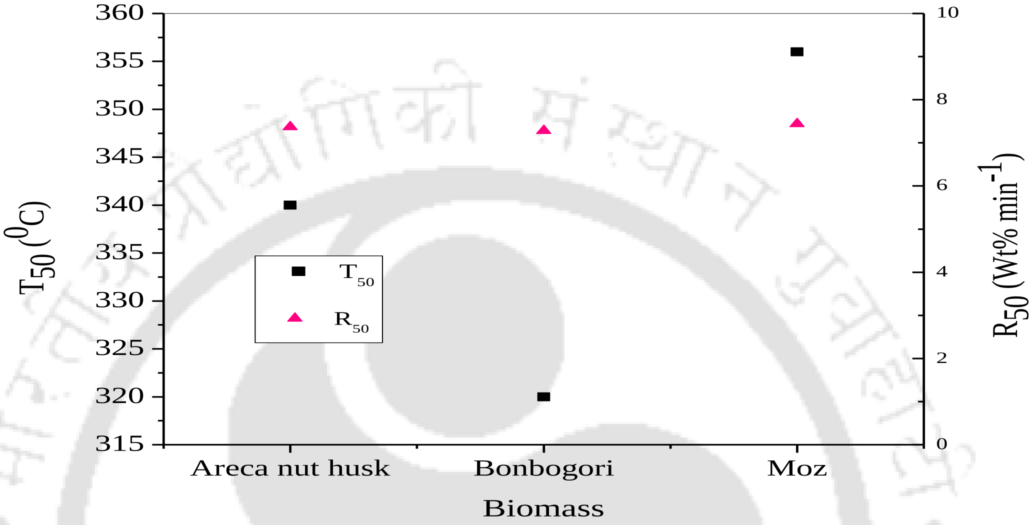

Fig.2.5. Outline of T_{50} and R_{50} of biomass samples

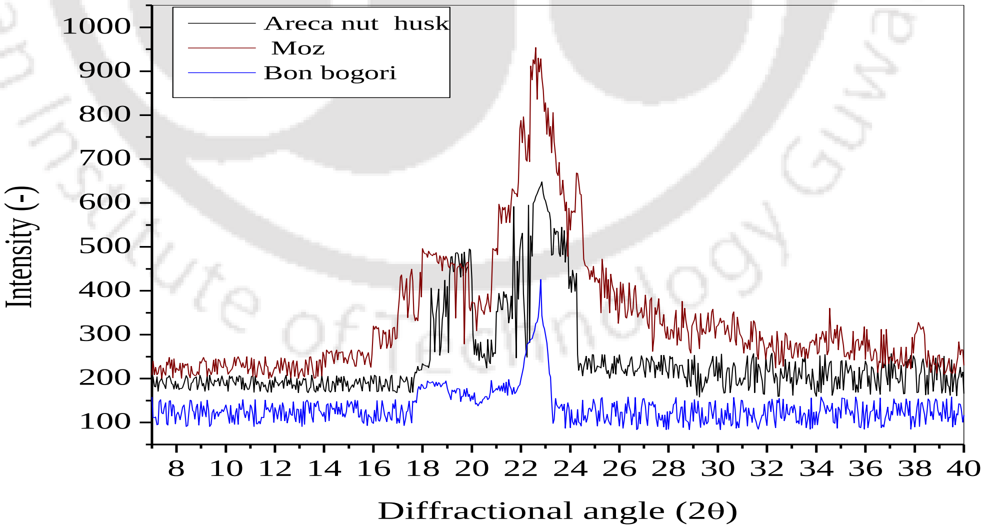

Fig. 2.6. X-ray diffraction analysis of biomass samples

The overall crystalinity of biomass depends on the matrix of lignin, cellulose and hemicellulose content[33]. Some researchers reported that wax (high molecular mass hydrocarbons and fatty acids) also affects the crystalinity of the biomass [34]. The maximum crystalinity was observed in Areca nut husk fiber (63.84%) followed by Moj (46.43%) and Bonbogori (42.46%) (Fig. 2.6).The results showed that Areca nut husk is less prone to enzymatic digestion than other two biomass materials. It can be noted that crystalinity and values of T_{50} and R_{50} of all the three biomass are reported for the first time in this study.

The fingerprint regions of the FTIR graph are illustrated in Fig 2.7. The main characteristics were attributed to the presence of different concentration of lignin, hemicellulose and cellulose present in the fibers. The peaks in the fingerprints are assigned in Table 2.4. The lowest transmittance was shown by Bonbogori followed by Moj and Areca nut husk in the range 2500-3500 $cm^{-1}$and 550 cm^{-1}-1700 cm^{-1}. This range represents the hydrogen bonded stretching bands of OH group, which are, suppose to come either from the glucoside linkages of cellulose or the hydroxyphenyl, guaiacyl and syringyl groups of lignin [35]. The Areca nut husk showed the highest transmittance among all the three biomass samples in the range of 1700 cm^{-1} -1730 cm^{-1}, which signifies the density of hemicellulose is more in Areca nut husk fiber than the lignocellulosic fibers of Moj and Bonbogori.

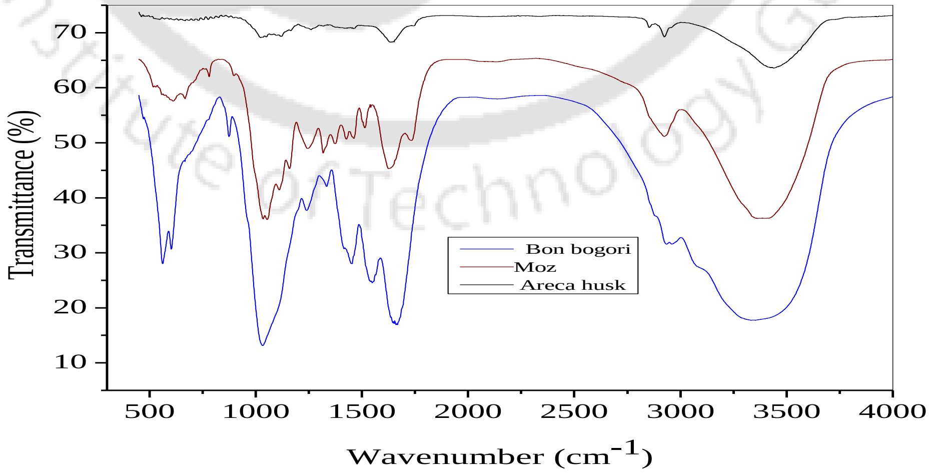

Fig. 2.7. Fourier transforms infrared spectroscopy analysis of biomass samples

Table 2.4. Assignment of the FTIR bands of functional groups in lignocellulosic biomass samples

Name of characteristics group	Wave number (cm^{-1})
OH	3200-3600
C-H stretch of aliphatic structure.	2960-2850
C-O-C vibration in cellulose and hemicellulose.	~1158
C=O (un-conjugated Xylan).	1700-1730
C=O at C-3 and C–C of cellulose.	~1058
Aromatic ring stretch in lignin.	1500-1600
C-H (Crystalline and Amorphous cellulose).	~1429 and 893
C–H methyl and methylene groups	2930-2910
CH_2 cellulose, lignin.	1462-1425
C–H deformation in cellulose, and hemicellulose.	1384-1346

The band in the range of 1500-1700 cm^{-1} represents the aromatic ring stretch (which is mostly due to lignin as the biomass contains negligible amount of protein) [36]. C=C of aromatic skeletal vibrations in lignin appears in the region of 1500–1700 cm^{-1} and C–H symmetric and asymmetric stretching bands in the region of 2900 cm^{-1}. Several researchers proposed different peak pairs for representation of crystalinity and amorphous characters, attributed mainly by cellulose molecules, among them two pairs (~1430 cm^{-1} for crystalline and 893 $cm^{-1}$for amorphous) were chosen for the present study to compare which biomass is more susceptible for cellulosic hydrolysis [37]. The peak ratio (amorphous/crystalline) was found as follows: Bonbogori (1.79), Moj (1.23) and Areca nut husk (1.02) (Fig 2.7). Presence of amorphous cellulose content in lignocellulosic fiber signifies the easier enzymatic hydrolysis, as described in previous section. This result shows that Bonbogori is more amenable for enzymatic hydrolysis than Moj and Areca nut husk. These results antagonize with the results obtained by X- ray diffraction analysis of lignocellulosic materials as described in previous section.

An ideal biomass for bio-energy production should have high calorific value along with high cellulose and hemicellulose concentration than lignin and ash content.The physicochemical characterization of all the three-biomass samples showed that Bonbogori and Moj both have almost similar calorific value and structural sugar (cellulose and hemicellulose) content but Moj is more accomplishable than Bonbogori due to its high cellulose and comparatively lower lignin content. The Arecanut husk was not apposite as a feedstock for bio-energy production due to its high ash and lower cellulose content. However, selection of suitable feedstock for bio-energy production based on mere physicochemical characterization is not a wise decision, several factors like availability, gross production volumes should be accounted for selection of biomass for biofuel production. The study of all these biomass from North-East India ensures that these biomasses can be utilized as a feedstock material, irrespective of the route of biofuel production process such as gasification or fermentation to meet the demand of second-generation biofuel.

2.4 Conclusions

The present study suggested that Areca nut husk, Moj and Bonbogori could be used as a source of lignocellulosic biomass for biofuel production. These lignocellulosic materials can compete favorably with other conventional sources such as sugar cane bagasse or corn for biofuel production. Lack of knowledge about the indigenous tree and shrub species located in the forests at different provinces of North-East India retards setting up biomass based energy plants. Preliminary information on physicochemical characterization of these biomasses surely opens opportunity of selecting future biofuel candidates in the North-East region of India. This is the first systematic report on characterization of lignocellulosic biomasses (Areca nut husk, Moj, Bonbogori) available in the provinces of North-East India.

References

[1]Garrett RH, Grisham CM. Biochemistry, 2010; 192-193.

[2] Heinze T, Liebert T. Unconventional methods in cellulose functionalization. Prog Polymer Science,2001;26:1689-762.

[3] Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulose biomass. BioresourceTechnology, 2009;100:10-18.

[4] Grabber JH. How do lignin composition, structure, and cross-linking affect degradability- A review of cell wall model studies. Crop Science, 2005; 45:820-831

[5] Jarimopas B, Niamhom S, & Terdwongworaku A. Development and testing of a husking machine for dry betel nut (*Areca catechu Linn*.). Biosystems Engineering,2009 ;102(1), 83-89.

[6] Nair, G. K. (Jan 26, 2009). The Hindu Business Line.

[7] Narayanmurthy G, Ramachandra YL, Rai P, Manohara YN, & Kavitha BT. Areca husk: an inexpensive substrate for citric acid production. Indian Journal of Biotechnology,2008;7, 99-102.

[8] Patwardhan PR, Satrio JA, Brown RC, Shanks BH. Influence of inorganic salts on the primary pyrolysis products of cellulose. BioresourceTechnology, 2010;101:4646-4655.

[9] Bin Y, Hongzhang C. Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw. Bioresource Technology, 2010;101:9114-9119.

[10] Determination of ash in biomass: laboratory analytical procedure. NREL/TP-510-42622; January 2008.

[11] Nimlos MN, Brooking E, Looker MJ, Evans RJ. Prepr. Pap. Am.Chem. Soc., Div. Fuel Chem, 2003;48:590-591.

[12] ASTM D 3173e3187. Standards test method for moisture in the analysis sample of coal and coke, in gaseous fuels. Coal and Coke; 1989.

[13] ASTM E711-87. Standard test method for gross calorific value of refuse-derived fuel by the bomb calorimeter; 2004.

[14] Rocha GJM, Martin C, Soares IB, Maior AMS, Baudel HM, Abreu CAM. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. Biomass and Bioenergy, 2011;35:663-670.

[15] Park S, Baker JO, Himmel ME, Parlla PA, Johnson DK. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. Biotechnology. Biofuel, 2010;3:10.

[16] Bansal P, Hall M, Realff MJ, Lee JH, Bommarus AS. Multivariate statistical analysis of X-ray data fromcellulose: a new method to determine degree of crystallinity and predict hydrolysis rates. Bioresource Technology,2010;101:4461-4471.

[17] Yoshida M, Liu Y, Uchida S, Ukagami Y, Kaneko S, Fukuda K. Effects of Cellulose Crystallinity, Hemicellulose, and Lignin on the Enzymatic Hydrolysis of Miscanthus sinensis to Monosaccharides. Biosci BioTechnology Biochem, 2008; 72(3):805–810.

[18] Segal L, creely JJ, Martin Jr, Canrad CM. An empirical method for estimating the degree of crystalinity of native cellulose using the X ray diffractometer. Textile Res Journal ,1959; 29:786-794.

[19] Kelley SS, Rowell RM, Davis M, Jurich CK, Ibach R. Rapid analysis of the chemical composition of agricultural fibers using near infrared spectroscopy and pyrolysis molecular beam mass spectrometry. Biomass Bioenergy, 2004; 27:77–88.

[20] Ledakowicz S, Stolerak P. Kinetics of Biomass Thermal Decomposition. Chemical Papers, 2002; 56(6):378-381.

[21] Saddawi A, Jones JM, Williams A, Wojtowicz MA. Kinetics of the Thermal Decomposition of Biomass.Energy Fuels, 2010; 24: 1274–1282.

[22] Ghaly AE, Ergudenler A. Thermal degradation of cereal-straw in air and nitrogen. J. Appl. Chemical BioTechnology, 1991; 27 (4): 111-126.

[23] Senelwa K ,Sims REH. Fuel characteristics of short rotation forest biomass. Biomass Bioenergy, 1999; 17:127–140.

[24] Determination of Extractives in Biomass; Laboratory Analytical Procedure (LAPNREL/TP-510-42619, National Renewable Laboratory, Golden, CO (2005).

[25] Naik SN, Goud VV, Rout PK, Kathlene J, Dalai AK. Characterization of Canadian biomass for alternative renewable biofuel. Journal of Renewable Energy, 2010; 35 (8): 1624–1631.

[26] Hornof V, Kokta BV, Valade JL, Fassen JL. Effect of lignin content on Thermal degradation of wood pulp. ThermodynamicaActa, 1977; 19: 63-68.

[27] Chen WH, Tu YJ , Sheen HK, Impact of dilute acid pretreatment on the structure of bagasse for bioethanol production. Int Journal of Energy Research, 2010; 34: 265–274.

[28] Kumar, A.; Jones, D.; Hanna, M. Thermochemical Biomass Gasification: A Review of the Current Status of the Technology Energies,2009, *2*, 556–581.

[29] Guimaraes JL, Silva CG, Satyanarayana KG. Characterization of banana, sugarcane bagasse and sponge gourd fibers of Brazil. Jr of InduCrop Product, 2009; 30: 407–415.

[30] Khalil HPSA, Khairul A, Bakare IO, Bhat IU. Thermal, spectroscopic, and flexural properties of anhydride modified cultivated Acacia spp. Wood Science Technology. 2010.

[31] Kakati R, Konwer D. Fuelwood characteristics of some indigenous woody species of North-East India. Biomass Bioenergy, 2001; 20:17-23.

[32] Abdullah SS, Yusup S. Method for screening of Malaysian biomass based on aggregated matrix for hydrogen production through gasification. J. Applied Sci ,2010; 10: 3301-3306

[33] Meshitsuka G, Isogai A. Chemical structures of cellulose, hemicellulose and lignin. In: Hon DN, editor. Chemical modification of lignocellulosic materials. New York: Marcel Dekker Inc; 1996: 11–34.

[34] Zhang LH, Dong L, Wang LJ, Wang TP, Zhang L, Chen XD. Effect of steam-explosion on biodegradation of lignin in wheat straw. Bioresource Technology, 2008; 99:8512–8515.

[35] Mothe CG and Miranda IC. Characterization of sugarcane and coconut fibers by thermal analysis and FTIR. J Therm Anal Calorim,2009; 97:661–665.

[36] Pandey KK, Nagveni HC, Rapid characterisation of brown and white rot degraded chir pine and rubberwood by FTIR spectroscopy. Erjrof wood products, 2007; 65**:** 477-481.

[37] Ciolacu D, Ciolacu F,Popa. Amorphous cellulose structure and characterization.CelluloseChem Technology, 2011; 45(1-2):13-21.

Chapter-III

Pretreatment of Lignocellulosic Biomass

To improvecompetence of cellulose hydrolysis, pretreatment is requiredtoefficiently break its recalcitrant structure. Pretreatment is an important cost-driver of lignocellulose conversion to biofuel and animportant step prior to enzyme hydrolysis. It disrupts the plant cell wall network and partially separates the major polymer components (lignin, cellulose and hemicellulose). However, pretreatment of lignocellulosic materials may also result in the release of inhibitors and deactivators of the enzymatic hydrolysis of cellulose. Development of enzyme processes for hydrolysis of cellulose to glucose must reduce inhibition and deactivation effects in order to enhance hydrolysis and reduce enzyme usage. Therefore, great attentions have paid design of pretreatment technologies to split recalcitrant characteristics of lignocellulose. In thisstudy,two types of pretreatment viz. dilute acid and ultrasound-assisted lime pretreatment was conducted over the Areca nut husk, Bonbogori and Moj. The study includes optimization of both the pretreatmentprocess using Taguchi methodology.

3.1 Introductionand review of literature

The complex structure of lignocelluloses acts as a protecting barrier of plants cell wall from attack by potential pathogenic organisms and purveys resistance against mechanical forces. The digestibility of cellulose or other polysaccharides present in lignocellulosicbiomasses into monomers or the fermentable sugars is hindered by many chemical, structural or physiochemical factors.Modern biotechnology research has designed enzymes or the cocktail of several enzymes to hydrolyze rawlignocellulosic material to yield monosaccharides but the process is time consuming and not economically feasible technology. To accelerate the hydrolysis process scientists are interested to incorporate an additional step, which is known as “Pretreatment”. Pretreatment process rapidly disintegrate the lignocelluloses to its mother components i.e. lignin, cellulose and hemicellulose. Therefore, the primary step towards bio-fuel production from lignocellulosic biomass is pretreatment. This step facilitates enzymatic hydrolysis process by altering the structural features, increasing surface areas and porosity of lignocellulosic biomasses. In general, the objectives of the pretreatment process are as follows,

1. Production of reactive cellulose fiber, which is suitable for enzymatic hydrolysis
2. Reduction of byproducts formation like furfural
3. Preserve the hemicellulose fraction in biomass
4. Minimum energy requirement
5. Consumption of fewer chemicals
6. Reducing the cost of feedstock size reduction

The pretreatment process can roughly be classified into physical, chemical and biochemical pretreatment process(Fig. 3.1).

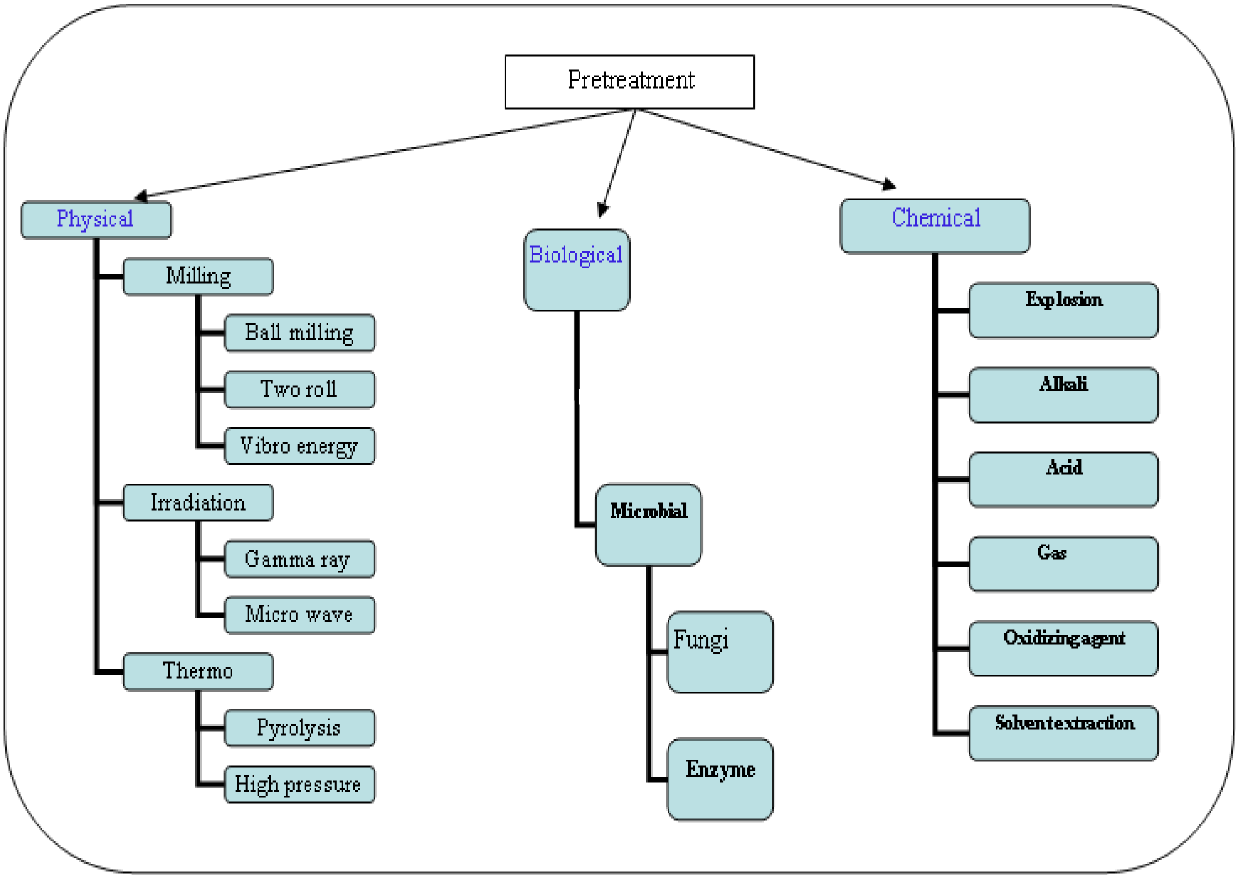

Fig. 3.1. Different methods of biomass pretreatment

Each pretreatment processhas their own advantages and disadvantages.Table 3.1 shows the advantage and disadvantages of different pretreatment processes. Among all the pretreatment processes the chemical pretreatment process results in rapid and good yield of sugar after enzymatic hydrolysis. The biological pretreatment sounds good but the slower rate of conversion and cost of enzymes does not make the process popular. Beside irradiation, other physical pretreatment processes demands high energy input to make the pretreatment process successful. The present study focused on two pretreatment processes to achieve high product yields in subsequent enzymatic hydrolysis and fermentation operations. The dilute sulfuric acid pretreatment process and ultrasound assisted limepretreatment process was conducted and compared accordingly.

Table 3.1. Comparison of different pretreatment process

Pretreatment		Advantages	Disadvantages	References
Chemical process	Dilute acid	Operation time is less, High yield of pentose sugar	Acid Recovery and formation of furfural	[1-2]
	AFEX	High yield of pentose sugar; No inhibitory compounds	Recovery of ammonia is not effective. Less effective process with increasing lignin content	[3-4]
	Lime	No Inhibitory compounds	Operation time is more	[5-6]
	Organosolvosis	High yield of pentose sugar	Solvent recovery is expensive	[7-8]
Physical process	Milling	Operation time is less	The overall yield is poor. Energy requirement is high	[9-10]
	Irradiation	High yield of sugar; No inhibitory compounds	Need special design of equipment and process	[11-12]
	High pressure	No Inhibitory compounds	Maintaining high-pressure itself is a challenge	[13]
Biological process	Microorganisms	Low energy requirement No production of inhibitory compound Mild operation conditions	Rate of reaction is slow	[14-15]

3.2 Dilute acid pretreatment process

For any pretreatment procedure, it is highly pertinent and presumably useful to elucidate the chemical-physical state of the biomass, especially the chemical structure and local mobility, and, hence, entanglement of the major chemical components of the biomass during or at least at the end of the pretreatment process. Acid pretreatment method includes application of dilute or concentrated acids to the lignocellulosic biomass material, which facilitates breaking the rigid lignocellulosic matrix. The most commonly used acid is dilute sulphuric acid (H_2SO_4). Dilute acid used to hydrolyze the hemicellulose fraction and increase the porosity of biomass, which will make the cellulose more accessible to enzymes. An advantage of the dilute acid pretreatment processes is that sugars hydrolyzed from hemicelluloses can be recovered. Upon treatment with dilute sulfuric acid, in addition to the hydrolysis/de-polymerization of hemicellulose, one might expect that a part of ether linkages are cleaved, and under severe enough treatment conditions, the cleaved linkages form other linkages, i.e., lignin re-condensation.

It is reported that more ether linkages are cleaved by acid treatment with increased temperature but that above ~130 °C, this treatment also results in lignin re-condensation, such that a maximum in the loosening of the biomass occurs between 120 and 130 °C. Therefore, the dilute acid pretreatment method may be defined as exposure of high temperature (e.g. 180 °C) during a short period of time; or at lower temperature (e.g. 120°C) for longer retention time (30–90 min) to the lignocellulosic materials [16]. At present, the dilute sulphuric acid treatment is receiving substantial attention over concentrated sulfuric acid [17]. Recent articles have reviewed that the dilute sulphuric acid pretreatment process facilitates enzymatic hydrolysis of lignocellulosic biomass [18-19]. Dilute sulfuric acid pretreatment process has been successfully applied with different types of lignocellulosic biomasses such as Switch grass [20], Corn stover [21], and Poplar [22]. Olive tree biomass was pretreated with 1.4% H_2SO_4 at 210 °C resulting in 76.5% of hydrolysis yields and cashew apple bagasse pretreated with diluted H_2SO_4 at 121°C for 15 min yielded ethanol as high as 0.47 gg^{-1}glucose [23, 24]. Dilute acid hydrolysis has many advantages compared with concentrated acid such as less corrosion, shorter reaction times, and higher reaction rate [25]. Lignin is known as a nuisance material for ethanol makers as it retards enzymatic hydrolysis procedure. Beside lignin the native crystalline structure (cellulose I) of cellulose is considered to be one of the major factors limiting its potential in terms of cost-competitive lignocellulosic biofuel production [26]. Lu, Xuebin*et al.*, (2009) used Rapeseed straw as a lignocellulosic material which has 60% carbohydrate. A central composite design of response surface method was used to optimize H_2SO_4-catalyzed hydrothermal pretreatment of rapeseed straw, in respect to acid concentration (0.5–2%), treatment time (5–20 min) and solid content (10–20%) at 180 °C. Enzymatic hydrolysis and fermentation were also measured to evaluate the optimal pretreatment conditions for maximizing ethanol production. The results showed that acid concentration and treatment time was more significant than solid content for optimization of xylose release and cellulose recovery. Pretreatment with 1% sulfuric acid and 20% solid content for 10 min at 180 °C was found to be the most optimal condition for pretreatment of rapeseed straw for ethanol production. This study has demonstrated that hemicellulose in the rapeseed straw can be removed efficiently by H_2SO_4-catalyzed hydrothermal pretreatment at high solid loading (20%). Concentration of inhibitory product like furfural, were reported less in this process [27].

Noureddini*et al.*(2010)studieddistiller’s grain and corn fiber as the biomass for pretreatment process.The total carbohydrate content of distillers’ grains and corn fiber were 57.7 ± 2.0 and 77.0 ± 1.0 wt %, respectively. In this study, dilute sulfuric acid hydrolysis for the conversion of distillers’ grains and corn fiber to monomer sugars and the formation of furfural were investigated. The extent of solubilization of biomass beyond monomer sugars was also monitored. Biomass loadings in the range of 5–20 wt % at 5% intervals, acid concentrations in the range of 0.5–1.5 vol% at 0.5% intervals, and temperatures of 120 and 140 °C were studied. The experimental results confirmed an increasing trend in the formation of monomeric sugars as a function of time. The highest yields of monomeric sugars were observed at the lower substrate loadings (5 and 10 wt. %), higher concentrations of sulfuric acid (1.0 and 1.5 vol. %) and when the temperature was 140 °C [28]. For the majority of the cases under consideration, the most effective period of hydrolysis appeared to be during the initial 20–30 min of the reaction. Formation of furfural during the course of hydrolysis was significantly lower at 120 °C and also lower for the distillers' grains samples compared with the corn fiber samples. The total amount of the solubilized matter during the hydrolysis was significantly higher than the amount of the monomeric sugars. Shi. *et al.* (2012) investigated dilute sulfuric acid pretreatment of com straw and rice straw and enzymatic hydrolysis of cellulose [29]. The straw was pretreated at 121 °C with different sulfuric acid concentrations (1, 2, 3, 4and 5%, vol/vol.) and residence times (30, 60, and 90 min).Pretreatment residence time plays a key role in increase the glucose concentration comparing to sulfuric acid concentration. Cellulose remaining in the pretreated feedstock was highly digestible by cellulase from *Trichodermaviride*. The result showed that the saccharification yield was 72.38% and 82.84% from wheat straw and rice straw respectively. The pretreatment conditions were as follows acid concentration 2% (vol/vol),temperature 121°C and time 60 min. To produce ethanol cost-effectively from herbaceous feedstocks such as corn stover, efficient xylan hydrolysis with good amount of monomeric xylose yield isrequired. Dilute acid pretreatment is well established as one of the pretreatment technologies for xylan hydrolysis; however, the accumulation of salts, production of toxic byproducts, and the release of acetic acid can inhibit enzymatic saccharification and fermentation, which leads to decrease yield of ethanol. Successful removal of acetyl groups from native corn stover by alkali de-esterification could potentially increase yield of monomeric xylose from pretreatment and enzymatic hydrolysis, improve cellulose digestibility, and reduce the cytotoxicity of the fermentation broth. Chen et al., (2012) reported that the dilute acid pretreatment process removed significant amount of acetyl groups from Corn stover. Dilute acid pretreatment improvedsaccharification process ofxylan and glucan hydrolysis by 15% and 30% over control samples. In whole slurry enzymatic hydrolysis, a 30% improvement in cellulose digestibility was obtained over the control [30].

3.2.1. Theory of acid hydrolysis process

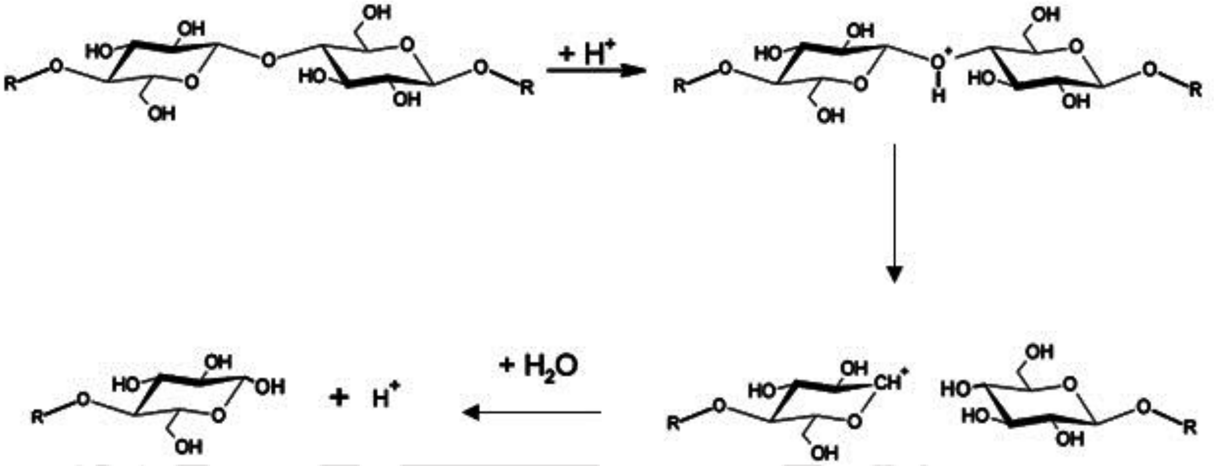

Fig. 3.2. Mechanism of acid hydrolysis of cellulose

The molecular mechanism of acid-catalyzed hydrolysis of cellulose (cleavage of β-1-4-glycosidic bond) follows the pattern outlined in Fig.3.2. The cellulosic substrate is made up of microcrystalline bundles attached to each other by para-crystalline regions and surrounded by a charged water boundary layer that results from dipole−dipole interactions caused by aligning of water molecules around the polar surface of the cellulose exterior [31]. Water molecules and H^+ ions (from acid) have to penetrate the cellulose fiber in order to precede the hydrolysis reaction effectively. Otherwise, hydrolysis takes place only on the surface of the cellulose. Acid catalyzes the breakdown of long cellulose chains to form shorter chain oligomers and then to sugar monomers that the acid can degrade. The hydrolysis of cellulose begins with the reaction of acidic proton and oxygen that bonds two glucose units, forming the corresponding conjugated acid. The cleavage of this C-O bond and breakdown of the conjugate acid to the cyclic carbonium ion then takes place, which adopts a half-chair conformation. Free sugar and a proton are liberated after rapid addition of water. The formation of the intermediate carbonium ion takes place more rapidly at the end than in the middle of the polysaccharide chain.

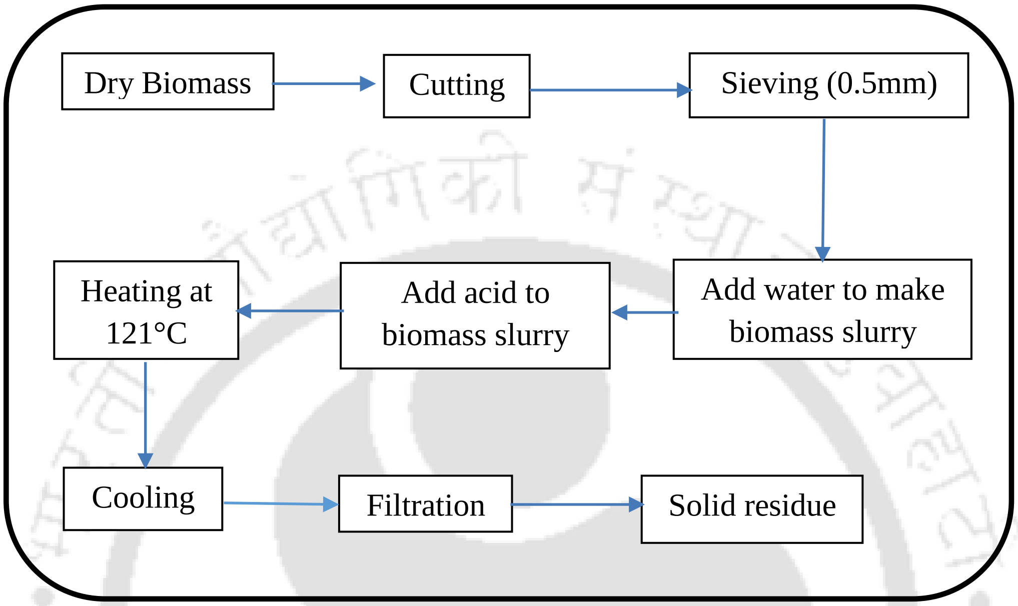

Fig 3.3. Process flow diagram of dilute acid pretreatment process

3.3 Ultrasound assisted lime pretreatment

Among the various existing pretreatment technologies, lime pretreatment has proven to be a useful pretreatment method because it demands less amount of energy and yields a good amount of fermentable sugar after enzymatic hydrolysis [32]. Moreover, it is a less expensive and non-hazardous chemical agent [33]. The beauty of lime pretreatment lies in the removal of lignin from biomass without any momentous loss of structural carbohydrate materials e.g. glucose, xylose, arabinose etc. It is also reported that alkaline pretreatments remove acetyl groups from hemicellulose, which improves saccharification by lowering steric-hindrance of enzymes [34]. The sole hitch of this pretreatment process is longer process operation time. Few researchers overcome this drawback by elevating the reaction temperature to curtail operational time [35]. However, lime can be more effective as a pretreatment agent at mild temperature as lime is more soluble at lower temperature, which leads to more alkalinity and may lead to more efficient pretreatment of lignocellulosic materials [36].

3.3.1 Theory of lime pretreatment

The major effect of alkaline pretreatment is delignification. Alkaline pretreatments successfully increase the lignocelluloses digestibility without the production of furfural and methyl furfural [37].The alkaline material reacts with biomass in three modes namely

1. Reaction with lignin
2. Neutralization of organic acids
3. Reaction with resins and waxes of biomass materials

Lignin can be described as three-dimensional macromolecules with high molecular weight in the range of 100 KD. It originates from phenyl-propanoid precursors such as coumaryl, coniferyl, and sinapyl alcohol C_6–C_3 and is present in vascular plants [38]. Depolymerization of lignin using alkaline materials (e.g. lime) depends on the cleavage of two types of aryl ether bonds: $C_{aliphatic}OC_{aromatic}$ and $C_{aromatic}OC_{aromatic}$(ordered from least to most stable). Oxidative agents prominently improve the effects of alkaline pretreatments. In alkaline media (pH>12) oxygen is reduced through the reaction with phenolic hydroxyl groups to superoxide radical (-O2•). Reactions involved in alkaline oxidative pretreatments are primarily single-electron (radical) reactions. Delignification reactions involve the formation of several different acids produced during the degradation of carbohydrates that introduce hydrophilic groups into the lignin structure [39]. Nucleophilic attack also occurs in some extend causing ring opening, which promotes further degradation and solubilization. The schematic diagram (Fig. 3.3) shows the typical delignification reactions (when OH^{-} anions involved).

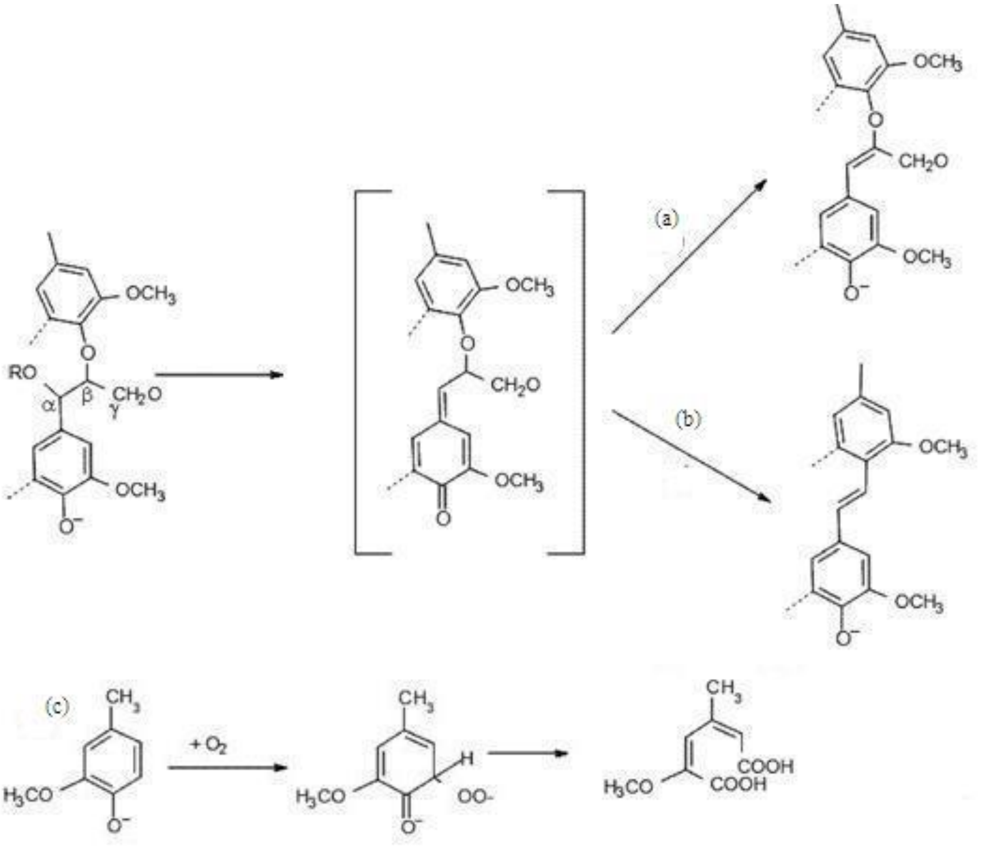

Fig. 3.4 Lignin degradation reactions in alkaline conditions involving - and -aryl ether linkages

In highly basic environment, acidic proton of phenols (in lignin) gave phenolateion. Then the phenolate ion eliminates the ether from benzylic position to produce an intermediate. This intermediate underwent two transformations either regain the aromaticity, or produce highly conjugated arylvinyl-phenol after cleavage of CH_2O group (Fig. 3.1 (a),(b)). In presence of molecular oxygen, phenolate moiety (Lignin) oxidized to 1,4-dicarboxylic acid which is soluble in basic solution(Fig. 3.1 (c)). Therefore, lime facilitates delignification steadily in presence of oxygen without any damage of the carbohydrates.

Rabelo*et al.* (2008) worked on pretreatment of sugarcane bagasse with lime (calcium hydroxide) followed by bioethanol production.Experiments were performed using the bagasse as it comes from an alcohol and sugar factory (non-screened bagasse) and bagasse in the size range from 0.248 to 1.397 mm (screened bagasse) (12-60 mesh).

It was observed that the particle size presented influence in the release of fermentable sugars after enzymatic hydrolysis using low loading of cellulase and β-glucosidase (3.5 $FPUg^{-1}$ dry pretreated biomass and 1.0 IUg^{-1} dry pretreated biomass, respectively) [40]. Chang *et al*. (1998) used lime as a pretreatment agent to enhance the enzymatic digestibility of two common crop residues: bagasse and wheat straw. A systematic study of pretreatment conditions suggested that for short pretreatment time (1-3 h), required high temperature (85-135°C) to achieve high sugar yields, whereas for long pretreatment times (e.g., 24 h), low temperatures (50-65°C) were effective. The recommended lime loading was 0.1 g $Ca(OH)_2g^{-1}$ dry biomass. Water loading had little effect on the digestibility. Under the recommended conditions, the 3-d reducing sugar yield of the pretreated bagasse increased from 153 to 659 mg Eq. $glucoseg^{-1}$ dry biomass, and that of the pretreated wheat straw increased from 65 to 650 mg Eq. $glucoseg^{-1}$ dry biomass. A material balance study on bagasse showed that the biomass yield after lime pretreatment was 93.6%. No glucan or xylan was removed from bagasse by the pretreatment, whereas 14% of lignin became solubilized. A lime recovery study showed that 86% of added calcium was removed from the pretreated bagasse by ten washings and could be recovered by carbonating the wash water with CO_2 at pH 9.5 [41]. Mass *et al.*(2008) reported a integrated pilot-scale process where lime-treated wheat straw with a high dry-matter content (around 35% by weight) is converted to ethanol via simultaneous saccharification and fermentation by commercial hydrolytic enzymes and bakers' yeast (*Saccharomyces cerevisiae*). After 53 hours of incubation, an ethanol concentration of 21.4 g lit^{-1} was detected, corresponding to 48% glucan-to-ethanol conversion of the theoretical maximum. The xylan fraction remained mostly in the soluble oligomeric form (52%) in the fermentation broth [42]. Cheng *et al.* (2010**)** used rice straw as a substrate for alkaline pretreatment.5 g H_2O g^{-1} straw using sodium hydroxide (NaOH) and compared to pretreatment at 10 g H_2Og^{-1} straw by hydrated lime ($Ca(OH)_2$).Reaction temperature was held constant at 95°C for lime pretreatment and 55°C for NaOH pretreatment. The range of delignification was 13.1% to 27.0% for lime pretreatments and was 8.6% to 23.1% for NaOH.Treatment at higher temperature also improved delignification; delignification with water alone ranged from 9.9% to 14.5% for pretreatment at 95°C, but there was little effect observed at 55°C. Post-pretreatment washing of biomass was not necessary for subsequent enzymatic hydrolysis. Maximum glucose yield was 176.3 mgg^{-1} dried biomass (48.5% conversion efficiency of total glucose) in lime-pretreated (unwashed biomass) and were 142.3 mgg^{-1} dried biomass (39.2% conversion efficiency of total glucose) in NaOH-pretreated (unwashed biomass) [43].

The carbon-carbon bonds are stable in alkaline conditions, the cleavage of oxygen-carbon bonds are the most significant reaction in the pretreatment at basic condition. This reaction will take place and is producing phenolic hydroxyl groups from the cleavage of the aryl-alkyl-ether bounds. Several researchers to explain the phenomena of delignification of lignocellulosic biomass materials [44] classified three different stages namely initial, bulk and residual phases. During the initial delignification stage in alkaline pulping with sodium hydroxide, phenolic α-O-4-linkages in lignin and some non-phenolic ß -O-4-linkages are cleaved. In the bulk stage, the major reaction is the cleavage of non-phenolic ß -O-4-linkages and at the residual delignification stage, carbon–carbon linkages in lignin are cleaved and carbohydrates are degraded.

3.3.2 Theory of ultrasonication

Ultrasound is sound with a sonic spectrum ranges between 20 kHz to 10 MHz therefore its pitch is above human hearing as human ear can detect up to 16KHz only. In practice, three ranges of frequencies are reported for three distinct uses of ultrasound (i) high frequency, for diagnostic purposes (2–10 MHz), (ii) low frequency or conventional power ultrasound (20–100 kHz), and medium frequency, or “sonochemical-effects” ultrasound (300–1000 kHz) [45]. There are two theory exists to explain the phenomena happened during ultrasonication process. Hot spot theory postulates the formation of micro-bubbles at the site of nucleation when high frequency sound waves (50-60 KHz) are applied to the liquid. The bubbles generate due to the decrease of boiling point caused by negative pressure and it collapses due to positive pressure created by the same wave. This alternating change of pressure is known as rarefaction. The positive pressure forced the bubble to implode which leads to formation of local hot spot with temperature and pressure around 5000K and500atm respectively [46]. Therefore, huge pressure and temperature, can be achieved which is required to break the polymeric biomass into their mother components without designing any high-pressure vessel. This method is not only cost effective but also safe to use.

The electrical theory explained the above phenomena of breaking bonds by means of generating huge electrical charges. The electrical double layer can be formed in any liquid on the surface of the cavitation bubbles at the zone of cavitations. This process is independent of the method of creation of cavitation [47].

Application of ultrasound technology exists in food processing technology for depolymerization of biopolymers, emulsification, tanning of vegetables etc [48]. Ultrasonic pretreatment of corn slurry was reported 20 fold reduction in corn size particle, which facilitated 30% more glucose yield at the time of enzymatic hydrolysis [49]. Cassava chips were pretreated using ultrasonic unit, which had maximum power output of 20kw and frequency of 20 kHz [50]. Ultrasonic assisted (250 W, 30 min) hydrogen peroxide pretreatment method followed by biological treatment (*Pleurotusostreatus*) was performed on rice hull yielded 31.8% and 32.2% more total sugar and glucose respectively than that of sole biological treatment [51]. Considering all the inputs from the literature, it was decided to combine the lime and ultrasound technique for the pretreatment of biomass.

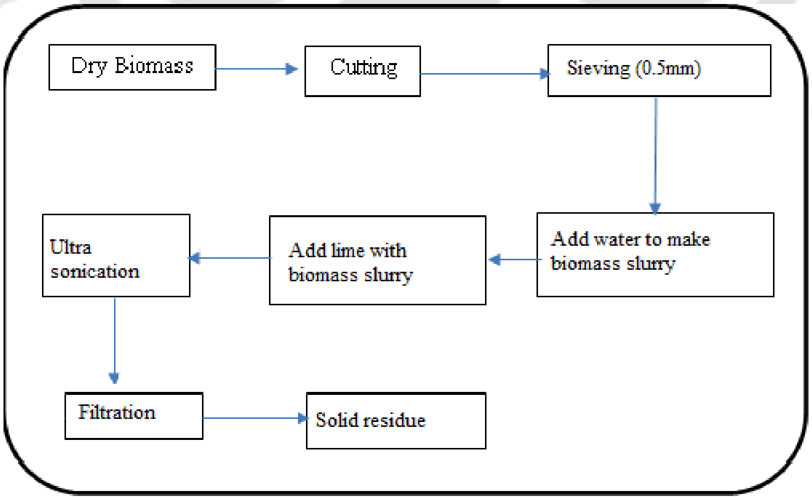

Fig 3.5. Process flow diagram of Ultrasound assisted lime pretreatment process

3.4 Materials and methods

All the biomass materials i.e. trunks of Bonbogori and Moj were collected from the local forest near IIT Guwahati and Areca nut husk were collected from the local market in Kamrup district of Assam. All the raw biomasses were air dried, milled, and finally screened to select the fraction of particles with a size lower than 0.5 mm, homogenized in a single lot, and stored in airtight polyethylene bags until needed. The present study was conducted fortwo pretreatment process i.e. dilute acid pretreatment process and ultrasound assisted lime pretreatment process.

3.4.1 Ultrasound Equipment

The biomasses mixed with lime were pretreated with the help of a probe type ultrasonic generator (Model UP 100H, Hielscher, Germany) with an operating frequency of 30 kHz. The amplitude maintained was 100%, and the temperature was controlled (35 ± 2 °C) using a water bath. The schematic of the experimental set up illustrated in Figure 3.4. The theoretical maximum intensity of this ultrasound equipment was 100 W. The actual energy input study of this ultrasound equipment was also determined. The energy study was conducted according to the method of calorimetry [52]. The input power was determined to be approximately 36 W.

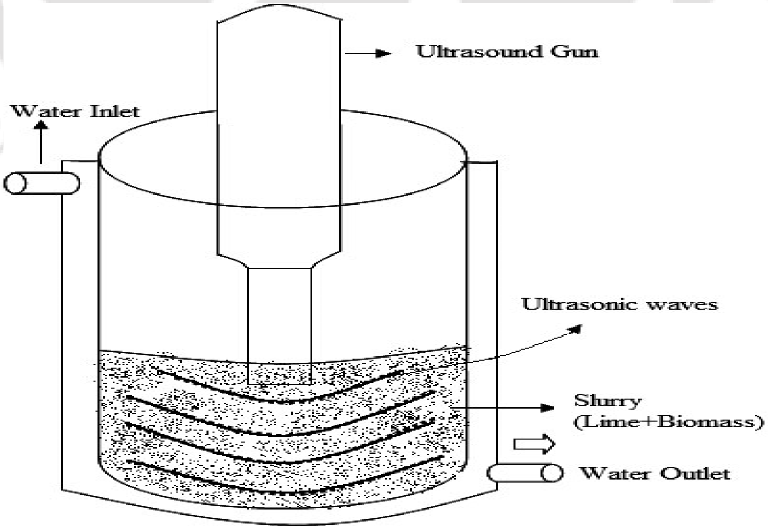

Fig. 3.6. The schematic diagram of experimental set up for ultrasound assisted lime pretreatment

3.4.2Experimental design and procedure

Based on the Taguchi method, an orthogonal array experiment (L9) was designed to determine the optimum conditions for both dilute acid and lime assisted pretreatment method.The experiments were designed with three factors in three different levels viz. sulfuric acid concentration(%; vol/vol), solid loading (g ml^{-1}) and time of operation (min)for dilute acid pretreatment process optimization (Table 3.2), and lime loading (weight of lime (g)/ weight of dry biomass (g), biomass loading (weight of water (g)/ weight of dry biomass (g)), duration of ultrasound irradiation (min) were chosen for ultrasound assisted lime pretreatment of biomasses respectively (Table 3.3).Each biomass samples were pretreated as per the design of experiments (DOE) illustrated in Table.3.4. Each row of the matrix represents one run. The three levels of each factor are represented by a "1" or a "2" or a "3" in the matrix.For analysis of the results and optimization of conditions for setting the control factors, MINITAB software was used. The dilute acid pretreatment experiments were conducted using 100 mL Erlenmeyer flask in autoclave at the designed conditions. The reaction volume of 50 mL was maintained for each experiment. The pressure of the autoclave for each experiment was maintained at 15 psi. After each set of experiment, the solid residues were collected and analyzed for the XRD. The crystalinity of the solid residue and the raw biomass was determined from the XRD graph, using Eq.2.1 (chapter 2).

The delignification index was used as the response for the ultrasound assisted lime pretreatment.After each set of experiments (ultrasound assisted lime pretreatment) the samples were collected and analyzed for the lignin content.The lignin content of the pretreated biomass material was measured by means of using NREL protocol. The dried samples were incubated with 72% sulphuric acid at 30 °C for an hour followed by dilution and autoclave at 121°C for 30 min. The residuals were collected by means of vacuum filtration followed by drying at 105°C for 24 h and subsequently weighed.

The delignification (fraction of lignin removed from biomass) index was calculated as per the Eq. (3.1).

$$Delignification = \frac{Ligninremovedintreatedbiomass\ (g)}{Ligninpresentinrawbiomass\ (g)} \qquad (3.1)$$

Table 3.2 Different Levels and factors of dilute acid pretreatmentof Moj, Bonbogori and Areca nut husk

Parameters	Level 1	Level 2	Level 3
Acid concentration (%)	0.5	1.5	3
Solid loading (%)	1	2.5	5
Time(min)	20	30	40

Table 3.3 Different Levels and factors of ultrasound assisted lime pretreatment of Moj, Bonbogori and Areca nut husk

Parameters	Level 1	Level 2	Level 3
lime loading (A)	0.25	0.5	0.75
biomass loading (B)	10	20	30
ultrasound duration (C)	60	120	180

Table 3.4 Design matrix (L_9 orthogonal array) for dilute acid pretreatment process: crystalinity index and S/N ratio

Acid concentration (%)	Solid loading (%)	Time (min)	Crystalinity (%)			S/N Ratio		
			Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj
1	1	1	56.92	46.21	53.21	-35.11	-33.29	-34.52
1	2	2	49.26	40.17	47.93	-33.85	-32.07	-33.61
1	3	3	59.84	50.71	62.81	-35.54	-34.1	-35.96
2	1	2	38.14	32.17	34.17	-31.63	-30.14	-30.67
2	2	3	42.21	35.56	44.68	-32.51	-31.01	-33.00
2	3	1	57.46	47.24	58.36	-35.19	-33.48	-35.32
3	1	3	31.68	23.14	26.64	-30.02	-27.28	-28.51
3	2	1	44.26	34.18	38.51	-32.92	-30.67	-31.71
3	3	2	36.47	28.19	32.19	-31.24	-29.1	-30.15

3.5 Results and discussion

3.5.1 Determination of optimal conditions using Taguchi robust design

Taguchi robust design method was used to identify the optimal conditions and to select the parameters having the maximum influence on both dilute acid and ultrasound assisted lime pretreatment process. Table 3.2 and Table 3.3shows the detail results of each run for each pretreatment process.

Table 3.4 shows the layout of the experimental design. Instead of conducting 27 (3^3=27) experiments each principle factor represents three levels i.e. acid concentration ranges from 0.5 to 3%, solid loading 1 to 5% and operation time 20 to 40 min. These factors along with their levels were selected and co-located by the L9 orthogonal array of Taguchi's method (Table 3.3).

Table 3.5 Design matrix (L_9 orthogonal array) for ultrasound assisted lime pretreatment process: delignification ratio and S/N ratio

Lime loading (%)	Biomass loading (%)	Time (min)	Delignification ratio			S/N Ratio		
			Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj
1	1	1	0.39	0.43	0.33	-8.18	-7.33	-9.63
1	2	2	0.51	0.55	0.44	-5.85	-5.19	-7.14
1	3	3	0.48	0.49	0.40	-6.38	-6.20	-7.96
2	1	2	0.52	0.52	0.49	-5.68	-5.68	-6.18
2	2	3	0.56	0.51	0.58	-5.04	-5.85	-4.73
2	3	1	0.41	0.48	0.36	-7.74	-6.38	-8.91
3	1	3	0.60	0.54	0.35	-4.44	-5.35	-9.02
3	2	1	0.57	0.61	0.54	-4.88	-4.29	-5.35
3	3	2	0.55	0.66	0.61	-5.19	-3.61	-4.29

Process operation with the highest S/N values ("Larger the better" characteristics) always results in the optimum quality with minimum variance or deviation. The aim of any design of experiment, therefore, is always to determine the highest possible S/N ratio for the experimental results [53]. Based on the result (Table 3.5) the S/N ratio of each parameter was calculated at each level as illustrated in (Fig.3.5 and Fig 3.6).

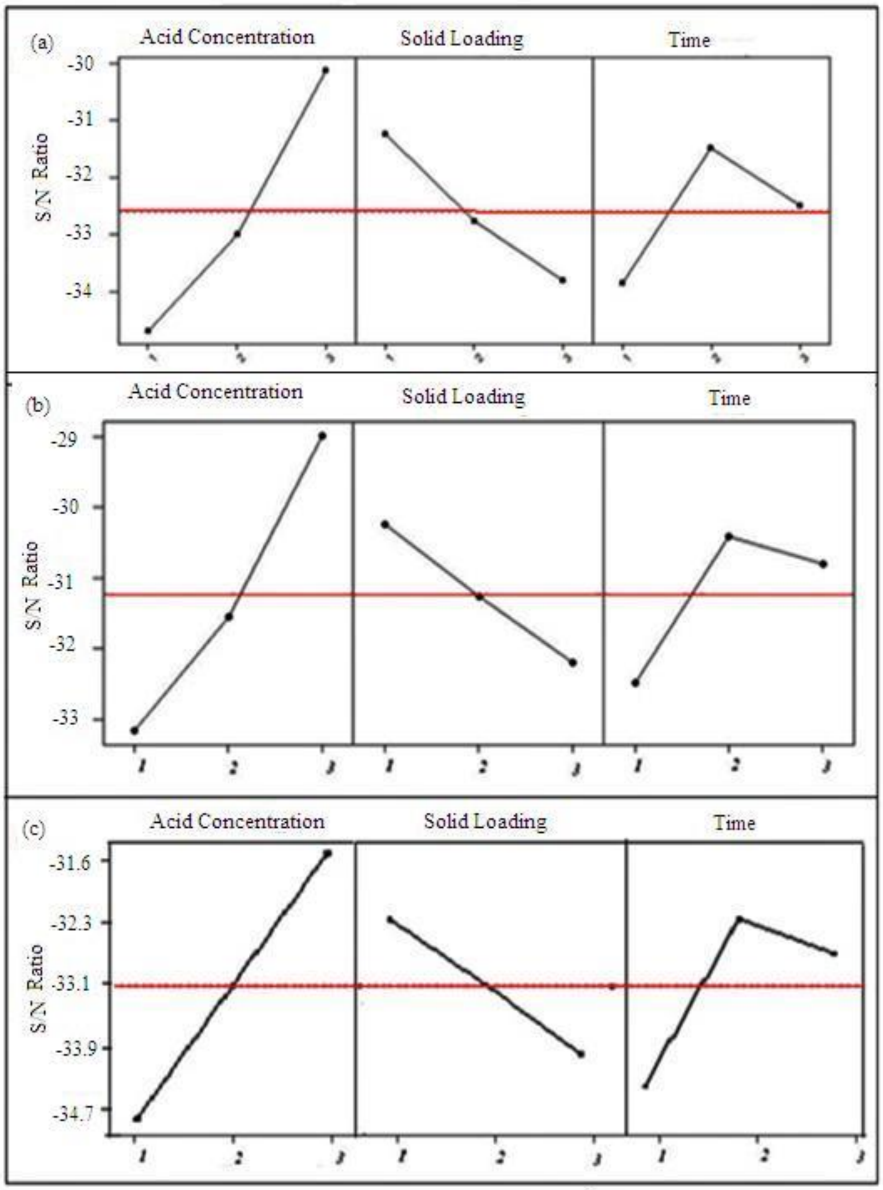

Fig. 3.7. Main Effects plot for S/N ratio for dilute acid pretreatment (a) Moj, (b) Bonbogori (c) Arecanut husk

The values of crystalinity obtained at different experiments were listed in Table 3.4 along with the S/N ratio for each experiment. The optimum conditions for this process were determined from the main effects plot depicted in Fig. 3.5. The optimum conditions (dilute acid pretreatment process) within the design conditions of the Areca nut husk, Bonbogori and Moj fiber was achieved at acid concentration (level 3), reaction time (level 2) and solid loading (level 1).

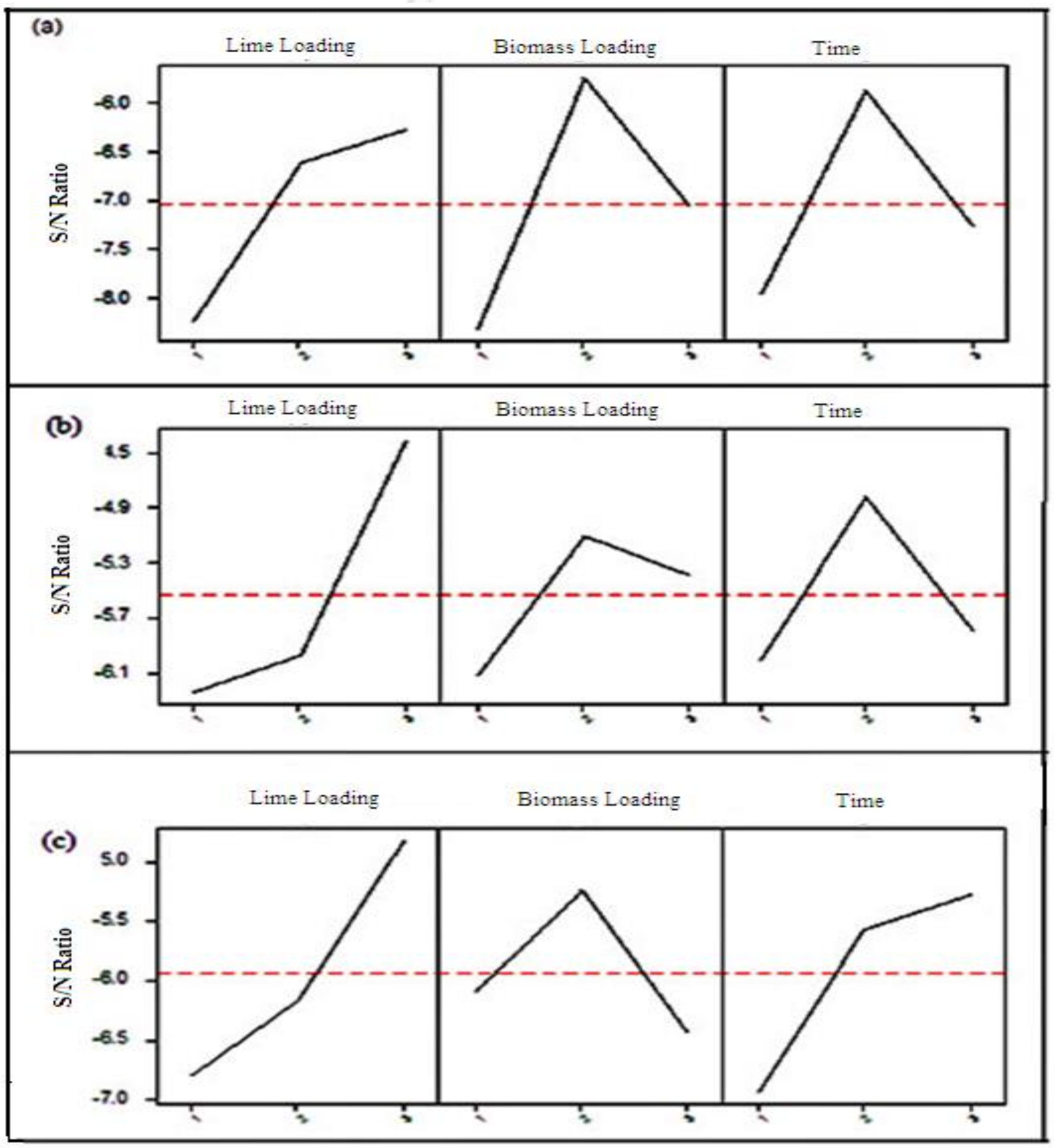

Fig. 3.8 Main Effects plot for S/N ratio for ultrasound assisted lime pretreatment (a) Moj, (b) Bonbogori (c) Arecanut husk

The optimum conditions for both Moj and Bonbogri were observed at A3, B2 and C2 but for Arecanut husk the pattern was little different A3, B2 and C3. The results implied that the longer ultrasound duration had an adverse effect on Arecanut husk pretreatment process (Fig. 3.6).

3.5.2 Analysis of variance (ANOVA)

The purpose of ANOVA was to find out the effect of individual parameter in entire pretreatment process for all the biomass samples.

3.5.2.1 Dilute acid pretreatment process

To investigate the effects of each parameter in entire pretreatment process over crystalinity of all the biomasses the analysis of variance (ANOVA) was carried out and the results were shown in Table 3.6.

Table 3.6 ANOVA chart of the results of dilute acid pretreatment

Factor	DOF	Sum of squares			Variance			F ratio		
		Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj
Acid concentration	2	479.44	446.4	749.1	239.72	223.2	374.52	64.72	43.13	153.41
Solid loading	2	126.32	104.43	259.4	63.16	52.21	129.7	17.05	10.09	53.13
Time	2	214.07	127.24	214.3	107.03	63.62	107.164	28.90	12.29	43.90
Error	2	7.4	10.34	4.88	3.7	5.17	2.44	-	-	-

The experimental data were used to estimate “F” value of Fishers test (“F” test). “F” test is performed to know that whether the difference in treatment means is random or statistically significant in different process. The calculated “F” value should be higher than the value obtained from statistical table at a pre defined confidence level to make a parameter statistically significant [53]. In this study all, the “$F_{0.9}$”values were higher than the tabulated value described in standard statistical table.

It can be observed that (Fig 3.7) dilute concentration played a major role (> 60%) followed by time of operation (>16%) and solid loading concentration (>10%).

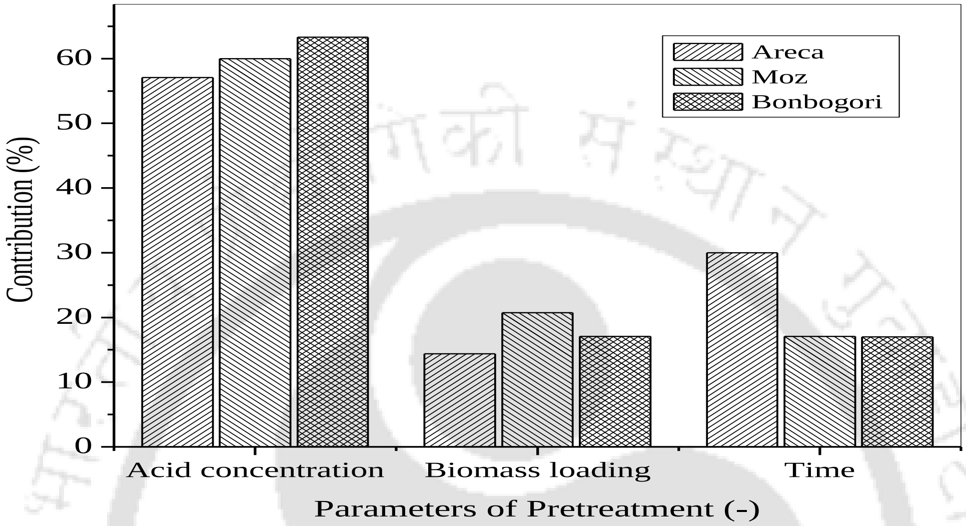

Fig. 3.9. Contribution of different parameters on pretreatment process.

There is a multitude of interdependent mechanisms exists to describe the acid catalyzed hydrolysis process of lignocellulosic pretreatment. Acid can penetrate the inner lignocellulosic matrix and liberate monomers from the cellulosic and hemi-cellulosic chains by breaking the 1-4 linkages; moreover, it also helps to swell the matrix, which indirectly reduces the crystallinity. Higher solid loading prevents acid to penetrate inside the matrix, as biomass components are hydrophobic in nature and agglomerates soon after addition of water. Elongated operation time has harsh effect on biomass, the sugar components starts forming furfural, hydroxy-methyl furfural and many other components, which do not support to form amorphous biomass.

3.5.2.2 Ultrasound assisted lime pretreatment process

The results of ANOVA calculated from the raw data of the experiments were illustrated in Table 3.7.

Table 3.7. ANOVA chart of ultrasound assisted lime pretreatment

Factor	DOF	Sum of squares			Variance			F ratio		
		Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj
Lime loading	2	0.02	0.02	0.03	0.01	0.01	0.02	23.15	54.53	35.7
Biomass loading	2	0.01	0.01	0.04	0.00	0.00	0.02	7.92	14.11	40.16
Time	2	0.01	0.01	0.03	0.01	0.00	0.01	15.46	21.21	31.55
Error	2	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-

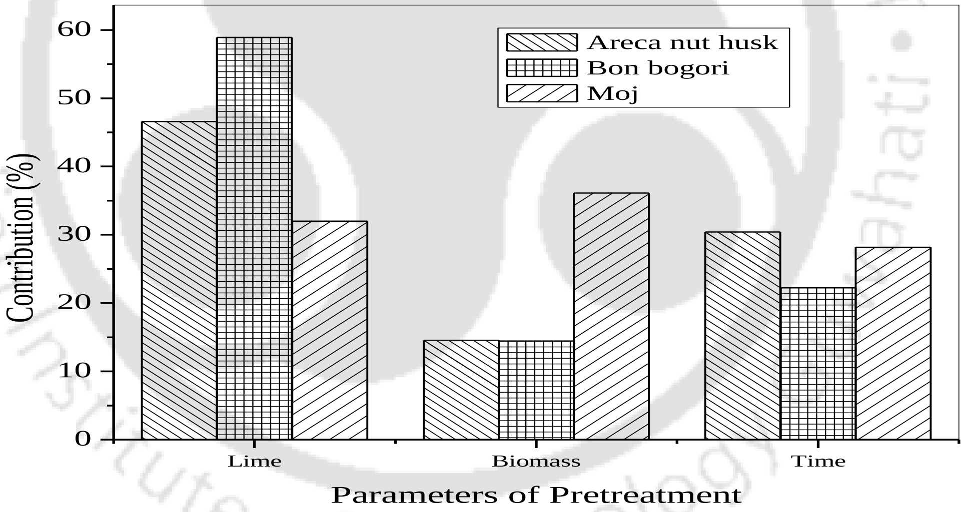

Fig. 3.10. Contribution of different parameters on ultrasound assisted lime pretreatment process

The result showed that lime loading ratio played a major role (>46%) followed by ultrasound irradiation time (>22%) and biomass loading (>14%) when pretreatment was conducted on Bon bogori and Arecanut husk but Moj had a different pattern, biomass loading ratio played a major role (36.12%) in the pretreatment process followed by lime loading (32.01%) and duration of ultrasound irradiation (28.18%) (Fig.3.8). The Fishers test (“F” test) helps to judge the statistical significance of parameters. The calculated “F” value should be higher than the value tabulated in statistical table at a pre defined confidence level [53].

3.5.3Verification test

The conclusive step of optimization is to compare the predicted or calculated value with experimental value of the quality characteristics i.e. crystalinity index, delignification ratio. Taguchi method of optimization helps to find out the optimum conditions of parameters to obtain the crystalinity or delignification ratio of lignocellulosic fibers of studied biomass. The final step is to predict and verify the improvement in the quality characteristics i.e. crystalinity and delignification ratio using the optimal level of the design parameters. The predicted S/N ratio using the optimal level of the design parameters can be calculated from Eq 3.2(the details of the equation are described in Appendix 1)

$$\frac{S}{N}_p = \frac{S}{N}_m + \sum_{i=1}^{n} \left(\frac{S}{N}_i - \frac{S}{N}_m\right) \tag{3.2}$$

where,

$\frac{S}{N}_p$ = PredictedS/Nratio; $\frac{S}{N}_m$ = Total mean S/N ratio; $\frac{S}{N}_i$ = Mean S/N ratio at the optimal level and *“n”* is the number of the main design parameters that affect the quality characteristics.

Table 3.8 illustrated the predicted value of the crystalinity and S/N ratio and the actual experimental value. The result showed that the experimental values were almost same as the predicted one and the experimental crystalinity value differs from the predicted value by 1.26% only.

Table 3.8 Results of confirmation experiment of dilute acid pretreatment

	Crystallinity (%)			S/N		
	Areca nut husk	Bon bogori	Moj	Areca nut husk	Bon bogori	Moj
Predicted	30.41	22.85	24.07	-29.66	-27.18	-27.63
Experimental	31.28±0.5	23.14±0.5	25.17±0.5	-29.91±0.1	-27.29±0.1	-28.02±0.1

Acid catalyzed pretreatment process helps to swell the lignocellulose fibers and lignin retards swelling of biomass mass [54-55]. Acid pretreatment removes acid soluble lignin from biomass materials, which makes the biomass wet and thus indirectly responsible for reduction of cellulose crystalinity. Lignocellulosic biomass materials agglomerate soon after addition of water, which is the sole cause of inefficient pretreatment process at higher solid loading. Prolong operation time has harsh effect on biomass, the sugar components starts forming furfural, hydroxy-methyl furfural and many other components which do not support to form amorphous biomass [56]. The enzymatic activity on acid pretreated biomass material will further increase, as acid pretreatment removes hemicellulose and makes the biomass porous [57]. The predicted values and experimental values of delignification ratios and corresponding S/N ratios were well agreed (Table 3.9) with each other for ultrasound assisted lime pretreatment.

Table 3.9 Results of confirmation experiment of ultrasound assisted lime pretreatment

Name of the biomass	Delignification ratio		S/N ratio	
	Predicted	Experimental	Predicted	Experimental
Moj	0.65	0.64	-3.79	-3.84
Bonbogori	0.69	0.68	-3.27	-3.35
Arecanut husk	0.67	0.65	-3.51	-3.74

Maximum difference between predicted and experimental value was found to be 2.99 % for Areca nut husk. The present study reports removal of 68%, 65% and 64% of the lignin present in the native lignocellulosic fiber of Bonbogori, Areca nut husk and

Moj respectively. The yield of total solid after through washing was quite high in all biomass except Arecanut husk. The lower bulk density of this biomass retarded the recovery process. The cellulose and hemicellulose yield after the ultrasound assisted pretreatment was also determined. The results (Table 3.10) indicate a higher recovery of hemicellulose and cellulose irrespective of all biomass materials, which can be inferred that ultrasound assisted lime pretreatment process, does not have any adverse effect over sugar materials. A thorough washing after the pretreatment process can help to improve the yield of both this materials. The hydroxyl group of lime reacts with phenolics, ether group of lignin molecules and calcium ion reacts with carbon di-oxide to form calcium carbonate, which directly deposits onto the surface of lignocellulosic fiber. Few researchers reported that deposition of calcium on the lignocellulose fiber retards the degradation of cellulosic material. The ratios of recovery yield in the present study for all the biomasses agreed with the previous reports.

Table 3.10 Yields and lime consumptionof biomass after the pretreatment

Name of the Biomass	Recovery Yield of Total solid[1]	Cellulose yield[2]	Hemicellulose yield[3]	Lime consumption [4]
Areca nut husk	0.85±0.05	1.19±0.05	1.30±0.04	0.22±0.06
Bonbogori	0.80±0.04	1.49±0.04	1.34±0.05	0.34±0.05
Moj	0.78±0.05	1.46±0.04	1.34±0.05	0.38±0.05

[1] $\frac{\text{weight (dry basis) of recovered (after pretreatment)total solid}}{\text{Initial weight (dry basis)ofbiomass}}$ [2] $\frac{\text{cellulose (\%)in biomass after pretreatment}}{\text{cellulose (\%)in biomass before pretreatment}}$

[3] $\frac{\text{Hemicellulose (\%)in biomass after pretreatment}}{\text{Hemicellulose (\%)in biomass before pretreatment}}$ [4] $\frac{\text{lime consumed (g)}}{\text{raw biomass (g)}}$

Lime consumption is a rough indication of delignification pattern. The amount of lime consumed during the pretreatment process was determined by pH neutralization with a standard solution of acid, 5(N) HCl. The detail calculation of lime consumption is given below.

$$Wc = \frac{Mol\ \text{wt of Ca(OH)}_2}{2mol\ \text{HCL}} \times \frac{N_{HCl} \times v}{1000} \qquad (3.3)$$

Wc = Weight of reacted lime (g) , Mol wt of $Ca(OH)_2$ = 74.092 g , N_{HCl} = Normality of HCl 5, v = volume of HCL required to neutralize the solution (mL)

The results (Table 3.10) showed that ultrasound assisted lime pretreatment process enhances the lime consumption irrespective of the type of biomass species. The lime consumption is directly proportional to the temperature and time. The ultrasonication process increases the local heating temperature abruptly, which enhances rate of lime consumption and results carbohydrate rich biomasses. Similar kinds of lime pretreatment of all the biomasses were conducted without any assistance of ultrasound. The results from the study of lime (sole) pretreatment with all the biomasses revealed that to get a dignified amount of lignin removal it took minimum 8 weeks (Fig. 3.9), whereas the ultrasound-assisted lime pretreatment can yield the same degree of delignification within 180 min. The bonding between the two carbon molecules in lignin are stable in alkaline conditions therefore, breaking of bonds between carbon and oxygen molecules are the most significant reaction in the pretreatment of lignocellulosic materials at alkaline condition. Lignin reacts with alkali and generates phenolic hydroxyl groups from the cleavage of the aryl-alkyl-ether bonds. Therefore, lime consumption is a rough indication of delignification pattern [58]. The amount of lime consumed during the pretreatment process was determined by pH neutralization with a standard solution of acid, 5 N HCl.

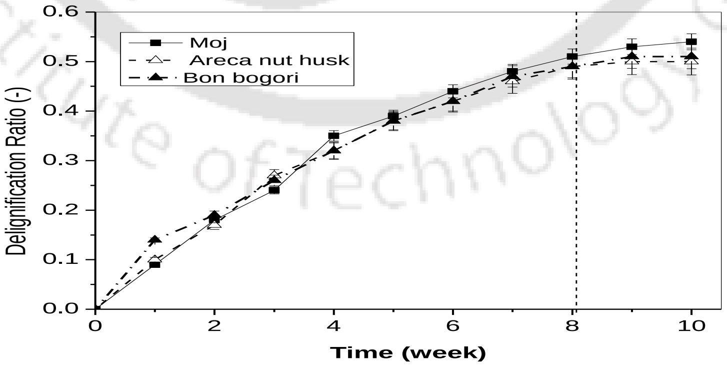

Fig.3.11. Lime pretreatment of biomass

3.6 Conclusions

The goal of the lignocellulosic biomass pretreatment optimization process is to facilitate the enzymatic hydrolysis process i.e. saccharification. Dilute acid pretreatment and ultrasound assisted lime pretreatment process was employed in this study.The recommended conditions for the dilute acid catalyzed pretreatment of all the three-studied biomass i.e.Moj, Bonbogoriand Arecanut huskwere: acid concentration (2.5%), solid loading (1%) and time of operation (30min) respectively. Under these optimized dilute acid pretreatmentconditions, the crystallinity of MojandBonbogorireduced from 64.14 % to 25.17% and 52.46% to 23.14% respectivelyand for Areca nut husk the degree of reduction of crystalinity was 49.8%.Whereas ultrasound assisted lime pretreatment removed 68%, 65%, and 64% of the lignin present in the native lignocellulosic fiber of Bonbogori, Areca nut husk, and Moj respectively. Acid can penetrate the inner lignocellulosic matrix and liberate monomers from the cellulosic and hemi-cellulosic chains by breaking the 1-4 linkages, moreover, it also helps to swell the matrix which indirectly reduces the crystallinity. Higher solid loading prevents acid to penetrate inside the matrix as biomass components are hydrophobic in nature and agglomerates soon after addition of water. Elongated operation time has harsh effect on biomass, the sugar components starts forming furfural, hydroxy-methyl furfural and many other components, which do not support to form amorphous biomass. The hot spot theory of ultrasonication postulates the formation of micro-bubbles at the site of nucleation when high frequency sound waves (50-60 KHz) were applied to the liquid. The bubbles generate due to decrease of boiling point caused by negative pressure and the same bubble collapses due to positive pressure created by the same wave. The positive pressure forced the bubble to implode which leads to the formation of local hot spot with temperature and pressure approximately 5000 K and 500 atm respectively.Therefore, it generates a high pressure and temperature, which is required to break the polymeric lignocellulosic biomass into smaller components. The results showed that ultrasound assisted lime pretreatment process enhances the lime consumption irrespective of the type of lignocellulosic biomass.

References

[1] Chen Ye , Stevens M A, Zhu Y ,Holmes J, Moxley G, Xu H. Journal of Industrial Microbiology & Biotechnology, 2012;39 (5):691-700.

[2] Sindhu R, Kuttiraja M, Binod P, Janu KU, Sukumaran RK, Pandey A. Dilute acid pretreatment and enzymatic saccharification of sugarcane tops for bioethanol production. Bioresour Technol, 2011; 102(23):10915-21

[3] Speers AM, Reguera G. Consolidated Bioprocessing of AFEX-Pretreated Corn Stover to Ethanol and Hydrogen in a Microbial Electrolysis Cell. Environ Sci Technol, 2012; 46(14):75-81.

[4] Jin M, Gunawan C, Balan V, Lau MW, Dale BE. Simultaneous saccharification and co-fermentation (SSCF) of AFEX(TM) pretreated corn stover for ethanol production using commercial enzymes and Saccharomyces cerevisiae 424A(LNH-ST). Bioresour Technol. 2012; 110: 587-94.

[5] Tao L, Aden A, Elander RT, Pallapolu VR, Lee YY, Garlock RJ, Balan V, Dale BE, Kim Y, Mosier NS, Ladisch MR, Falls M, Holtzapple MT, Sierra R, Shi J, Ebrik MA, Redmond T, Yang B, Wyman CE, Hames B, Thomas S, Warner RE. Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. Bioresour Technol. 2011;102(24):11105-11114

[6] Sierra-Ramírez R, Garcia LA, Holtzapple MT. Selectivity and delignification kinetics for oxidative short-term lime pretreatment of poplar wood, part I: Constant-pressure 2011 ;27 (4): 976-985.

[7] Zhao X, Cheng K, Liu D. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biotechnol. 2009 ; 82(5):815-27.

[8] Arato C, Pye EK, Gjennestad G. The lignol approach to biorefining of woody biomass to produce ethanol and chemicals. Appl Biochem Biotechnol. 2005;121-124:871-82

[9] Inoue H, Yano S, Endo T, Sakaki T, Sawayama S. Combining hot-compressed water and ball milling retreatments to improve the efficiency of the enzymatic hydrolysis of eucalyptus Biotechnol. Biofuels, 2008;1: 2-4.

[10] Craeyveld VV, Delcour JA, Courtin CM.Ball milling improves extractability and affects molecular properties of psyllium (Plantago ovata Forsk) seed husk arabinoxylan.J. Agric. Food. Chem, 2008; 56:11306–11311.

[11] Yong J, Soo S. Compositional changes in industrial hemp biomass induced by electron beam irradiation Pretreatment. Biomass and Bioenergy, 2011;35 (7):67–3270.

[12] Khan F, Ahmad SR, Kronfli E.Gamma-radiation induced changes in the physical and chemical properties of lignocelluloses. Biomacromolecules, 2006; 7: 2303–2309.

[13] Valery B Agbor, Nazim C, Richard S, Alex B, David L. Biomass pretreatment: Fundamentals toward application Biotechnology Advances,2011;29(6): 675-685.

[14] Bak JS, Kim MD, Choi IG, Kim KH. Biological pretreatment of rice straw by fermenting with Dichomitus squalens. New Biotechnology, 2010 ; 27(4):424-34.

[15] Singh D, Chen S. The white-rot fungus Phanerochaete chrysosporium: conditions for the production of lignin-degrading enzymes. Appl Microbiol Biotechnol. 2008;81(3):399-417.

[16] Alvira P, Tomás-Pejó E, Ballesteros M , Negro MJ.Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology,2010; 101: 4851–4861.

[17] Kobayashi T, Kohn B, Holmes L, Faulkner R, Davis M, Maciel GE. Molecular-Level Consequences of Biomass Pretreatment by Dilute Sulfuric Acid at Various Temperatures, Energy and Fuels, 2011; 25: 1790-1797.

[18] Himmel ME, Ding SY, Johnson DK, Adeny WS, Nimlos MR, Brady, JW, Foust TD. Biomass Recalcitrance: Engineering Plants and Enzymes for Biofuels Production, Science, 2007;315: 804-807.

[19] Kumar P, Barrett DM, Delwiche MJ, and Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial and Engineering Chemistry Research, 2009 ; 48(8): 3713–3729.

[20] Digman MF, Shinners KJ, Casler MD. Optimizing on-farm pretreatment of perennial grasses for fuel ethanol production, Bioresource Technology, 2010; 101: 5305–5314.

[21] Xu J, Thomsen MH, Thomsen AB. Pretreatment on corn stover with low oncentration of formic acid, Journal of Microbiology and Biotechnology, 2009; 19(8): 845–850.

[22] Wyman CE, Dale BE, Elander RT. Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies, Biotechnology Progress, 2009; 25(2): 333-339.

[23] Cara C, Ruiz E, Oliva,JM, Sáe, F, Castro E. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresour. Technol. 2008; 99: 1869–1876.

[24] Rocha,MV, Rodrigues TH, De Macedo G.R, Gonçalves LR. Enzymatic hydrolysis and fermentation of pretreated cashew apple bagasse with alkali and diluted sulfuric acid for bioethanol production. Appl. Biochem. Biotechnol, 2009; 155:407–417.

[25] Huang HJ, Lin W L, Ramaswamy S, Tschirner U, Process modeling of comprehensive integrated forest biorefinery: An integrated approach. Appl. Biochem. Biotechnol. 2009; 154 (1–3): 205– 216 .

[26] Cheng G, Varanasi P, Li C, Liu,H, Melnichenkos BY, Simmons BA, Kent MS, Sing S. Transition of Cellulose Crystalline Structure and Surface Morphology of Biomass as a Function of Ionic Liquid Pretreatment and Its Relation to Enzymatic Hydrolysis. Biomacromolecules, 2011;12(4): 933-941.

[27] Lu Xuebin, Yimin Z, and Irini A. Optimization of H2SO4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: focusing on pretreatment at high solids content. Bioresource Technology, 2009;100(12):3048-3053.

[28] Noureddini H, and J Byun. Dilute-acid pretreatment of distillers' grains and corn fiber. Bioresource Technology, 2010; 101(3):1060-1067.

[29] Shi Y, Yan Tao, Wang Y, Zhao J, Zhou S . The study of the dilute acid pretreatment technology of corn stover and rice straw. 2012; 550-553.

[30] Chen X, Shekiro,J, Elander R, Tucker M.Improved Xylan Hydrolysis of Corn Stover by Deacetylation with High Solids Dilute Acid Pretreatment.Ind. Eng. Chem. Res., 2012, 51 (1), 70–76.

[31] Roland DP .Cellulose: Pores, Internal Surfaces, and the Water Interface. Textile and Paper Chemistry and Technology. ACS Symp. Ser.**1976**, *49*, 20

[32] Xu J and Cheng JJ. Pretreatment of switchgrass for sugar production with the combination of sodium hydroxide and lime. Bioresource Technology. 2011; 102(4): 3861-3868.

[33] Chang V S, Nagwani M, Holtzapple MT. Lime pretreatment of crop residues bagasse and wheat straw. Appl. Biochem. Biotechnol. 1998; 74: 135–159.

[34] M. Falls , RS Ramirez, MT Haltzapple. Oxidative Lime Pretreatment of Dacotah Switchgrass. Appl Biochem Biotechnol (2011) 165:243–259

[35] Chang. V.S, Nagwani. M., Kim. CH, Holtzapple.MT , Oxidative lime pretreatment of high-lignin biomass. Appl. Biochem. Biotechnol. 2001;94: 1-28.

[36] J.Xu, Cheng JJ, Sharma RR, and Burns JC. Lime pretreatment of switchgrass at mild temperatures for ethanol production. Bioresource Technology. 2010; 101 (8): 2900-2903 .

[37] Harmsen P, Huijgen W, Bermúdez L, Bakker R. (2010). Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass (1st ed.), ISBN 9789-085-857-570,

[38] Sun R, Tomkinson J. Comparative study of lignins isolated by alkali and ultrasound-assisted alkali extractions from wheat straw. *Ultrasonics sonochemistry* 2002, *9*, 85–93.

[39] Klinke HB. Ahring BK, Schmidt AS, Thomsen AB. Characterization of degradation products form alkaline wet oxidation of wheat straw. Bioresource Technology. 2002; 82: 15-26.

[40] Rabelo SC, Filho RM, Coasta AC. Lime Pretreatment of Sugarcane Bagasse for Bioethanol Production, Appl Biochem Biotechnol. 2009; 153:139–150.

[41] Chang VS, Nagwani M, Holtzapple MT. Lime Pretreatment of Crop Residues Bagasse and Wheat Straw, Applied Biochemistry and Biotechnology. 1998; 74: 135-159.

[42] Mass RH, Bakker RR, Boersma AR, Bisschops I, Pels JR, Jong ED, Weusthuis RA,Reith H. Pilot-scaleconversion of lime-treated wheat straw into bioethanol:quality assessment of bioethanol and valorization of side streams byanaerobic digestion and combustion, Biotechnology for Biofuels.2008; **1**:14.

[43] Cheng YS, Zheng Y, .Dooley CW, .Jenkins BM, VanderGheynst JS. Evaluation of High Solids Alkaline Pretreatment of Rice Straw, Appl Biochem Biotechnol.2010; 162: 1768–1784.

[44] Kim S, Holtzapple MT. Delignification kinetics of corn stover in lime pretreatment. Bioresource Technology. 2006; 97:778–85.

[45] Ince N H, Tezcanli G, Belen RK. Ultrasound as a catalyzer of aqueous reaction systems : the state of the art and environmental applications. Applied Catalysis .2001;*29*: 167–176.

[46] Thompson LH, Doraiswamy LK. Sonochemistry : Science and Engineering. Ind. Eng. Chem. Res. 1999; 38:1215–1249.

[47] Margulis M A, Margulis IM. Contemporary review on nature of sonoluminescence and sonochemical reactions. Ultrasonics Sonochemistry. 2002; *9*: 1–10.

[48] Sun R, Tomkinson J. Comparative study of lignins isolated by alkali and ultrasound-assisted alkali extractions from wheat straw. Ultrasonics Sonochemistry 2002; *9*: 85–93.

[49] Khanal SK, Montalbo,M, Leeuwen JHV, Srinivasan G, Grewell D. Ultrasound Enhanced Glucose Release From Corn in Ethanol Plants. Biotechnology Bioengineering. 2007; 98: 78–85.

[50] Nitayavardhana S, Rakshit SK, Grewell D, Leeuwen JHV, Khanal S K. Ultrasound Pretreatment of Cassava Chip Slurry to Enhance Sugar Release for Subsequent Ethanol Production. 2008,;101: 487–496.

[51] Yua J, Zhanga J, Hea J, Liua Z, Yua Z. Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull. Bioresource Technology. 2009; 100(2): 903–908.

[52] Sivasankar, T.; Paunikar, A. W.; Moholkar, V. S. Mechanistic features of the sonochemical degradation of organic pollutants. AICHE J. 2008; 54 (7):2206-2219.

[53] Xiansheng N, Zhenggan Z, Xiongwei W, Luming L. The use of Taguchi method to optimize the laser welding of sealing. Optics and Lasers in Engineering. 2011; *49*: 297–304.

[54] Yang B, Wyman E. Effect of xylan and lignin removal by batch and flow through pretreatment on the enzymatic digestibility of corn stover cellulose, Biotechnol. Bioeng, 2004; 86: 88-95.

[55] Taherzadeh MJ, Karimi K.., Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review, Int. J. Mol. Sci. 2008; 9: 1621-1651.

[56] Kootstra AMJ, Mosier NS, Scott EL, Beeftink HH, Sanders JPM. Differential effects of mineral and organic acids on the kinetics of arabinose degradation under lignocellulose pretreatment conditions, Biochemical engg Journal. 2009; 43: 92-97.

[57] Laxman RS, Lachke AH. Bioethanol from Lignocellulosic Biomass: Part I Pretreatment of the Substrates, Hand book of biofuels , 2009 CRC press.

[58] Dobrzañski L A, Domagaa J, Silva, J F. Archives of Materials Scienceand Engineering. 2007; *28*: 133-140.

Chapter IV

Saccharification of pretreated biomass

The conversion of lignocellulosic materials into biofuel involves hydrolysis of cellulose and hemicellulose to fermentable sugars and the subsequent fermentation of these sugars into biofuel. The hydrolysis process was carried out by cellulase enzymes that break down structural carbohydrates into monomeric sugars such as glucose and xylose. Lignocellulosic materials contain cellulose, hemicellulose and lignin that are closely associated in a complex crystalline structure. As a result, the efficiency of the hydrolysis is reduced due to limited accessibility of the enzymes to the substrate i.e. cellulose. Therefore, the degree of saccharification process can be considered as a yardstick to compare the different pretreatment process. The present study compared the previously examined pretreatments techniques such as dilute sulfuric acid pretreatment, lime (sole) and ultrasound assisted lime pretreatment process by the yield of sugar after saccharification. The results revealed that both lime and ultrasound assisted pretreatment process yields maximum amount of sugar than dilute acid pretreatment process. Apart from the mere comparisons, the study also includes the optimized enzyme loading with the biomasses to provide the sugar rich solution for simultaneous ethanol and bio-hydrogen production.

4.1. Introduction

Cellulase enzymes are at present one of the leading industrial enzyme, because of their wide applications in several industries such as textile, pulp and paper, agriculture, laundry and food industry [1]. However, cellulases may become the largest volume industrial enzyme, if ethanol from lignocellulosic biomass through enzymatic route becomes a major transportation fuel. Efforts are being made to use biomass, in the form of simple sugars. Unfortunately, most sugars in biomass are wrapped in long cellulose molecules that are effectively protected against chemical processing by tight packing. Unlocking cellulose to provide renewable chemical feedstock can be achieved by pretreatment process followed by enzymatic hydrolysis or saccharification. Cellulolytic enzymes accomplish enzymatic hydrolysis of lignocellulosic biomasses [2].

Cellulases are inducible enzymes synthesized by large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials and generally, they are unable to use protein or lipids as energy source. They are present in 13 of the 82-glycoside hydrolase families identified by sequence analysis [3].The lists of microorganisms producing cellulase are listed below in Table 4.1.

Table 4.1 List of cellulase producing microorganisms

Fungi			Bacteria	
Soft rot	Brown rot	White rot	Aerobic	Anaerobic
Aspergillus niger, *Penicillium brasilianum* *Chaetomium cellulyticum*	*Coniophora puteana;* *Lanzites trabeum;* *Poria placenta;* *Tyromyces palustris*	*Phanerochaete chrysosporium;* *Sporotrichum thermophile;* *Trametes versicolor*	*Eubacterium cellulosolvens;* *Geobacillus sp.;* *Microbispora bispora*	*Acetivibrio cellulolyticus;* *Butyrivibrio fibrisolvens;* *Clostridium thermocellum*

Among the different sources of cellulase, fungal cellulases are most popular [4]. Fungal cellulases typically have two separate domains: a catalytic domain (CD) and a cellulose binding module (CBM), which is joined by a short polylinker region to the catalytic domain at the N-terminal.A typical cellulolytic complex includes a variety of hydrolytic and oxidative enzymes. Hydrolytic enzymes includes endoglucanase [1,4-*ß*-D glucan glucanohydrolase], cellobio hydrolase(CBH)[1,4-*ß*-D-glucan-cellobiose-hydrolaseEC3.21.91] and *ß*-gulcosidase[1,4-*ß*-d-glucosideglucohydrolase].

Cellulase enzyme, however, has been projected as a major cost contributor to the lignocellulose-to-ethanol technology, it costs around 15–20 cents per gallon ethanol as compared to only 2–4 cents per gallon ethanol for amylases used in the starch-to-ethanol process [5]. The biotechnology based multinational companies; Genencor International and Novozymes Biotech have reported the development of technology that has reduced the cellulase cost for the cellulose-to-ethanol process. Both the companies has played a significant role to reduce the cost of cellulase several folds by their active research and are continuing to bring down the cost by adopting novel technologies. Recently, Genencor has launched Accelerase®1500, a cellulase complex intended specifically for lignocellulosic biomass processing industries. Accelerase®1500 is produced with a genetically modified strain of *T. reesei*as stated in Genencoreinc. s bulletin. This enzyme preparation is claimed to contain higher levels of β-glucosidase activity than all other commercial cellulases available today, to ensure almost complete conversion of cellobiose to glucose [6].It has an optimal pH of 4.6–5.0 but it inactivates below 4.0 or above pH 7.0. Usually 50 °C temperature is employed for hydrolysis by cellulases but Accelerase®1500 works well in temperature ranging from 50 to 65 °C though at lower ends of the temperature range, the effective lifetime of enzyme is higher. The enzyme was reported well with hydrolysis of lignocellulosic biomass such as aspen,switch grass [7], sweet sorghum, bagasse [8],corn stover [9],fiber sludge [10], sugarcane bagasse [11].The present study was conducted by employing Accelerase®1500 for saccharification and this enzyme was gifted from Genencore International, B.V. (Netherlands) (Material Number A30173).

4.2. Mechanism of enzymatic hydrolysis

Cellulose is a linear condensation polymer consisting of D-anhydro glucopyranose connected together by β-1,4-glycosidic bonds. The degree of polymerization (DP) ranges between100 to 20,000 [12]. The cellulose molecule is very stable, with a half-life of 5–8 million years for β-glucosidic bond cleavage at 25°C [13].There are several enzymes, which are necessary for complete hydrolysis of biomass such as cellulase, xylanase, ligninase, pectinase, etc., among them cellulase is the most significant one as the principle component of biomass (>40%) is cellulose. Cellulase is a multi-enzyme complex of three different enzymes; exoglucanase, endoglucanase and β - glucosidase (BGL) which acts synergistically for complete hydrolysis of cellulose. The mechanism of cellulose hydrolysis is explained in details below (Fig.4.1).

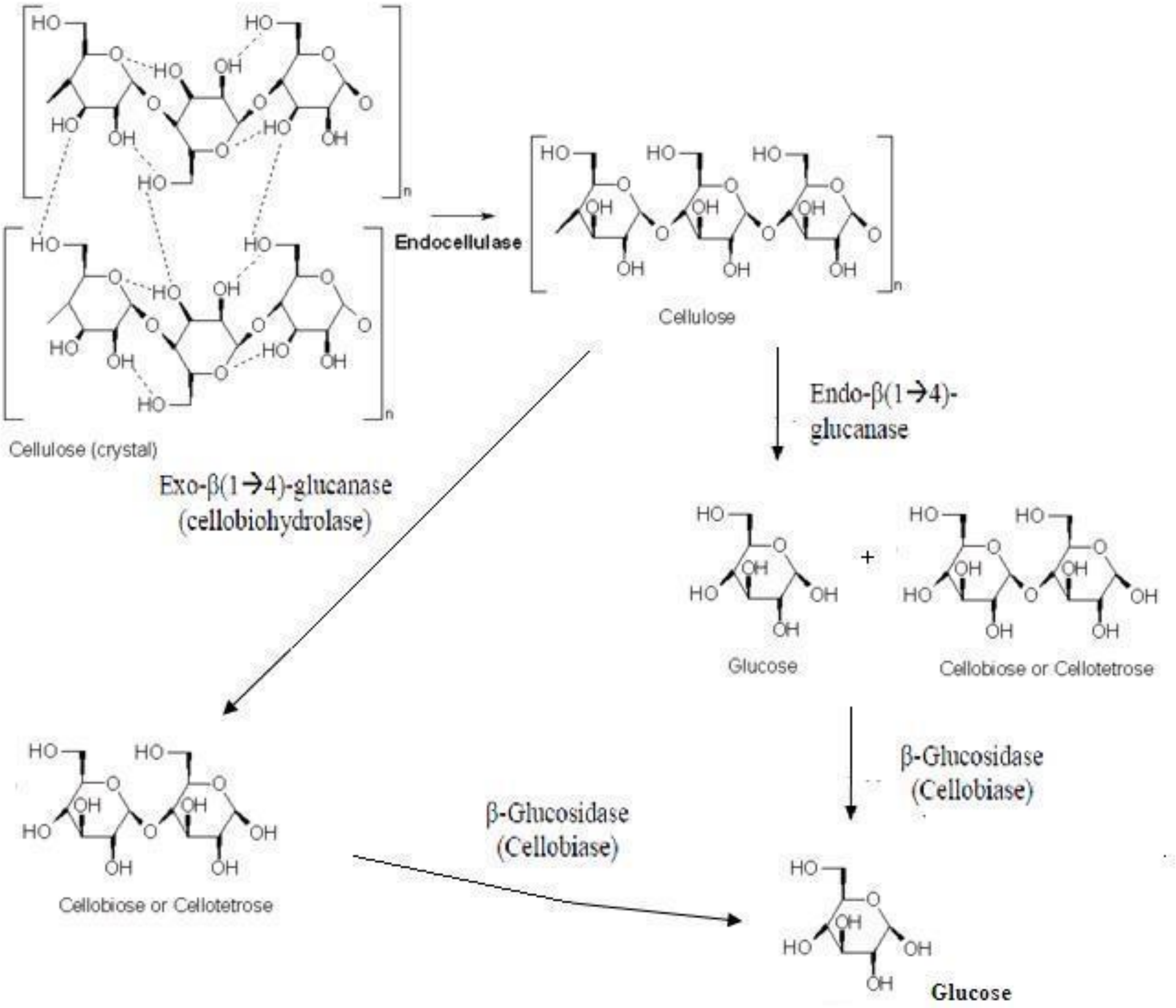

Fig. 4.1. Mechanism of enzymatic hydrolysis of cellulase

The various steps are as follows,

1. Endoglucanases (1,4-β-d-glucan-4-glucanohydrolases, EC 3.2.1.4) decrease the degree of polymerization of macromolecular cellulose by attacking accessible sites and breaking

the linear cellulose chainand their activities are often measured on a soluble high DP cellulose derivative, such as carbxymethyl cellulose.

2. Cellodextrinases (1, 4-β-d-glucanglucanohydrolases, EC 3.2.1.74) attack the chain ends of the cellulose polymers, liberating glucose.

3. Cellobiohydrolases (1,4-β-d-glucancellobiohydrolases, EC 3.2.1.91) produce cellobiose by attacking cellulose from chain ends ((Cel7A (cellobiohydrolase I), acts from the reducing ends, and Cel6A (cellobiohydrolase II) acts from the non-reducing ends of the cellulose chains).

4. Finally, β-glucosidases (EC 3.2.1.21) hydrolyze soluble cellodextrins (1,4- β-d-glucans) and cellobiose to glucose.

The breakdown of hemicellulose is facilitated by series of enzymes together,they are called as hemicellulase. The fraction of hemicellulose is generally made of two types of pentose sugars i.e. xylose and arabinose.

For breaking the polymer xylan

1. Endo xylanase [EC 3.2.1.8]: Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone.
2. Exoxylanase [EC 3.2.1.37] : Hydrolyzes the terminal β-1,4-xylose linkages releasing xylobiose
3. β-xylosidases [EC 3.2.1.32] : It attacks on xylooligosaccharides and releases xylose from xylobiose and short chain xylooligo-saccharides.

The other hemicellulase enzymes which are also known as accessory enzymesare listed in Table 4.2.

Table 4.2.Hemicellulase enzymes and their mode of action [14]

Enzyme	Location of action	Mode of Action
α-Arabinofuranosidase [EC 3.2.1.55]	α- L-arabinofuranosyl compounds attached to the xylan main chain	Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans.
α-Glucuronidase [EC 3.2.1.31]	α- 1,2-linked glucuronic or 4-O-methylglucuronic acid substituents attached to xylan main chain	Releases glucuronic acid from glucuronoxylans.
Acetylxylan esterase [EC 3.1.1.6]	*O*-Acetil groups attached to the side ends of xylan main chain	Hydrolyzes acetylester bonds in acetyl xylans, liberating acetic acid.
Ferulic acid esterase [EC 3.1.1.1]	Feruloyl group on the arabinofuranosyl side chain attached to the terminal non-reducing xylose	Hydrolyze the ester linkages between arabinose side chain residues and phenolic acids (ferulic acid).

4.3. Materials and method

The cellulase enzyme (Accelerase 1500, Material Number A30173) used in this study was kindly supplied by Genencore International, B.V (Netherlands). The carboxy-methyl cellulose activity (CMC) and filter paper activity (FPU) of the enzyme was determined according to the standard procedure recommended by the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (IUPAC) [15]. The cellobiase activity of the enzyme in cellobiase units (CBU) was assayed according to the method described by Cibulaska*et al*. (2010) [16].

All the pretreated lignocellulosic biomass materials (dilute acid, lime and ultrasound assisted lime pretreated biomass of Arecanut husk, Bonbogori and Moj) were washed thoroughly and then used for the hydrolysis process. The pretreated lignocellulosic biomass hydrolysis process was conducted at 50°C, 150 RPM for 96 hours (Fig. 4.2) at different enzyme loading (5, 10, 20 and 25 FPU $g^{-1}$of cellulose). The pH of the medium was maintained at 4.6 by automatic addition of 3M solution of NaOH. The samples were collected at different time intervals and centrifuged. The supernatant was filtered through 0.2 µm sterile filter and analyzed using HPLC (Repromer H+, 9µm, 300× 7.8 mm; temperature 25 °C; eluant: 9 mM sulfuric acid; flow 1mL min^{-1}; pressure: 90 bar)for the estimation of sugars.

4.4. Results and discussion

The carboxy-methyl cellulose activity (CMC) of the enzyme was 2481 ± 0.5 CMC U mL^{-1} and filter paper activity (FPU) of the enzyme was estimated to be 45 ± 0.2 FPU mL^{-1}. The cellobiase activity of the enzyme in cellobiase units (CBU) was found to be 134 ± 0.03 CBU mL^{-1}.

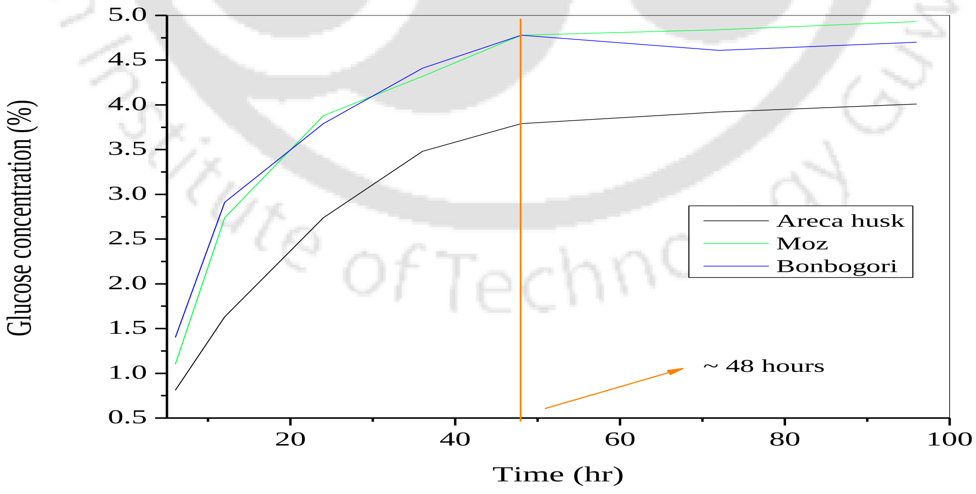

Fig. 4.2. Time profile of Saccharification

The profile of saccharification is shown in Fig 4.2. The graph showed that the equilibrium reached within 48 hours of operation and the maximum glucose concentration was found to be 4.8% with Moj. After 48 hours of operation, the glucose concentration in the saccharified broth became static. The possible reason for decrease in cellulase activity after 48 hours is due to the generation of cellobiose as one of the products from saccharification of cellulose present in the biomass, which is reported to inhibit cellulase activity [17].

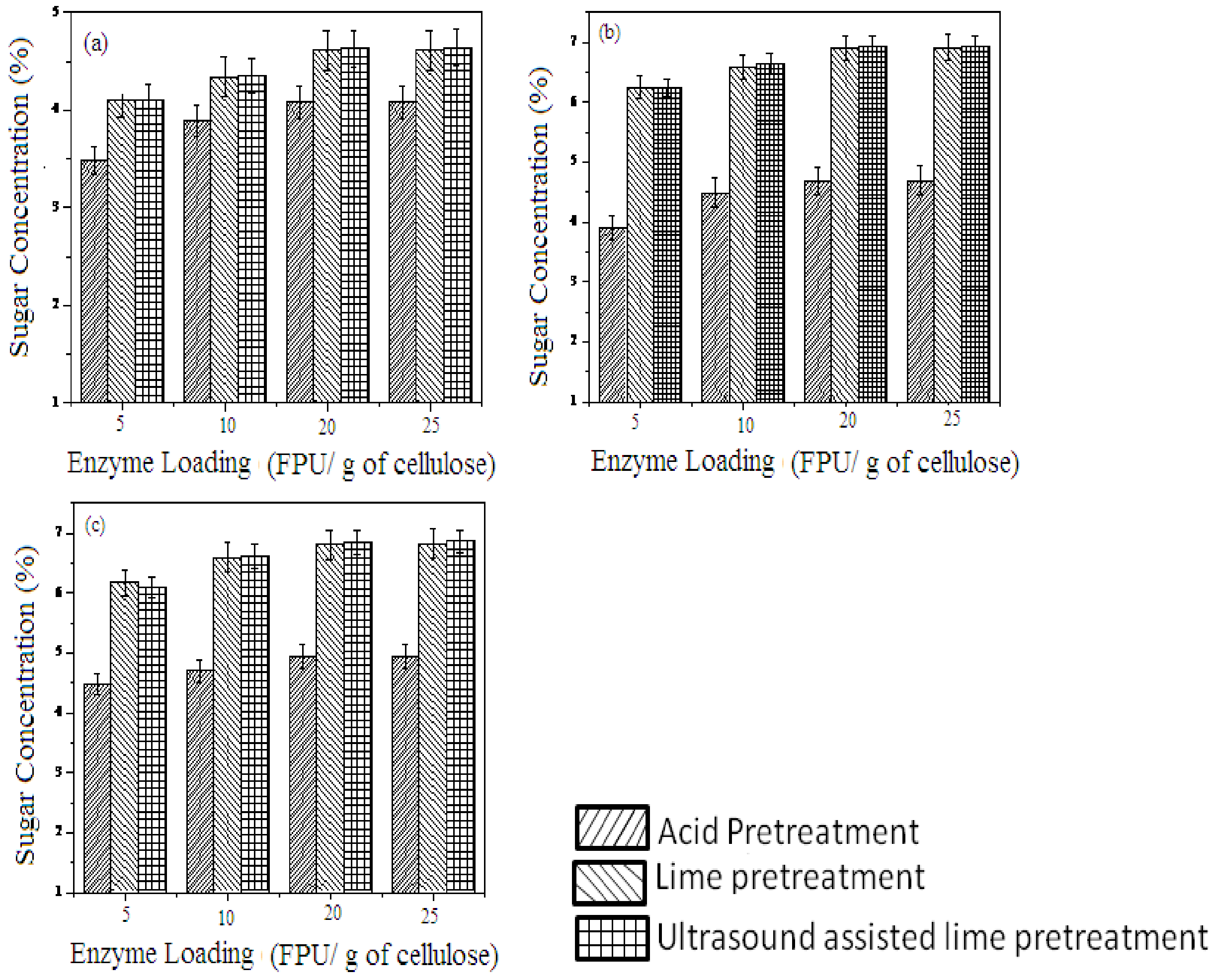

Fig.4.3. Comparison of pretreatment methods (a) Areca nut husk, (b) Bonbogori and (c) Moj.

The saccharification experiment was carried out for 96 hours and the final sugar concentration is reported in Figure 4.3.The maximum sugar concentration in the saccharified broth was reached 4.6%, 6.89% and 6.83% for Arecanut husk, Bonbogori and Moj respectively. The results also revealed that acid pretreated biomass samples were less prone towards enzymatic hydrolysis than the lime or ultrasound assisted lime pretreated biomass samples. The yield of sugar for using ultrasound-assisted biomass improved 13.05% to 50.54% than dilute acid pretreated biomass. Ultrasound assisted lime pretreatment removes the lignin, which facilitates the enzymatic hydrolysis, this may be the possible reason for obtaining more sugar from ultrasound assisted lime pretreated biomass samples.

4.5. Conclusions

During the study, it was observed that both ultrasound assisted lime pretreatment and lime pretreatment yield almost similar amount of sugar irrespective of biomass than acid pretreatment method. However, the yield of sugar was almost similar for both lime and ultrasound assisted lime pretreatment process, but the ultrasound assisted lime pretreatment process was much faster than the sole lime pretreatment process. The data of sugar hydrolysis revealed that the sugar yield became constant with enzyme loading of 20 and 25 FPU $g^{-1}$of cellulose. It may be considered that 20FPU g^{-1} of cellulose are sufficient enough for saccharification of biomass. The saccharification efficiency for ultrasound assisted lime pretreated biomass samples was highest and it was in the order of Bonbogori (82.5%) followed by Moj (80.1%) and Arecanut husk (79.5%). Therefore, it can be concluded that the ultrasound assisted lime pretreated biomass (Bonbogori) can be used (as it gave saccharification efficiency) for the next step of biofuel production i.e. fermentation process. 20 FPU g^{-1}cellulose recommended as the optimum enzyme loading for enzymatic hydrolysis or saccharification process.

References

[1] Tabka MG, Herpoël I, Sigoillot JC. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulasexylanasetreatment. Enzyme Micro Tech. 2006; 39:897–902.

[2] Lynd LR, Weimer PJ, Van WH, Pretorious IS. Microbial cellulaseutilizationFundamentals and biotechnology. Microbiology Molecular Biology Review.2002; 66: 506-577.

[3] Schulein M. Protein engineering of cellulasesBiochimBiophysActa-Protein StructMolEnzymol. 2000; 1543 (2): 239–252.

[4] Percival Zhang YH, Himmel ME, and Mielenz JR. Outlook for cellulase improvement: screening and selection strategies,Biotechnology Advances, 2006; 24: 452– 481.

[5] Schubert C, Can biofuels finally take center stage? Nature Biotechnology.2006; 24: 777–784.

[6] Technical report Genencoreinc 2009.

[7] Campbell MB, klinger J, and Sohonnard D. Feedstock mixture effects on sugar monomer recovery following dilute acid pretreatment and enzymatic hydrolysis. Bioresource Technol. 2012; 116: 320–326.

[8] Yesurf JN. Evaluation of cellulolytic enzymes from a newly isolated *Bacillus sp*. (2012) doctoral thesis Southern Illinois University.

[9] Choudhury R, Umagiliyage AL, Haddock J. Aqua-ammonia pretreatment of corn stover for enhancing enzymatic saccharification. Int J Agric&Biol Eng. 2012;5:56-61.

[10] Holm J, Lassi U, Romar H, Lahti R, Karkkainen J, Lajunen M. Pretreatment of fibre sludge in ionic liquids followed by enzyme and acid catalysed hydrolysis. Catalysis Today. 2011;196 (1):11-15.

[11] Badshah M, Liu J, Mattis B. Use of an Automatic Methane Potential Test System for evaluating the biomethane potential of sugarcane bagasse. Bioresource Technol. 2012;114: 262–269.

[12] Zhang Y-HP, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexedcellulase systems. BiotechnolBioeng 2004b;88:797–824.

[13] Wolfenden R, Snider MJ. The depth of chemical time and the powder of enzyme as catalysts. AccChem Res 2001;34: 938–45.

[14] Banerjee G, Car S, Scott-Craig JS, Borrusch MS, Aslam N. Synthetic Enzyme Mixtures for Biomass Deconstruction: Production and Optimization of a Core Set. BiotechnolBioeng. 2010; 106: 707-720.

[15] Brown, L. Torget R. Laboratory Analytical Procedure LAP 009, National Renewable Energy Laboratory, Golden, CO, 1996**.**

[16] Cybulska, I. Lei, H.Julson, J. Hydrothermal Pretreatment and Enzymatic Hydrolysis of Prairie Cord Grass Energy & Fuels. 2010; *24*: 718-727.

[17] Kim Y, Ximenes E, Mosier NS, Ladisch MR.Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme and Microbial Technology. 2011, 48 (4-5): 408–415.

Chapter V

Characterization of Wastewater Sludge

Wastewater Sludge can be described astwo-phase slurry consisting of water and solids. Wastewater sludge are generally used for land filling or as fertilizer. However, this material can be used as an excellent source of microorganisms. Several researchers reported a good amount of ethanol and hydrogen production by using wastewater sludge as a seed material from several cellulosic or lignocellulosic substrates. Therefore,the constituents of sludge materials should be properly characterized for the further downstream processing i.e. pretreatment of sludge followed by immobilization.In thisstudy,wastewater sludge was collected from the wastewater treatment plant of IIT Guwahati and analyzed for the several physical and chemical characteristics like proximate and ultimate analysis, thermo-gravimetric analysis, FTIR analysis etc.

5.1. Introduction

Sludge production is an unavoidable problem arising from the wastewater treatment plant. The sludge remained after municipal wastewater treatment contains considerable amounts of various contaminants and if the sludge is not properly handled and disposed to the environment, it may produce extensive health hazards. On the other hand, this sludge has benefits for plants and soils if it is utilized as fertilizer.

Wastewater treatment plant produces large amount of sludge as a result of various wastewater treatment methods such as flocculation, filtrationand coagulation. The general practice is to usethis sludge for land filling but wastewater sludge is a novel, compact consortium of self-immobilized bacteria that characteristically contains higher biomass concentrations. Other main advantages of sludge for using as a source of seed material for fermentation process are high biomass retention time in the reactor, good settling properties and the capacity to withstand high organic loading rate[1].The feasibility of anaerobic mixed cultures to enhance ethanol and hydrogen production from non-sterile organic substrates i.e. wastewater sludge has been studied thoroughly by several researchers [2, 3].Wastewater sludge is a potential seed for biohydrogen and ethanol production [4]. The yield of hydrogen and ethanol using wastewater sludge as seed material varies between 1.7 mole H_2mole^{-1} glucose to 2.0 mole H_2 mole^{-1}glucose [5, 6].

In this study, effort has been made to demonstrate the possibility of utilizing immobilized sewage sludge for simultaneous ethanol and hydrogen production in a bioreactor, which sought to investigate the characteristics of wastewater sludge.Conventional characterization parameters of wastewater sludge can be grouped asphysical, chemical and biological parameters. Physical parameters give general information on sludge processability.Chemical parameters are relevant to the presence of nutrients and toxic or hazardousmaterials. Biological parameters give information on microbial activity, organic mattersand presence of pathogens, which allows determining the degree of safety of use. The findings obtained from this study are expected to provide valuable information for the development of optimal immobilized-cell systems for simultaneous production of bio-hydrogen and ethanol in the bioreactors.

5.2 Materials and Method

5.2.1Wastewater sludge

Wastewater sludge sample was collected from IIT Guwahati wastewater treatment plant at Guwahati. The sampling was conducted three times at a regular interval from the same batch to make a composite sample. The wastewater sludge sample used in this work was first dried at room temperature and then separated from non desirable physical impurities, such as small fraction of leafs and plastic materials etc. The sample was then dried at 36 °C for seven days and ground to pass through 1mm sieve, the powdered samples was then stored in air tight plastic bags until use. The composition of the wastewater sludge sample used in this study was assessed on air-dried basis using proximate and ultimate analysis tests according to the methods described below.

5.2.2 Sedimentation

Settleability of sludge may be measured as sludge volume (SV) in a 1000 mL measuring cylinder (height of 36 cm) after 30 minutes sedimentation and is expressed for a known initial sludge concentration as sludge volume index (SVI).

5.2.3Proximate analysis

Total C and N of dry sludge were measured with an elemental analyzer (CHNS-mod. EA 1110, Carlo Erba, Italy). The percentage of C in the sample was calculated using acetanilide as a certified standard containing 71.09% of carbon.

5.2.4 Total Solids

The total solids concentration of sample can be obtained directly by weighing the sample before and after drying at 103^{o}C. The pH, temperature, specific gravity were measured off-line after taking samples from wastewater treatment plant according to the protocol of American Public Health Association(APHA) [7]. The procedure is described below.

Three evaporating dishes for each samplewereprepared by cleaning and ignition at 500^{o}C for one hour in a muffle furnace.The samples were then transferred to the prepared dish and the weight (weight of wet sample plus dish)was measuredin a drying (hot) oven. Volume of sample was chosen in such a way that the yield of residue lie between 2.5 mg and 200 mg. If necessary, successive sample portions to the same dish were added. During evaporation in a drying oven, the temperature was lowered to approximately 98^{o}C to prevent splattering. After this step the evaporated sample was dried for at least one hour at 103 to 105^{o}Cand theweight of the dish in the desiccator was noted (weight of dry solids). The cycle of drying at 103 to 105^{o}C, followed by cooling, desiccating, and weighing was continued until a constant weight is obtained, or until weight less than 4% of previous weight was obtained.

Calculation:

$$\text{Total solids, \% by weight} = \frac{A-C}{B-C}\times 100 \tag{5.1}$$

where:

A = weight of dried residue plus dish, mg

B = weight of wet residue plus dish, mg and

C = weight of dish, mg

5.2.5Volatile and Fixed Solids

After the total solids analysis was completed, the sample in a dish was kept for 20 minutes at 500°C in a muffle furnace and then cooledin a desiccator. During coolingthe top cover of the desiccator was opened for about two minutes to let off the hot gases. The desiccator was covered for complete cooling. When the temperature of the dish reached room temperature, the weight of the cooled dish was noted.

Calculation:

$$\text{Volatile solids, as \% total solids} = \frac{A-B}{A-C}\times 100 \tag{5.2}$$

$$\text{Fixed solids, as \% total solids } \frac{B-C}{A-C}\times 100 \tag{5.3}$$

where:

A = weight of residue plus dish before burning, mg

B = weight of residue plus dish after burning, mg

C = weight of dish, mg

5.2.6 Total Nitrogen

The total nitrogen in wastewater sludge is variable and may range from values between<0.1 to 18% [8]. The estimation of the amount of total nitrogen in the wastewater sludge was determined by Kjeldahl method. Total nitrogen is the combination of Total kjeldahl nitrogen (TKN) and nitrate nitrogen in the sample. In the presence of sulfuric acid,potassium sulfate and cupric sulfate (catalysts), the nitrogen of organic matter as well as free ammonia is converted to ammonium sulfate on digestion at 360°C –410°C. An excess of alkali is then added to liberate ammonia and distilled. The liberated ammonia is then absorbed in boric acid solution (mixed with indicator) and TKN is determined by titration with standard sulfuric acid or hydrochloric acid.

Calculation:

$$TKN = \frac{(A-B)\times N\times 1.4}{Wt}\times 10 \tag{5.4}$$

where

A= Standard H_2SO_4 (mL) used for sample
B = Standard H_2SO_4 (mL) used for blank
N= Normality of standard H_2SO_4
Wt = Weight of the sample (g)

5.2.7Total Phosphorous

Phosphorous is an essential nutrient used by organisms for growth. Phosphates are classified as orthophosphates, polyphosphates and organic phosphates. Phosphorus exists in several distinct forms in wastewater samples, in this procedure orthophosphates can be determined directly by colorimetric analysis. Other types require a digestion step to convert the "combined" phosphate to ortho form for analysis. This gives the amount of total phosphorus content.

Approx 50mL of sample was diluted to 1:1 ratio with distilled water. The diluted sample was then mixed with and 0.1mL phenolphthalein indicator, 1 mL of sulfuric acid solution and 0.4 g of ammonium persulfate. After vigorous mixing, the solution was boiled for 30 to 40 minutesfor digestion followed by coolingand neutralizationby 1(N) sodium hydroxide and made up the volume to 50 mL with distilled water. The digested sample was then analyzed for total phosphate by measuring absorbance at 880 nm using a reagent blank to zero the spectrophotometer. The value of total phosphate present in the sample was calculated using Eq. 5.5.

Calculation:

$$\text{Total phosphate (mg/L)} = \frac{A \times V}{1000 \times Wt} \qquad (5.5)$$

A= Conc. of phosphorous (mg/L) obtained from the calibration curve

Wt= Weight of the sample taken for digestion

V= Total volume of the digested solution

5.2.8Nutrient and Trace elements

Wastewater sludge is a byproduct of the wastewater treatment process and is enriched with metals, such as Cr, Cd, Cu, Ni, Se and Zn [9].Greater the amount of trace elements present in the sludge, less is the requirement of fortifying salts with it to support the growth of microorganisms.

The standard solutions of metals were prepared and calibrated using the Atomic Absorption Spectroscopy (AAS), to analyze the trace elements and other nutrients e.g. calcium, potassium and sodium present in the sludge sample. Approx 0.2 g of grounded sludge sample was digested in a digestion tube by addition of 10 mL of mixed acid solution (perchloric acid+ sulfuric acid; 1:5) for 30 to 40 minutes. The digested sample was filtered and analyzed for AAS using flame photometry.

5.2.9 Biological Oxygen Demand (BOD)

Organic matters are usually quantified as BOD (Biochemical Oxygen Demand).The Biological oxygen demand (BOD) test measures the dissolved oxygen consumed by microorganisms while assimilating and oxidizing the organic matter under aerobic conditions. The BOD values indicate the amount of biodegradable organic material (carbonaceous demand) and the oxygen used to oxidise inorganic material such as sulphides and ferrous iron. It may also measure the oxygen used to oxidise reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor [10].The BOD value, usually expressed in milligrams of oxygen consumed per liter of sample during five days of incubation at 20 °C, is most commonly used as a robust surrogate of the degree of organic pollution of water.

There are two recognized methods for the measurement of BOD, i.e., the dilution method and the manometric respirometric method. In the dilution method, the BOD test is carried out by diluting the sample with oxygen-saturated water and inoculating it with a fixed aliquot of "seed"; i.e., a small amount of micro-organisms that is typically generated by diluting a known concentration of microorganisms with buffered water. The sample is measured for dissolved oxygen (DO) and then sealed to prevent the entrance of any additional oxygen. The sample is kept at 20 °C in the dark (to prevent photosynthesis and, thereby, the addition of oxygen) for five days, after which the dissolved oxygen is measured again. The difference between the initial and final DOs is the BOD. The DO measurement in these samples is usually based on a manual iodometric titration, a procedure that is complicated and time-consuming.

In the manometric respirometric method, the tested sample is kept in a sealed container fitted with a pressure sensor. A substance (typically lithium hydroxide) that absorbs carbon dioxide (CO_2) is introduced in the container above the sample level before incubation. The sample is maintained in the dark for five days at 20 °C. The total amount of gas, and thus the pressure, decreases because the carbon dioxide released in microbial respiration is absorbed. The sensor electronics use the measured drop in pressure to compute and display the quantity of oxygen consumed. This method not only is simpler than the dilution method (i.e., no dilution of sample, no blank sample, and no titration required), but it can also be used performing shorter-term (less than 5 days) BOD testing.

Approx 20±0.5 g sludge sample was poured in a conical flask and 200 mL distilled water added into it followed by incubation for 2 h at 20°C. After completion of incubation period, the sample was centrifuged and the supernatant of the sample wascollected and analyzed for BOD test using digital DO probe. The BOD value was calculated using Eq. 5.6.

Calculation:

$$BOD\ (mg\ /\ ml) = \frac{D_1 - D_2}{P} \tag{5.6}$$

Where,

D_1 =DO of diluted sample immediately after preparation, mgL^{-1}

D_2 = DO of diluted sample after incubation at 20°C, $mg\ L^{-1}$

P= Sample volume diluted to 300 mL

5.2.10 Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) is an important parameter widely used to estimate the organic content of wastewater. The BOD analysis measures the amount of biodegradable organic compounds in terms of the equivalent oxygen concentration required for their decomposition. As COD determination (2–4 h) is simpler and faster than BOD (5 days), it is the preferred method for estimating the overall amount of the organic matters in wastewater.

The standard COD determination method requires a reflux process in acidic potassium dichromate solution at high temperature (148 °C). Typically, nearly all organic compounds can be fully oxidized to carbon dioxide with this method. The value of COD indicates the oxygen equivalent of the organic content that can be oxidised by potassium dichromate ($K_2Cr_2O_7$) using silver sulfate (Ag_2SO_4) as a catalyst under acidic conditions (H_2SO_4). The standard method (APHA, 1999) is well established and is used by the majority of researchers. A brief description of the process is stated below [11].

Approx 20±0.5 g sludge sample was poured in a conical flask and 200 mLdistilled water was added into it. The whole solution was kept in a shaking incubator for 2 h at room temperature followed by centrifugation at 6500 rpm for 10 min and the supernatant was collected. 1.5 mL of $K_2Cr_2O_7$ + 2.5 mL of sample (supernatant) +3.5 mL of sulfuric acid reagents were added to a COD vial and themixture was digested for 2 h in a digester at 150°C. The digested sample was then titrated against a standard FAS solution [Solution C: 0.12 mol L^{-1} $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$; 0.360 mol L^{-1} H_2SO_4]. Asharp change of the colour of the sample from bluish green to wine red signified the end of titration. A reagent blank simultaneously refluxed with the sample under identical conditions. The value of COD was calculated by using Eq. 5.7 as stated below.

Calculation

$$COD\ (mg/ml) = \frac{(V_1 - V_2)}{V_0} \times 8000M \tag{5.7}$$

Where

V_1= volume of $(NH_4)_2Fe(SO_4)_2\cdot 6H_2O$ requiredfor titration against the blank, in mL

V_2 = volume required for titration against the sample, in mL

M= Molarity of $(NH_4)_2Fe(SO_4)_2\cdot 6H_2O$

V_0 = volume of sample taken for testing in mL

5.2.11Thermal analyses

The thermo-gravimetric analysis (TGA) is one of the techniques used to study the primary reactions in the decomposition of solids since the heating rate is low and the cracking of primary products is negligible. The interpretation of the experimental data can provide information on the composition of the material, order of reaction, number of different processes that take place in the reaction. Thermogravimetric (TGA) analyses were carried out using a METTLER TOLEDO, Switzerland (Model no TGA 851e/ LF/1100), details on these procedures are described by Montecchio *etal*. (2006) [12]. In each test, one kg of wastewater sludge was pretreated. The sample was heated to 105 °C for 24 h and then kept indesiccators for further use. Sample of around 5 mg was used for each analysis. All the experiments were repeated three times to evaluate their reproducibility. The heating rate was maintained throughout the experiment (20 °C min^{-1}) with nitrogen flow rate 40 mL min^{-1}. Pure nitrogen was used as inert gas to prevent the presence of air. A constant flow rate of 100 mL min^{-1}nitrogen was used. The nitrogen flowrate preventsthe volatile products to remain close to the devolatilizing particles in the crucible, thus ensuring an inert atmosphere during the runs.

5.2.12 Microbiological analysis

The objectives of this study were to investigate the presence of active microbes in the sludge. In order to obtain a microbial count in the sludge, 0.1 g of wet sludge was weighed under sterile conditions and then suspended in 1.0 mL of deionized water. The samples were then mixed by vortexing for five minutes. 10 µL of the suspension samples were diluted into 50 mLdeionized water and shaken by hand for approximately two minutes. 100 µL of the diluted samples were spread onto different types of agar plates and incubated at 30 ^{o}C for two days. The details of each culture plate are illustrated herewith. Plate count agar (PCA) was designed for detection of bacteria, potato dextran agar (PDA) was used for cultivation of fungi possibly present in the sludge, malt extract agar (MEA) was used mainly for cultivation of potential yeasts grown in the sludge, and anaerobic agar (AA) was designated for observation of microorganisms that could grow under anaerobic conditions. PCA contained pancreatic digest of casein, yeast extract, dextrose, and agar; PDA contained potato starch, dextrose, and agar; MEA contained maltose, dextrose, glycerol, peptone, and agar; and AA contained agar with casein peptone, sodium chloride, dextrose, sodium thioglycollate, soy peptone, L-cystine, agar, sodium sulfoxyl formaldehyde, and methylene blue.

5.2.13 FTIR analysis

For spectroscopic investigations, the dried sludge material (fraction <0.55 mm) was mixed with FTIR grade KBr (1:100). The measurements were carried out in the mid-infrared range from 4000 cm^{-1} to 400 cm^{-1} with a FTIR spectrometer (Szimazdu IR Affinity 1). The reproducibility of the spectra of all the samples was verified on two sample preparations; from 64 to 100 scans with a resolution of 4 cm^{-1}. Baseline and other necessary corrections forpenetration depth and frequency variations were applied using Shimadzu IR solution 1.5 software supplied with the equipment.

5.3 Results and discussion

Water in sludge appears to exist in four forms[13].*Free water*: water that is not attached to sludge solids and that can be separated by simple gravitational settling and it was found to be 78% of the total volume. The sludge volume index (SVI)value was found to be 90, which shows that the sludge was well settled. *Interstitial water*: water that is trapped within the floc structure and travels with the floc or perhaps water trapped within a cell. This water can be released when the floc is broken up or the cell is destroyed. Some interstitial water might be removed by mechanical dewatering devices such as centrifuges. It was found to be around 20% of the total volume. *Vicinal water*: water that is associated with solid particles. This water is held on particle surfaces by virtue of the molecular structure of the water molecules and cannot be removed by centrifugation or other mechanical means. Vicinal water will not be free and it will exist as long as there is a surface. It was found to be about 1.5% of the total volume. *Water of hydration*: water that is chemically bound to the particle and can be released only by thermo-chemical destruction of the particles. It was found to be about 3% of the total volume.The specific gravity of the wastewater sludge was determined as 1.04 and the total solid concentration was 17.8%. Among this total solid the fixed carbon ash and other elements are also determined quantitatively (Table5.1). The volatile solids, and fixed solids as total solid (%)were found to be 53.21 % and 46.79% respectively. Sewage sludge contains considerable amounts of nitrogen and phosphorus, which can replace the need of nutrients for proliferation of microorganisms. Nutrient content of sludge has been found to varyconsiderably and is one of the sludge properties that have to be taken into consideration [14]. The quantity of nutrients in sewage sludge varies considerably butin general, average values of total Nand P are 3.8%and 2.2%,respectively on a dry matter basis [15].A range of trace elements present in the sludge were also quantified and shown in Table 5.2.BOD values indicate the amount of biochemically degradable organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulphides and ferrous iron. It can also be a measure of the oxygen used to oxidise reduced forms of nitrogen (nitrogenous demand), unless their oxidation is prevented by an inhibitor.Chemical oxidation test (COD) reflects the sum of the chemically oxidisable organic and inorganic compounds in the samples.An ideal biodegradability index is a BOD: COD ratio close to 1.0 [16]. Therefore, a bacterial seed that gives a ratio close to this value can be considered as efficient for degrading the particular system. In this case the value was found to be 0.46 and is well within the limit of biodegradability index. The values of BOD and COD of the present studied waste water sludge sample were illustrated in Table 5.1.

Table 5.1. Important parameters of raw wastewater sludge

Parameters	Value	Parameters	Value	Parameter	Value *	Parameter	Value*
Temperature	22	Moisture (%)	82.2	K	0.45	C	32.3
pH	6.48	Ash (%)	39.4	Na	0.68	H	4.01
Total Nitrogen (%TS)	1.5	Fixed Carbon (%)	6.9	Ca	4.4	N	4.2
Total Phosphorous (%TS)	2.3	Volatile Matter (%)	50.2	Mg	0.54	S	0.04
BOD (mg L^{-1})	234	Particle size	< 100 µm	Ba	0.04	Al	1.2
COD (mg L^{-1})	501	Specific Gravity	1.02	Fe	1.5	O_{CAL}	50.64

*Mean concentration (%TS)

Table 5.2. Metal concentration present in wastewater sludge

Metals	As	Ni	Cr	Cd	Se	Pb
Concentration (mg kg^{-1})	2.05	65	79	2.06	6.00	84.2

5.3.1. Thermogravimetric analysis

Thermogravimetric analysis provides a prior knowledge of initial and final temperatures for thermal degradation of sludge. The results of TGA/DTG are shown in Fig. 5.1.The most abundant release of volatile matter was observed in the temperature range 200–400 °C. Another peak was observed in DTG curveat temperature range of 635–665°C. The first peak might be due to decomposition and devolatilization of less complex organic structures. The second peak was caused by decomposition of more complex organic structures. The height of both the peak suggested that the amount of complex organic materials present in the sample were less than the less complex organic materials.

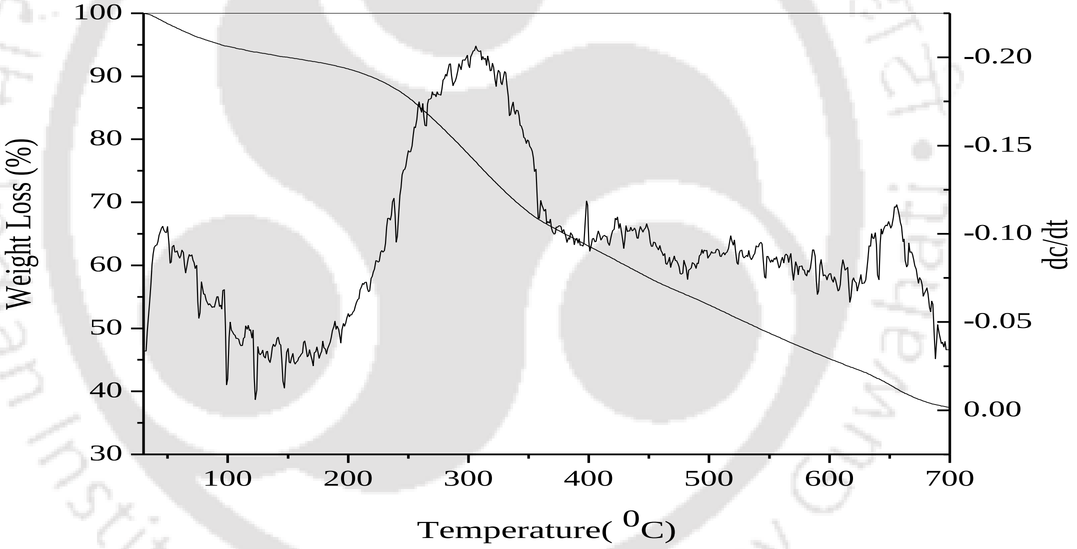

Fig. 5.1. Thermogravimetric analysis (weight loss and material degradation rate with respect to temperature) of wastewater sludge

5.3.2. Characterization of the sludge by FTIR

The chemical data provide information about individual compounds;FTIR spectral shapes of the wastewater sludge material describe the chemistry of the system in a general way. Band identification is based on the literature data. The most important bands found in the spectra of waste materials and their usual behaviors are described below.The region between 1150 and 950 cm^{-1}, with an intense band around 1117 cm^{-1} attributed to OH vibration of mineral compounds present in the sludge, but hydrocarbon and silicate compounds also absorb in this region as reported by Francioso *et al.* (2010)[17].

The stretching vibration of bonded and non-bonded hydroxyl groups and water causes the broad band at about 3400 cm^{-1} in each waste material. OH-bonding vibrations from water (1635 cm^{-1}), which is absorbed by functional groups in organic matter like lignocelluloses and inorganic constituents, can overlap vibrations from some functional groups [18].There is an absorption band related to the stretching of the N–H bond of organic compounds in this first region of thespectra, at 3428 cm^{-1}similar kind of results was reported by Nanny *et al.* (2002) [19]. Other absorption bands in the region from 3000 to 2800 cm^{-1} are attributed to the presence of hydrocarbon chains and bands in 2932 cm^{-1} is attributed to the asymmetrical stretching of C–H bonds from mehyl and methylene groups respectively similar kind of results was observed by Silva*et al.* (2012) [20].

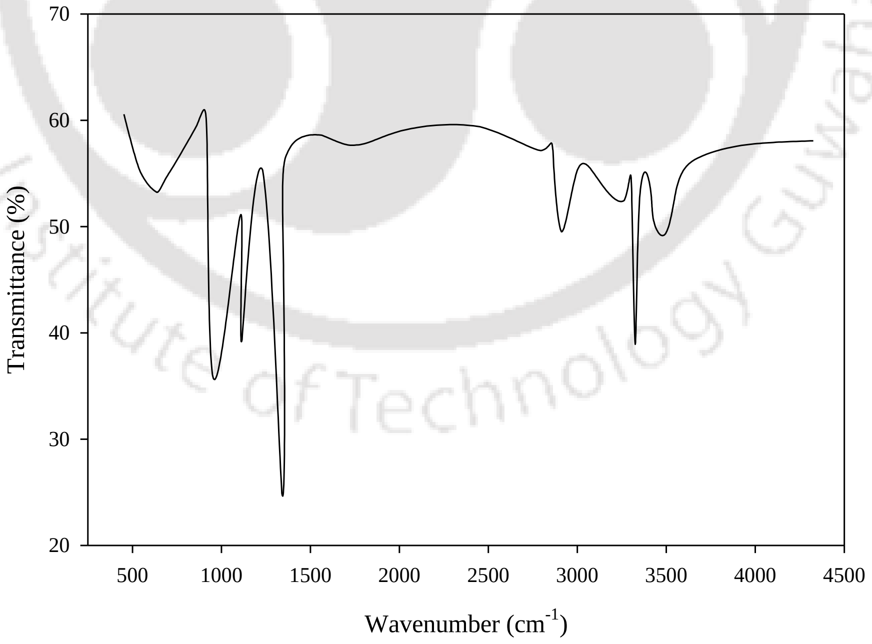

Fig5.2. FTIR analysis of wastewater sludge

5.3.3Microbiological analysis

Large numbers of bacterial colonies grew on each type of agar plates. However, few fungi or yeast colonies were observed on the plates (PDA and MEA). Thus, bacteria were the dominant microorganisms in the sludge. The results of quantitative analysis by cellcount. The bacteria also grew well in both anaerobic and aerobic conditions without a significant difference. The Microbial cell number per gram of wet sludge was 6.14 $\times 10^7$ and 2.68 $\times 10^8$ foranaerobic (AA) and aerorobic condition (PDA) respectively.

5.4. Conclusion

In the present study, the chemical and physical features of wastewater sludge indicates that the sludge contains good amount of nitrogen (1.5% of TS), phosphorous (2.3% of TS) and other essential trace elements. These elements along with nitrogen and phosphorous facilitate microbial growth and reduce the need for addition of supplementary nutrient elements in the fermentation medium. Therefore, the results ensure the usability of sludge as a seed material for simultaneous ethanol and hydrogen production.The number of bacteria in the sludge was counted around 10^8 per gram of wet sludge. Bacteria in the sludge could be grown in both anaerobic and aerobic conditions in the culture media.

Reference

[1] Beun JJ, Hendriks A, Loosdrecht MCM, Morgenroth E, Wilderer PA, Heijnen JJ Aerobic granulation in a sequencing batch reactor. Water Research,1999; 33 (10): 2283–2290.

[2] Fan Y, Li C, Lay JJ, Hou H, Zhang G. Optimization of initial substrate and pH levels for germination of spurring hydrogen-producing anaerobes in cow dung compost. Bioresource Technology, 2004; 91:189–193.

[3] Chen CC, Lin CY, Lin MC. Acid-base enrichment enhances anaerobic hydrogen production process. Appl Microbiol Biotechnology, 2002; 58: 224–228.

[4]Han W, Wang,Z, Chen H, Yao X, Li Y. Simultaneous biohydrogen and bioethanol production from anaerobic fermentation with immobilized sludge. Journal of biomedicine & biotechnology, 2011; 2011: 343-791.

[5]Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnology, 2001; 57:56–64.

[6]Wang A, Cao G, Liu W. Biohydrogen Production from Anaerobic Fermentation. Adv Biochem Eng Biotechnology, 2012;128:143-63.

[7] APHA (American Public Health Association), Standard Methods for the Examination of Water and Wastewater. 20th ed. American Public Health Association, Washington, DC, USA, 1998.

[8]Sommers LE .Chemical composition of sewage sludges and analysis of their potential use as fertilizers Journal of Environmental Quality, 1977; 6 (2): 225–232.

[9] He YD, Zhai YB, Li CTL, Yanf F, Chen L, Fan XP, Peng WF, Fu ZM. The fate of Cu, Zn, Pb and Cd during the pyrolysis of sewage sludge at different temperatures Environmental Technology, 2010; 31: 567–574

[10] Liu J and Mattiasson B. Microbial BOD sensors for wastewater analysis. Water Research. 2002; 36: 3786–3802

[11] Standard Methods for the Examination of Water and Wastewater American Public Health Association, Washington, DC (1999)

[12] Montecchio D, Francioso O, Carletti P, Pizzeghello D, Chersich S, Previtali F, Nardi S Thermal analysis (TG-DTA) and DRIFT spectroscopy applied to investigate the

evolution of humic acids in forest soil at different vegetation stages J. Therm. Anal. Calorimetry, 2006; 83 : 393–399.

[13] Vesilind PA, Martel C.J. Thermal conductivity of sludges. Water research,1989; 23 : 2393-2398.

[14] G. Tchobanoglous and F.L. Burton (Eds.), 1820 New York: McGraw-Hill.

[15] Smith S.R. 1996**.** Agricultural recycling of sewage sludge and the environment. CAB International. 382.

[16] Kumar A, Dhali P, Kumar R. Redefining BOD:COD ratio of pulp mill industrial wastewaters in BOD analysis by formulating a specific microbial seed. International Biodeterioration & Biodegradation,2010; 64(3): 197–202.

[17] Francioso O, Rodriguez-Estrada MT, Montecchio D, Salomoni C, Caputo A, Palenzona D

Chemical characterization of municipal wastewater sludges produced by two-phase anaerobic digestion for biogas production .J. Hazard. Material, 2010; 175: 740–746.

[18] Faix O. Classification of lignins from different botanical origins by FTIR spectroscopy.Holzforschung, 1991; 45: 21–27.

[19] Nanny MA, Ratasuk N. Characterization and comparison of hydrophobic neutral and hydrophobic acid dissolved organic carbon isolated from three municipal landfill leachates. Water Research, 2002; 36:1572–1584.

[20] Silva JO. Filho GR, Meireles CS, Riberio SD, Vieiria JG, Silva CV, Cerqueira DA. Thermal analysis and FTIR studies of sewage sludge produced in treatment plants. The case of sludge in the city of Uberlândia-MG, Brazil. Thermochimica Acta, 2012; 528(20): 72–75.

Chapter VI

Simultaneous Ethanol and Hydrogen Production through Fermentation

Fermentation is the ultimate step of biofuel production. Cellulosic ethanol is one of the most promising technological options available to reduce transportation sector greenhouse gas emissions. Bio-ethanol produced from plant starch and simple sugars has been the most successful biofuel to date. This study utilized the fermentable sugars produced from lignocellulosic biomass materials after saccharification of ultrasound-assisted lime pretreatment followed by fermentation using wastewater sludge as a source of microorganisms to produce bioethanol and biohydrogen. The study includes optimization of fermentation process using Taguchi methodology. Four important parameters such as type of reactors, temperature and pH of operation and organic loading rate were considered for optimization process. The optimum yield of both ethanol and biohydrogen were found to be 0.21 mol, and 30.8± 0.5-mmol respectively.

6.1 Introduction and literature review

The word fermentation is derived from a latin verb "*fevere*" which means to boil. However, events of boiling come into existence from the fact that during alcoholic fermentation, the bubbles of gas disintegrate at the surface of a boiling liquid and give the warty appearance. The fermentation process can be described as processes in which cellular energy is generated from the breakdown of nutrient molecules where there is no net change in the oxidation state of the products as compared with that of the reactants [1]. This definition of fermentation had the little meaning until the metabolic processes were known. Almost in all organisms, the metabolic pathways generating energy are fundamentally similar. The oxidation reaction for generating ATP may be accomplished in the presence of oxygen (in aerobes) or in absence of oxygen (in anaerobes). Thus in aerobic microorganism the process of ATP generation is referred to as cellular respiration, whereas in anaerobes or aerobes functioning under anaerobic condition is known as anaerobic respiration or fermentation.

Although fermentation (e.g. brewing and wine production) was done for many hundred years but it was L. Pasteur who observed microorganisms associated with fermentations. In 1815, Gay Lussac formulated the conversion of glucose to ethanol. After 1940, rapid development took place in the production large industrial solvent fermentation and distillation plant. Biological route is an alternative way to produce biofuels. Due to rising environmental concerns and to the periodic crises in some of the larger oil exporting countries, biofuels become a viable and realistic alternative in the energy market. Production of ethanol and other biofuels from cellulosic biomass is a major focus, with additional biotechnological paths to producing energy also receiving attention [2]. All lignocellulosic biomass are biodegradable and, to varying degrees, may be converted biologically in anaerobic digestion processes to several biofuels such as ethanol, hydrogen.

Valuable and interesting works have been published on the theme of fuel ethanol and biohydrogen production especially from lignocellulosic biomass. To produce biofuels from starch it is necessary to breakdown the chains of this carbohydrate for obtaining glucose syrup, which can be converted into ethanol by yeasts. This type of feedstock is the most utilized for ethanol production in North America and Europe. Corn and wheat are mainly employed with these purposes [3]. In tropical countries, other starchy crops as tubers (e.g. cassava) can be used for commercial production of fuel ethanol. Gnansounou *et al.* (2005), have examined the possibility of producing fuel ethanol from the juice of sweet sorghum in the conditions of North China [4]. However, the analysis of integration features of the overall process for fuel ethanol production from different feedstocks along with bio-hydrogen production has not been the main objective of those reports. Therefore, the objective of this work is to production of both bio-ethanol and bio-hydrogen simultaneously and explores integration as an important avenue for process improvement in the production of this liquid biofuel.

6.1.1 Bio-ethanol

Alcohol based fuels have been important energy sources since the 1800s. It has been used as fuels in three main ways: as a fuel for combustion engine, as a fuel additive to achieve octane boosting effects, and as a fuel for direct conversion of chemical energy into electrical energy in a fuel cell. The ideal biofuel needs to be produced from nonfood feed stocks, to be readily avail-able throughout the year, to serve as a drop-in replacement into existing infrastructure, and to be as energy dense as gasoline (petrol) or diesel. The production of bioethanol from different feedstock in different countries is tabulated in Table 6.1.

Table 6.1. Feedstock for bioethanol production in different countries

Feed stock	Country	Reference
Sweet sorghum	China	[5]
Sugar beet	Germany	[6]
Sugarcane	Brazil	[7]
Corn	USA	[8]

Ethanol as well as hydrogen is widely recognized as a green fuel for substitution or partial substitution of petroleum based fuels such as gasoline [9]. Bio-ethanol is ethyl alcohol, grain alcohol, or chemically C_2H_5OH or EtOH. Fermentation of sugars gives ethanol, CO_2 and heat. One mole of glucose yields two moles of C_2H_5OH and two moles of CO_2. One kg of glucose will theoretically produce 0.51 kg of ethanol and 0.49 kg of CO_2 [10]. The production of bio-ethanol from biomass is widely studied process. The fermentative routes has advantages than other chemical routes for production of ethanol such as the process is non-hazardous, less energy intensive and capable of ferment wide spectrum of lignocellulosic materials as a substrate for fermentation [11]. The baker's yeast (*Saccharomyces cerevisae*) is known for the production of bio-ethanol and it was proved to be more robust than bacteria and other yeasts with respect to both the tolerance to ethanol and inhibitors present in hydrolysates. However, due to its metabolic limitations, it cannot produce hydrogen [12]. The yeast (*S. cerevisiae*) has shown crucial limitations in relation to the pentose fermentation. This is due to the following reasons, (1) inability of *S. cerevisiae* to utilize pentose [13], (2) low levels of expression of the xylose-fermenting genes that codify for xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulose kinase (XK) [14], (3) low expression of XK limits the rate of the xylose fermentation and (4) imbalance of the redox cofactors (NAD^+/NADH and $NADP^+$/NADPH ratios) that reduces rates and ethanol yield from pentose [15].

6.1.2 Bio-hydrogen

The term bio-hydrogen derives from the latin words '*hydrogenium*', from Ancient Greek translated as hydro: which means "water" and genes means “born of” or “forming". The concept of bio-hydrogen production is based on the exploitation of bacteria to produce hydrogen as a by-product during the growth of biomass and the majority of the biologically produced hydrogen in the biosphere is derived from microbial fermentation processes [16].

It is well known that carbohydrates are the main source of hydrogen during fermentative processes and therefore wastes/wastewater or agricultural residues rich in carbohydrates can be considered as potential sources for hydrogen production [17]. Glucose and sucrose are the fermentation substrates mostly studied in the laboratory [18-19].

The hydrogen yield varies proportionally to the final metabolic products. Production of acetic and butyric acids favors production of hydrogen with the fermentation to acetic acid giving the highest theoretical yield of 4 mol H_2/mol hexose, while low H_2 yields are associated with more reduced end products, i.e. propionic and lactic acids and ethanol [20]. However, mixed acid fermentation producing butyrate in excess of acetate occurs upon biological degradation of glucose by clostridia-type microflora [21-23].

There are five possible routes exists for the production of microbial hydrogen viz. photolysis, photo fermentation, dark fermentation, water gas shift reaction and microbial fuel cells.

6.1.2.1 Photolysis

In this reaction, hydrogen is produced from water using light as a source of energy (direct photolysis (Eq. 6.1) or via production of glucose (indirect photolysis; Eq. 6.2 and Eq. 6.3) by green algae or cynobacteria.

Direct photolysis

$$2H_2O \xrightarrow{LIGHT\ ENERGY} 2H_2 + O_2 \tag{6.1}$$

Indirect photolysis

$$6H_2O + 6CO_2 \xrightarrow{LIGHT\ ENERGY} C_6H_{12}O_6 + 9O_2 \tag{6.2}$$

$$C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2 \tag{6.3}$$

Advantages

1. Has the ability to fix N_2 from atmosphere so there is no need to supplement the nitrogen rich foods to the organisms
2. Can produce H_2 directly from water and sunlight

Disadvantages

1. Requires high intensity of light
2. Low photochemical efficiency

6.1.2.2 Photo fermentation

Purple photosynthetic microbes are able to produce hydrogen from organic substrates through photo-fermentation route. This pathway is one of the promising routes of biohydrogen production, due to its higher rate of hydrogen production [24]. Hydrogen production by purple non sulfur bacteria was predominating in this process. Presence of nitrogenase enzyme in purple non sulfur bacteria helps to reduce substrates under oxygen-deficient conditions (Eq. 6.4).

$$C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2 \qquad (6.4)$$

Advantages

1. The process can be versatile in terms of types of feed to the microbes [25]
2. They can utilize organic acids produced by dark fermentative microbes as the substrate for photo fermentation

Disadvantages

1. Low volumetric rates of production
2. Low photosynthetic conversion efficiencies

6.1.2.3 Dark fermentation

Dark fermentation process is the fermentative conversion of organic substrate to biohydrogen. This process uses primarily anaerobic bacteria, although some algae are also used. The microorganisms grow on carbohydrate rich substrates as the name indicates, in the dark and produces hydrogen along with different metabolic products. The pathways are dependent on the type of bacteria used [26]. Standard fermentative pathway theoretically gives maximum four moles of hydrogen per one mole of glucose (Eq. 6.5) [27].

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2 \qquad (6.5)$$

Advantages

1. It produces valuable metabolites along with hydrogen such as butyric, lactic and acetic acids as by products
2. It is anaerobic process, so there is no O_2 limitation problem

Disadvantages

1. Relatively lower achievable yield of H_2

2. As yield, increases H_2 fermentation becomes thermodynamically unfavorable

6.1.2.4 Water gas shift reaction

Few photo heterotrophic bacteria in the family *Rhodospirllacae* can grow in dark environment. These microorganisms need only CO and water for their survival. It was reported that CO supports both cell growth and ATP synthesis, in dark and same ATP can be used as a catalyst for water gas shift reaction. Since it occurs at low temperature and pressure, water gas shift reaction favor a high conversion of CO to CO_2 and H_2 (Eq. 6.6) [28]. Organisms which grow and produce hydrogen are both the gram-negative bacteria, such as *R. rubrum* and *Rubrivax gelatinosus*, and the gram-positive bacteria, such as *Carboxydothermus hydrogenoformans*.

$$CO + H_2O \longrightarrow H_2 + CO_2 \qquad (6.6)$$

Advantages

1. Compared with thermo-chemical water gas shift processes, the cost of biological water gas shift processes are lower due to the elimination of reformer and associated equipment
2. Few microorganisms can convert 100% Carbon monoxide to non stoichiometric amounts of Hydrogen [29]

Disadvantages

1. The common objectives of these works were to identify suitable microorganisms that had high CO uptake and to estimate the hydrogen production rate
2. The reaction rate is slower than thermo-chemical process

6.1.2.5 Microbial fuel cells

A microbial fuel cell is an electrochemical device which converts the chemical energy of fuel to electrical energy by the catalytic actions of microorganisms [30]. Many microorganisms such as *Actinobacillus succinogenes, Clostridium beijerinckii, Alcaligenes faecalis, Enterococcus gallinarum, Pseudomonas aeruginosa* etc. possess the ability to transfer the electrons derived from the metabolism of organic matters to the anode. Various microbial or biochemical fuel cells have been developed using *Desulfovibrio desulfuricans, Proteous vulgaris, Escherichia coli, Pseudomonas* species and redox enzymes as biocatalysts [31].

Biotechnology-based MFC approaches hold great promise as methods for renewable energy production [32]. Under normal operating conditions, protons released by the anodic reaction migrate to the cathode to combine with oxygen to form water. Hydrogen generation from the protons and the electrons produced by the metabolism of microbes in an MFC is thermodynamically unfavorable. Researchers applied an external potential to increase the cathode potential in a MFC circuit to overcome the thermodynamic barrier. In this mode, protons and electrons produced by the anodic reaction are combined at the cathode to form hydrogen. The required external potential for an MFC is theoretically 110 mV, much lower than the 1210 mV required for direct electrolysis of water at neutral pH because some energy comes from the biomass oxidation process in the anodic chamber. MFCs can potentially produce more than 8–9 mol H_2 mol^{-1} glucose (Eq.6.7) compared to typical 4-mol H_2 mol^{-1} glucose achieved in conventional fermentation [33].

$$C_6H_{12}O_6 + 6H_2O \xrightarrow{\text{ELECTRIC } ENERGY} 12H_2 + 6CO_2 \tag{6.7}$$

Advantages

1. Can be operated at ambient conditions
2. High conversion efficiency

Disadvantages

1. Costly to operate
2. Sensitive to breakdown and decay

6.1.3 Simultaneous ethanol and hydrogen production

The fermentative routs of hydrogen and ethanol production are possible through either butyric acid type fermentation or ethanol type fermentation [34]. Many researchers' supports for butyric acid type fermentation process for hydrogen production as it yields more hydrogen but it lacks the stability of $NADH^+$ and H^+ accumulation during fermentation, whereas ethanol type fermentation process yield neutral products such as ethanol which reduces the acidic products, generated during fermentation process and leads to acidic fermentation process smoothly [35]. The summary of possible equation along with change in Gibbs free energy (ΔG) for simultaneous ethanol and hydrogen production is illustrated in Eq.6.8.

$$C_6H_{12}O_6 + 3H_2O \rightarrow C_2H_5OH + CH_3COO^- + 2HCO_3^- + 2H_2 + 3H^+ \qquad (6.8)$$

$$\Delta G_0 = -97 \text{ kJ mol}^{-1}$$

The stoichiometry of the equation describes that it can produce two mol of hydrogen and one mol of ethanol from one mole of glucose.

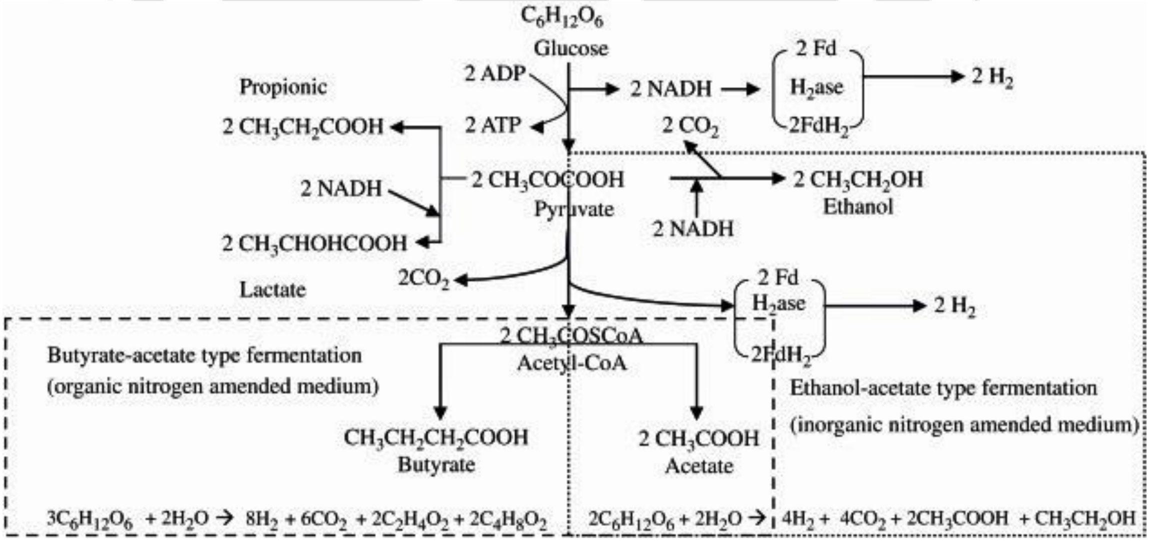

Fig. 6.1 Major metabolic pathways for glucose fermentation in mixed cultures [36-37]

The optimum pH for hydrogen production was reported in various literatures to be between 5.5 and 6.0 [38-39]. pH between 6.0 and 6.5 can produce an excessive amount of propionic acid. However, pH values lower than 4.5 are conducive to the production of H_2, CO_2, acetic acid, butyric acid, and ethanol [40]. As also indicated by the authors, the bacterial community structure of the mixed culture producing H_2 and EtOH at an equally important amount could be markedly different from the culture producing mainly H_2, because H_2 and EtOH production seem to be competitive events from the perspective of metabolic electron transfer and energy generation. A decreased pH gives origin to a phase of solvent production, such as EtOH, toward the end of the anaerobic fermentation. Gottschalk *et al.* (1986) reported that volatile fatty acids are no longer produced toward the middle of the batch growth of *C. acetobutyricum* and that n-butanol, acetone, and EtOH appear as new products toward fermentation [41]. According to the studies conducted by Wu *et al.* (2007), Koskinen *et al.* (2008), and Wang *et al.* (2008), yield of hydrogen decreases with increasing ethanol yield. In general, hydrogen yield and ethanol yield are inversely correlated irrespective of the type of sugar substrate or bioreactor used [42- 44].

As stated by Hwang *et al.* (2004) and Zhu *et al.* (2009), there are several pathways (Fig 6.1) for the production of organic acids, EtOH and hydrogen which can be explained by (Eqs. (6.9)–(6.11)) [45-46].

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2 \tag{6.9}$$

$$C_6H_{12}O_6 \rightarrow C_2H_5OH + \frac{1}{2}CH_3COOH + 2CO_2 + 2H_2 + 3H^+ + \frac{3}{4}CH_3(CH_2)_2COOH \tag{6.10}$$

$$C_6H_{12}O_6 \rightarrow + \frac{2}{3}CH_3COOH + \frac{2}{3}CO_2 + \frac{2}{3}H_2O + \frac{4}{4}CH_3CH_2COOH \tag{6.11}$$

From Eq. (4), it is possible to verify that hydrogen and EtOH can be produced because this pathway presents no competition between these two biofuels; they are produced concomitantly. Additionally, HAc and HBu may be produced via other metabolic pathways, as indicated in Eqs. (6.10) and (6.11).

6.2 Materials and Method

6.2.1 Preparation of Substrate for Fermentation

The biomass material (Bonbogori) was collected from the local forest near IIT Guwahati of Assam. All the biomass samples were chopped; air-dried, milled and prepared samples were used for ultrasound assisted lime pretreatment process. The ultrasound-assisted lime pretreatment was conducted according to the recommended design (Lime loading ratio 0.75; Biomass loading ratio 20 and duration of ultrasound 180 min). The residual pretreated biomass samples were then washed and followed by saccharification process as described in Chapter 4 (Temperature –50 °C; RPM 150, pH 4.5; Enzyme loading 20 FPU g^{-1} of cellulose). The saccharified sugar solution was then used as the substrate of fermentation process.

6.2.2 Bioreactors

Fig 6.2 (a) and (b) shows the schematic diagrams of both fluidized bed bioreactor and stirred tank bioreactor respectively. Fluidized bed bioreactor (FBBR) was constructed of glass with the following dimensions: 55 cm height, an internal diameter of 5 cm, and a total volume of 1500 mL. The substrate was recycled through a recycling pump. The stirred tank bioreactor (STBR) was made of glass with the following dimensions: 27 cm height, an internal diameter of 11 cm, and a total working volume of 1500 mL. The temperature of both types of reactors was maintained by recirculating heated water from a thermostatic bath through the column water jackets. A gas liquid separator was used at the outlet to collect gaseous and soluble products separately. The hydrogen gas was collected by means of a water displacement method. The liquid flow rate velocity maintained throughout the process for each experiment was 95 to 110 L h^{-1}

to fluidize the static bed materials. The biogas (CO_2 and H_2) was measured by means of liquid dispersing method and the gas components were analyzed by gas chromatography.

6.2.3 Immobilization of Sludge and Media Preparation

The wastewater sludge was collected from the wastewater treatment plant of IIT Guwahati. Hydrogen productivity of the sludge was enhanced by pretreating with HCl (0.1 N) at pH 3.0 for 24 h to eliminate the methanogenic activity [47].

50 mL of pretreated sludge was mixed with 100 g polyethylene–octene elastomer (POE). The resulting mixture was cell-entrapped colloid beads (1.5 mm diameter). After being rinsed with deionized water, the colloid bead was immersed into a solution containing 0.5 g sodium alginate, 0.5 g zirconium oxide and 5 g of pretreated sludge and was then transferred to 0.1M $CaCl_2$ for solidification. The resulting immobilized-cell beads showed an average density of 1.4 g cm^{-3}.

The medium used for this study was hydrolysate of pretreated biomass (bon bogori) along with the wastewater sludge. The other inorganic supplements to support the growth of microorganisms for both ethanol and hydrogen production was: $CaCl_2{\cdot}2H_2O$, 100 mg; $MnSO_4{\cdot}6H_2O$, 1.5 mg; NH_4Cl, 2600 mg; K_2HPO_4, 250 mg; $MgCl_2$, $6H_2O$, 125 mg; $FeSO_4$,$7H_2O$, 5 mg; $CoCl_2$, $6H_2O$, 2.5 mg; $MnCl_2$, $4H_2O$, 2.5 mg; Na_2MoO_4 , $2H_2O$, 0.5 mg; H_3BO_4, 0.5 mg; $NiCl_2$, $6H_2O$, 0.5 mg; $ZnCl_2$, 0.5 mg antifoaming agent. The chemical oxygen demand (COD) value of the prepared substrate solution was found to be in the range of 30-50 kg COD/L.

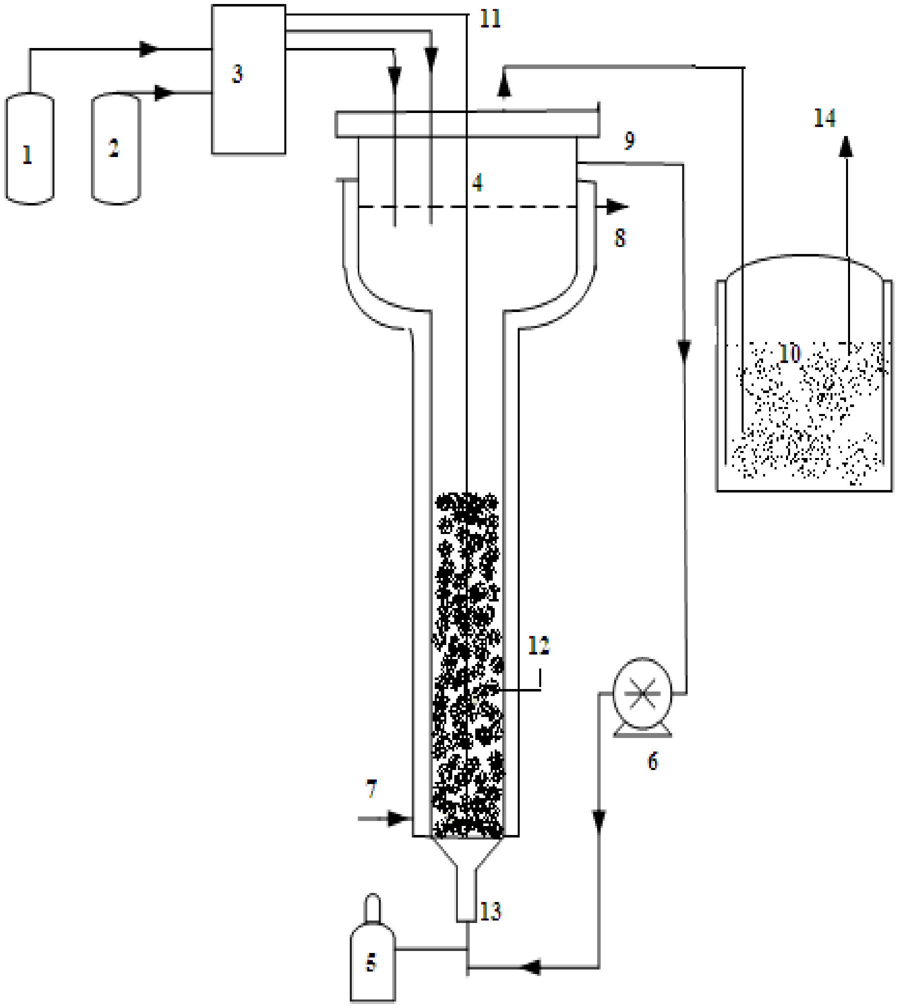

Fig. 6.2. (a) Schematic diagram of fluidized bed bioreactor

1. acid tank, 2. base tank, 3. controller 4. fluidized reactor, 5. media storage tank, 6. circulation pump, 7. hot water inlet at jacket of fluidized bed reactor, 8. hot water outlet from jacket of fluidized bed reactor, 9. media outlet, 10. water displacement tank, 11. provision of gas inlet (nitrogen for making the environment anaerobic), 12. beads of waste water sludge, 13. medium inlet to fluidized bed, 14. provision to collect the gas.

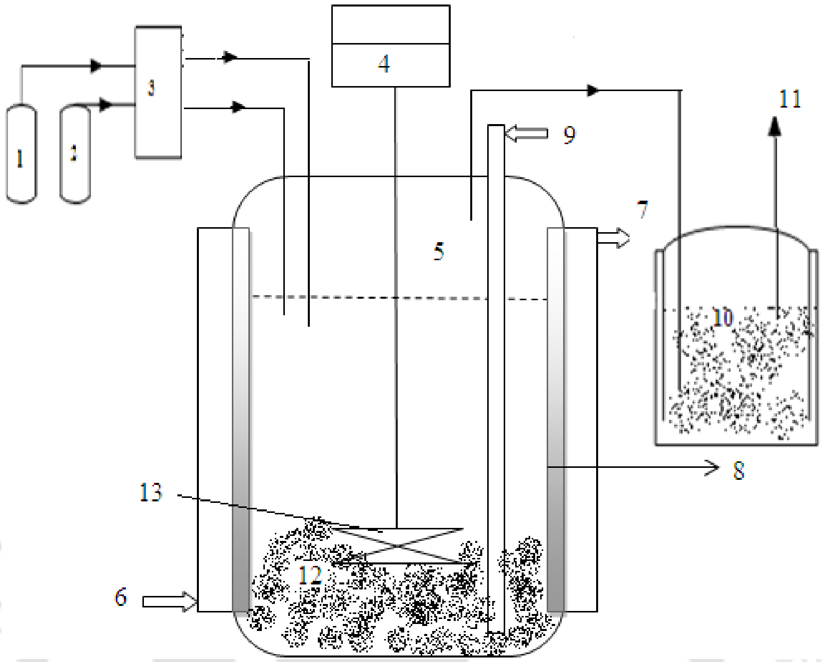

Fig. 6.2. (b) Schematic diagram of Stirred Tank Bioreactor

1. acid tank, 2. base tank, 3. Controller, 4. Motor, 5. Reactor, 6. hot water inlet at jacket of fluidized bed reactor, 7. hot water outlet from jacket of fluidized bed reactor, 8. Baffles, 9. media/sampling port, 10. water displacement tank anaerobic, 11. provision to collect the gas, 12. beads of wastewater sludge, 13. impeller.

6.2.4 Fermentation

The simultaneous ethanol and bio-hydrogen optimization experiments were conducted according to the Taguchi method of design experiment. The experiments were designed with four factors in combination of two and three different levels viz. type of reactor, temperature, pH and organic loading rate and their different levels that will be optimized were tabulated in Table 6.2. Selection of this parameter was based on the through literature survey of various researchers. “1”, “2” or “3” represent the three levels of each factor in the matrix. For analysis of the results and optimization of conditions for setting the control factors, MINITAB software was used.

50 g (approximately) of immobilized acid treated wastewater sludge was poured into the reactor to make a static bed. The height of this static bed was about 5 cm. The broth was fed from the bottom of the reactor along with the nitrogen gas thoroughly to create the anaerobic condition. The same amount of (50 g) of immobilized acid treated wastewater sludge was poured into the STBR. The broth was fed from the top of the reactor along with the nitrogen gas thoroughly to create the anaerobic condition. The speed of stirrer was maintained at 150 RPM. The pH of the medium was adjusted by using pH controller as described in diagrams (Fig. 6.2 (a) and (b)). The detail design matrix of experimental design and results for simultaneous ethanol and hydrogen production was described in Table 6.3.

Table 6.2 Different levels of the parameters used for experimental design

Factor	Parameters	Level 1	Level 2	Level 3
A	Reactor	FBBR*	STBR**	-
B	Temperature (°C)	30	40	50
C	pH	4.5	5.5	6.5
D	Organic Loading Rate	30	40	50

- * FBBR = Fluidized Bed Bioreactor
- ** STBR = Stirred Tank Bioreactor

Table 6.3. The Design matrix for simultaneous ethanol and hydrogen production

Run	A	B	C	D
1	1	1	1	1
2	1	1	2	2
3	1	1	3	3
4	1	2	1	1
5	1	2	2	2
6	1	2	3	3
7	1	3	1	2
8	1	3	2	3
9	1	3	3	1
10	2	1	1	3
11	2	1	2	1
12	2	1	3	2
13	2	2	1	2
14	2	2	2	3
15	2	2	3	1
16	2	3	1	3
17	2	3	2	1
18	2	3	3	2

6.2.5 Analytical Methods

The analysis of gas samples was carried out by gas chromatography (GC) using a thermal conductivity (TCD) detector and molecular sieve column with argon as a carrier gas. The injector, detector and column temperatures were 30 °C, 200 °C and 230 °C respectively. The liquid samples were analyzed by HPLC (Repromer H+, 9 µm, 300 × 7.8 mm; Temperature 25 °C; Eluant: 9 mM sulfuric acid; Flow 1 mL min^{-1}; Pressure: 90 bar). Standard method was used to determine the COD, VSS, TS of the wastewater sludge [48].

6.3 Results and Discussion

The simultaneous production of hydrogen and ethanol from the ultrasound assisted lime pretreated lignocellulosic biomass (Bonbogori) was performed accordingly to the design of experiment (Table 6.3) in both stirred tank and fluidized bed bioreactor.

Table 6.4. The results for simultaneous ethanol and hydrogen production

Run	Ethanol (mol/substrate)	Hydrogen (mmol/substrate)	$\hat{D}$ avg
1	0.14	11.2	0.125
2	0.2	16.4	0.181
3	0.12	26.9	0.180
4	0.19	16.5	0.177
5	0.29	22.3	0.254
6	0.2	34.5	0.263
7	0.21	10.1	0.146
8	0.17	26.1	0.211
9	0.08	26.8	0.146
10	0.13	9.5	0.111
11	0.09	14.5	0.114
12	0.11	17.1	0.137
13	0.22	10.1	0.149
14	0.18	18.5	0.182
15	0.09	26.8	0.155
16	0.14	13.1	0.135
17	0.08	16.5	0.115
18	0.11	19.1	0.145

The results of the experiments were tabulated in Table 6.4. In this study, two important outcomes were measured simultaneously. Therefore, the production of both ethanol and hydrogen was considered here as two responses. The experimental data presented in Table 6.4 were the average values obtained from triplicate runs. The individual S/N plots for ethanol, hydrogen and their combinations were presented in Fig 6.3 (A) & (B).

According to the Taguchi method of optimization the optimal combination for maximizing the yield of ethanol and hydrogen were A_1, B_2, C_1, D_2 and A_1, B_2, C_3 and D_3 respectively. The subscript after each factor is the optimal level. The result showed that the optimal level of pH (factor C) and organic loading rate (factor D) for maximizing the ethanol yield antagonize with the maximization of hydrogen yield. The conventional method of Taguchi may not suitable for optimizing this duel response problem. The desirability function was applied to optimize this duel response problem. The detail of the desirability function was described elsewhere in previous section of this article. The combined response of ethanol and hydrogen was represented by overall desirability. The desirable ranges are from zero to one (least to most desirable, respectively). The mean overall desirability ($\hat{D}$) of each experimental run based on L_{18} orthogonal array was calculated and represented in Table 6.4. A new S/N plot (Fig 6.3. (C)) was prepared considering mean overall desirability ($\hat{D}$) as quality index.

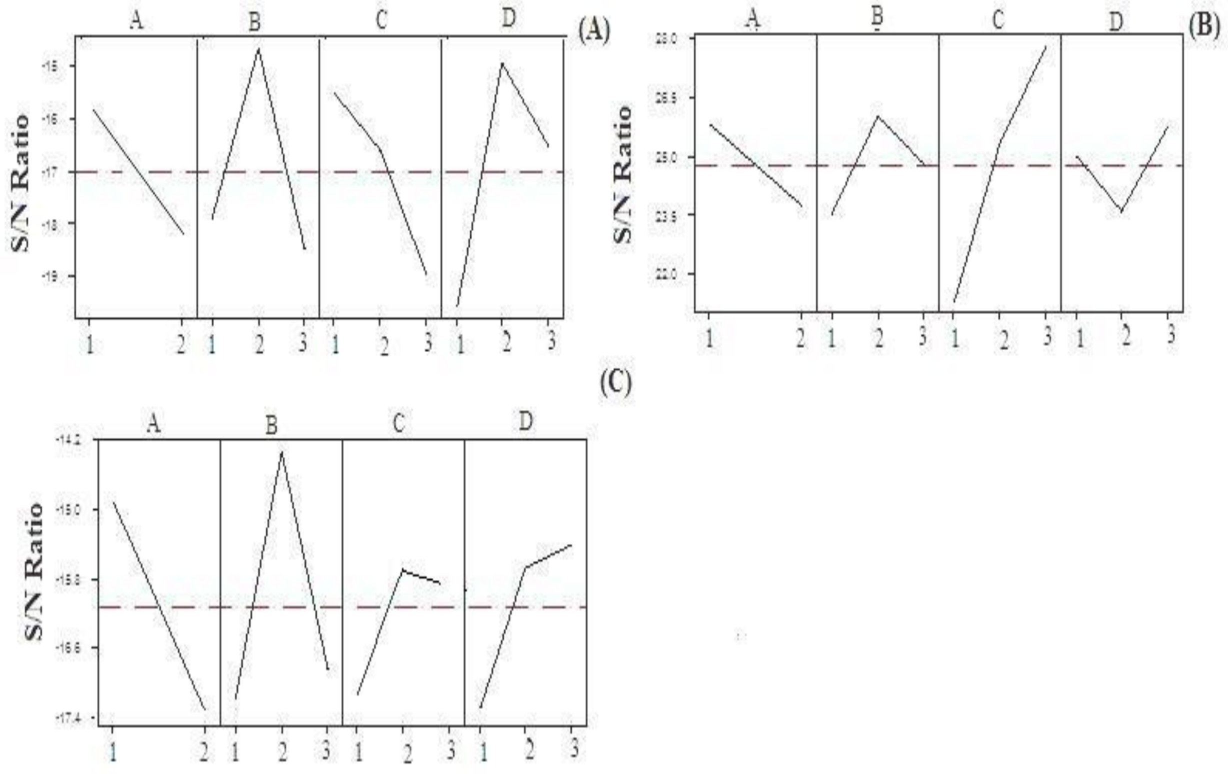

Fig 6.3. S/N plots, Ethanol (A), Hydrogen (B) and $\hat{D}$ (C).

The optimal factor combination for both ethanol and hydrogen production was achieved at A_1, B_2, C_2, D_3 respectively, i.e. optimized conditions are Reactor type – FBBR, Temperature 40 °C, pH 5.5 and OLR 50 g L^{-1}.

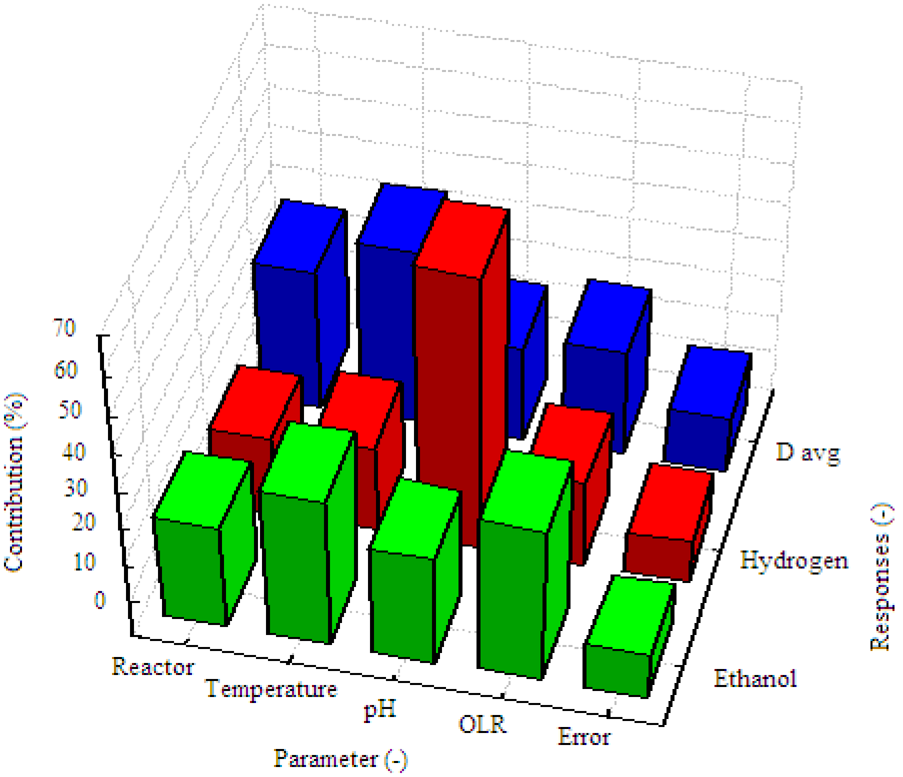

Fig 6.4. Contribution of Factors

Analysis of variance (ANOVA) was applied on the experimental results to find out the percentage of contribution of each factor against a stated level of confidence. Thus, information about how important the effect of each controlled factor can be obtained using ANOVA (Table 6.5). The contribution of each factor was illustrated in Fig 6.4.

The data showed that effect of factor B for simultaneous production of ethanol and hydrogen was more (41.18%) followed by A D and C. The individual Taguchi approach of optimization revealed that to maximize only hydrogen pH had the major contribution (63.22%) where as for maximizing ethanol temperature played a major contribution (32.76%). The organic loading rate also influenced volumetric biogas production. The microorganisms present in a mixed culture used to produce hydrogen during acidification phase of fermentation process if the pH raises above 7.0. The methanogens used these hydrogen molecules as electron donor for production of methane [49]. The methanogenic activity of the sludge observed in this study was unexpected as the reactor pH was controlled at a value of 5.5, which falls outside the range reported for methanogenic activity (6.5-7.6) [50].

Table 6.5 ANOVA chart for all the parameters

Parameter	DOF	SS			Variance			F value		
		Ethanol	Hydrogen	D_avg	Ethanol	Hydrogen	D_avg	Ethanol	Hydrogen	D avg
A	1	0.010	96.142	0.009	0.010	96.142	0.009	121.817	132.084	93.072
B	2	0.018	115.368	0.013	0.009	57.684	0.006	103.397	79.248	62.562
C	2	0.011	548.874	0.005	0.006	274.437	0.003	66.426	377.032	26.191
D	2	0.019	117.454	0.007	0.009	58.727	0.003	109.977	80.682	32.703
Error	10	0.001	7.278	0.001	0.000	0.727	0.000			
Total	17									

The wastewater sludge used in this study was pretreated with acid alkali to suppress the growth of this methanogens, which facilitated the increasing yield of hydrogen. The microorganism present in the sludge was mainly mesophilic in nature, which grows well, and yield products around temperature at 40 °C. At higher temperature, only thermopiles can grow and yield products but population of those microorganisms were not dominated in this study. Fluidized bed bioreactor performed better than the stirred tank reactor for production of both ethanol and hydrogen. Fluidized bed bioreactor has the better provision of mixing than stirred tank reactor which may be the reason for this performance. Absence of stirrer in fluidized bed bioreactor helps microorganisms to grow in less shear free environment than stirred tank reactor, which leads to improve yield of both the products. The experimental F values were quite higher than the tabulated F values [$F_{0.05, 1, 10}$ = 4.96 (for factor A) and $F_{0.05, 2, 10}$ = 4.10 (for factor B, C and D)]. The results consequently concluded that all the parameters studied here are statistically significant at 5% level of significance.

Once the optimal level of the design parameters has been selected, the final step is to predict theoretically and verify the improvement of the quality characteristics practically using the optimal level of the design parameters. The predicted (S/N) values were calculated by using the A4. The predicted values and experimental values were illustrated in Table 6.6.

The experimental results using the theoretically optimized values produced 0.21 ±.04 mol ethanol and 30.8± 0.5 mmol hydrogen. These values showed the S/N ratio and D_{avg} values around -12.39 and 0.25 respectively (Table 6.4).

Table 6.6. Results of confirmation experiment

	$\hat{D}$ avg	S/N
Predicted	0.268	-11.42
Experimental	0.25±.05	-12.39

The other soluble metabolite products detected by HPLC were lactic acid (1104 ±10 mg L^{-1}), propionic acid (1.4±0.5), acetic acid (11±2 mg L^{-1}) and butyric acid (9±2 mg L^{-1}). The residual glucose was observed to be 0.2 g L^{-1}.

6.4. Conclusions

A sustainable alternative for production of traditional petroleum refining products such as chemicals and fuels is lignocellulosic biomass. The present study emphasizes the production of ethanol and hydrogen from the saccharified lignocellulosic biomass. In this study the Taguchi method of optimization (multi response) was applied to optimize various parameters within a predetermined design space. The present study used "desirability function" to solve the multi response (Ethanol and Hydrogen production) problem. The optimal factor combination for both ethanol and hydrogen production was achieved by using fluidized bed bioreactor at temperature 40 °C, pH 5.5 & OLR 50 gL^{-1}.

References

[1] Encyclopedic Dictionary of Polymers, 2011; 892

[2] Lynd LR , Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J,Wyman CE. How biotech can transform biofuels. Nature biotechnology, 2008; *26*: 169–72.

[3] Cardona C, Sánchez OJ. Fuel ethanol production: process design trends and integration opportunities. Bioresource technology, 2007, 98, 2415–57.

[4] Gnansounou E, Dauriat A, Wyman CE. Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China. Bioresource Technology, 2005; 96: 985–1002.

[5] Biofuels Annual Report China, 2011: 11039. http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Beijing_China%20-%20Peoples%20Republic%20of_7-21-2011.pdf

[6] Industrial uses of sugar from sugar beet increasing in the EU. 2011: E60026.

[7] Angelo C. Growth of ethanol fuel stalls in Brazil. Nature news, 2012. 491:646-647.

[8] Carter C, Rausser G, Smith A. The Effect of the U.S. Ethanol Mandate on Corn Prices. Presentation at Fifth Berkeley Bioeconomy Conference.2012.

[9] Pan C, Zhang M, Fan Y, Xing Y. Hou H. Journal of agricultural and food chemistry, 2009; *57*: 2732-2738.

[10] Ritslaid K, Küüt A, Olt J. State of the Art in Bioethanol Production. Agronomy Research, 2010; 8: 236–254.

[11] Wu KJ, Chang CF, Chang JS. Simultaneous production of biohydrogen and bioethanol with fluidized-bed and packed-bed bioreactors containing immobilized anaerobic sludge. Process Biochemistry, 2007; 42(7): 1165-1171.

[12] Olsson L, & Hahn-Hägerdal, B. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates .Process Biochemistry, 1993; 28: 249–257.

[13] Toivari, M. H., Salusjârvi, L., Rouhonen, L., & Penttilä, M. Endogenous Xylose Pathway in *Saccharomyces cerevisiae* Applied and Environmental Microbiology, 2004; 70: 3681–3686.

[14] Matsushika A, Inoue H, Kodaki T, and Sawayama, S. Ethanol production from xylose in engineered Saccharomyces cerevisiae strains: current state and perspectives. Applied Microbiology and Biotechnology,2009; 84: 37–53.

[15] Verho R, Londesborough J, Penttilã M, and Richard P. Engineering redox cofactor regeneration for improved pentose fermentation in *Saccharomyces cerevisiae***.** Environmental Microbiology, 2003; 69:5892–5897.

[16] Gordon J M. Tailoring optical systems to optimized photobioreactors. Int J Hydrogen Energy, 2002; 27: 1175–1184.

[17] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microbial. Technology, 2006;38 (5): 569–582.

[18] Chen CC, Lin CY, Lin M. Acid–base enrichment enhances anaerobic hydrogen production process. Appl. Microbiol. Biotechnology, 2002; 58 (2): 224–228.

[19] Zoetemeyer R.J, van den Heuvel JC, Cohen A. pH influence on acidogenic dissimilation of glucose in an anaerobic digester. Water Research, 1982; 16: 303–311.

[20] Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: challenges for process optimisa- tion. Int. J. Hydrogen Energy, 2002; 27 (11–12): 1339–1347.

[21] Chen CC, Lin CY. Start-up of anaerobic hydrogen producing reactors seeded with sewage sludge. Acta Biotechnology, 2001; 21 (4): 371–379.

[22] Mizuno O, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresource Technology, 2000; 73 (1): 59–65.

[23] Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technology, 2002;82 (1): 87–93.

[24] Wang YZ, LiaoQ, Zhu X, Tian X, Zhang C. Characteristics of hydrogen production and substrate consumption of Rhodopseudomonas palustris CQK01 in an immobilized-cell photobioreactor. Bioresource.Technology,2010;101 (11): 4034–4041.

[25] Redwood MD, Paterson B M., Macaskie LE. Integrating dark and light bio-hydrogen production strategies: towards the hydrogen economy. Rev. Environ. Sci. Biotechnology, 2009; 8 (2), 149–185.

[26] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme and Microbial Technology, 2006; 38 (5): 569–582.

[27] Kovacs KL, Maroti G, Rakhely G. A novel approach for biohydrogen production. International Journal of Hydrogen Energy, 2006; 31 (11): 1460–1468.

[28] Levin DB, Pitt L, Love M. Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy, 2004; 29 (2): 173–185.

[29] Maness PC, Huang J, SmolinskiS, Tek V and Vanzin G. Energy generation from the CO oxidation-hydrogen production pathway in *Rubrivivax gelationsus*, Appl Envioronment Microbiology, 2005; 71: 2870-2874.

[30] Turner AFT, Karube I, Wilson GS (Eds.), Biosensors fundamentals and applications, Oxford University Press, Oxford .1987; 291–314.

[31] Himmel ME, Baker JO, Overend RP (Eds.), Enzymatic conversion of biomass for fuels production, American Chemical Society, Washington, D.C .1994; 271–290.

[32] Kaparaju P, Serrano M, Thomsen AB, Kongjan P, Angelidaki I. Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology, 2009; 100: 2562–2568.

[33] Liu H,Grot S, Logan BE. Electrochemically assistedmicrobial production of hydrogen from acetate. Environ Sci Tchnology, 2005; 4317–20.

[34] Barros AR , Silva E L. Hydrogen and ethanol production in anaerobic fluidized bed reactors: Performance evaluation for three support materials under different operating conditions Biochemical Engineering Journal, 2012; 61: 59-65.

[35] Guo WQ, Ren NQ, Wang XJ, Xiang, WS, Meng ZH, Ding J, Qu YY, Zhang LS. Biohydrogen production from ethanol-type fermentation of molasses in an expanded granular sludge bed (EGSB) reactor.International Journal of Hydrogen Energy, 2008; 33: 4981-4988.

[36] Turcot J, Bisaillon A, Hallenbeck PC: Hydrogen production by continuous cultures of *Escherchia coli* under different nutrient regimes. Int J Hydrogen Energy, 2008; 33**:**1465-1470.

[37]Angela A, Dimitar K, Irini A, Diana Z and M M Alves. Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. Biotechnology for Biofuels, 2012; 5:6.

[38] Infantes A, Campo, Villaseñor J, Fernández FJ Influence of pH, temperature and volatile fatty acids on hydrogen production by acidogenic fermentation, International Journal of Hydrogen Energy, 2011;36(24):15595–15601.

[39] Antonopoulou G, Gavala HN, Skiadas IV. Angelopoulos, K.; Lyberatos, G. Biofuels generation from sweet sorghum: fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresource Technology,2008; 99:110–119.

[40] Ren N, Wang B, Huang JC. Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor, Biotechnology. Bioengineering, 1997; 54: 428–433.

[41] G Gottschalk. Bacterial Metabolism, Springer-Verlag, Berlin/Heidelberg/New York, 1986.

[42] Wu KJ, Chang CF, Chang JS. Simultaneous production of biohydrogen and bioethanol with fluidized-bed and packed-bed bioreactors containing immobilized anaerobic sludge, Process Biochemistry, 2007; 42: 1165–1171.

[43] Koskinen PEP, Beck SR, Orlygsson J, Puhakka LA. Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from icelandic geothermal areas, Biotechnology Bioengineering, 2008; 101: 679–690.

[44] Wang B, Wang J. Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production, Int. J. Hydrogen Energy, 2008; 33: 7013–7019.

[45] Hwang MH, Jang NJ, Hyum SH, Kim IS. Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH, J. Biotechnology, 2004; 111: 297–309.

[46] Zhu H, Parker W, Basnar R, Proracki A, Falletta P, Béland M, Seto P, Buffer requirements for enhanced hydrogen production in acidogenic digestion of food wastes, Bioresource Technolpgy, 2009; 100 : 5097–5102.

[47] Hsiung WC and Chang JS. Continuous Biohydrogen Production from Starch with Granulated Mixed Bacterial Microflora, 2008; 16: 93-97.

[48] Clescerl LS, Greenberg AE, Eaton AD. Standard Methods for Examination of Water & Wastewater Washington, DC: American Public Health Association. ISBN 0-87553-235-237.

[49]Fang HH, Liu HE. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technology, 2002; 82(1): 87-93.

[50] Rittmann BE , McCarty PL. Environmental Biotechnology Principles and Applications, 2nd edition, McGraw-Hill Publications, New York, 2001; 755.

Chapter VII

Conclusions and Future Scope

The main theme of the thesis is to investigate the potential of different lignocellulosic biomass available in North East part of India for biofuel production. This chapter discusses the major conclusion drawn from the present investigation and the future works, which can be done as an advancement of the current study.

7.1 Conclusions

Since last few decades, reliable and sustainable energy supply has been a major cause of concern for the global community. To respond this energy crisis, a lot of effort has been made around the world to explore renewable and sustainable energy technologies. The present study was focused on unraveling the lignocellulose fermentation for the production of bio-ethanol and bio-hydrogen.

Lack of knowledge about the indigenous tree and shrub species located in the forests at different provinces of North-East India retards setting up biomass based energy plants. Preliminary information on physico-chemical characterization of these biomasses surely opens opportunity for selecting future biofuel candidates in the North-East region of India. This is the first systematic report on characterization of lignocellulosic biomasses (Areca nut husk (*Areca catechu*), Moj *(Albizia lucida)*, Bonbogori *(Ziziphus rugosa),*) available in the provinces of North-East India. The study includes several physical characterization like proximate and ultimate analysis, thermogravimetric analysis, crystalinity and chemical characterization that embraced FTIR and Raman spectroscopy. The maximum crystalinity was observed in Areca nut husk fiber (63.84%) followed by Moj (46.43%) and Bonbogori (42.46%).

The preliminary step for the conversion of these solid biomass samples to liquid fuels need pretreatment. To improve competence of cellulose hydrolysis, pretreatment is required to efficiently break its recalcitrant structure. Two types of pretreatment processes viz. dilute acid and ultrasound-assisted lime pretreatment was carried out with Areca nut husk, Bon bogori and Moj. In case of dilute acid pretreatment process the crystallinity of biomass sample such as *Albizzia lucida* and *Ziziphus rugosus* reduced from 64.14 % to 25.17% and 52.46% to 23.14% respectively, while for Areca nut husk the reduction in crystallinity was 49.8%. Similarly both the lime and ultrasound assisted lime pretreatment reports removal of lignin ranged between 64% to 68% present in the native lignocellulosic fiber of studied biomass i.e. Bonbogori, Areca nut husk, and Moj respectively.

The hydrolysis or saccharification of the pretreated biomass sample was carried out by cellulase enzymes which break down structural carbohydrates into monomeric sugars such as glucose and xylose. The degree of saccharification process was considered as the yardstick to compare different pretreatment processes. After comparing the saccharification efficiency of different pretreatment process it was noticed that ultrasound assisted lime pretreated samples gave the highest saccharification efficiency and it was in the order of Bon bogori (82.5%) followed by Moj (80.1%) and Arecanut husk (79.5%). It was also pointed out that sugar yield became almost constant with enzyme loading of 20 and 25 FPU/g of cellulose. Therefore, from the saccharification study it can be concluded that biomass samples pretreated with ultrasound assisted lime pretreatment could be used for the next step of biofuel production i.e. fermentation process and 20 FPU /g cellulose are recommended as optimum enzyme loading for enzymatic hydrolysis or saccharification process.

The possibility of utilizing immobilized sewage sludge for simultaneous ethanol and hydrogen production in a bioreactor was studied. Wastewater sludge is a potential seed for bio-hydrogen and ethanol production. The characterization study showed sludge contains good amount of nitrogen (1.5% of TS), phosphorous (2.3% of TS) and other essential trace elements which facilitates the growth of microbes and reduces the need of supplementary nutrient elements in fermentation broth. The number of bacteria in the sludge was counted around 10^8 per g of wet sludge. Bacteria in the sludge could be grown in both anaerobic and aerobic conditions in the culture media. The fermentable sugars obtained after saccharification of ultrasound-assisted lime pretreated lignocellulosic biomass consequently increased bio-ethanol as well as bio-hydrogen yield in the fermentation process using wastewater sludge as a source of microorganisms. Four important parameters such as the type of reactors, temperature, pH of operation and organic loading rate was considered for optimization process. In this deigned conditions the optimum amount of both ethanol and bio-hydrogen yield was found to be 0.21 mol and 30.8± 0.5-mmol of ethanol and bio-hydrogen respectively. The optimal combination of parameters within the design conditions for both ethanol and hydrogen production was achieved by using fluidized bed bioreactor at temperature 40 °C, pH 5.5 and OLR 50 Kg L^{-1}.

This work demonstrates systematic report on characterization of three abundant lignocellulosic biomasses available in the North-East region of India for the production of biofuels. The present study improved the traditional lime pretreatment process by introducing ultrasound with it. However, the yield of both bio-ethanol and bio-hydrogen, after the fluidized bed fermentation were less than the values reported in other literatures but this study paved the avenues of simultaneous bio-ethanol and bio-hydrogen production from lignocellulosic biomass using wastewater sludge.

7.2 Scopes for Future Work

North–East India is well known for its wide range of several bio-resources. Present study focused on only three lignocellulosic biomass materials but in future, there are scopes to exploit more biomasses available in different states of North–East India for the production of biofuels. This study put focus on dilute acid pretreatment and lime pretreatment process but other pretreatment methods such as ionic liquid based treatment method can be applied and embellishment the pretreatment process. This work concludes with outlines of related research directions that would be worthy of future investigation. The wastewater sludge can be treated as a source of mixed culture; present work successfully dominates the methanogenic bacteria for improving the yield of both hydrogen and ethanol. By using the genome-scale reconstructed metabolic network models for each ethanol and hydrogen-producing microorganism, a combined metabolic network model for any potential mixed-culture system that captures the dominant metabolic interactions among strains can be readily built. The pathway analyses for the combined metabolic network model can give important information, such as which genes are essential for producing ethanol, hydrogen and which enzymes would most probable be regulated for improving growth conditions, what kind of metabolic interactions would come into view among the strains in the mixed culture system. This research work could be one of the interesting directions for mixed culture systems in the future as well.

Appendix -A

Taguchi methodology

Introduction

Dr. Genechi Taguchi, a Japanese scientist, made a new era in optimization process after Second World War, which is known, as Taguchi method. Dr.Taguchi is an internationally renowned quality consultant, and his contributions have made a significant breakthrough in product and process quality through the use of statistical design of experiments. Taguchi proposed several new approaches for experimental designs that are now called 'Taguchi Methods'. They were introduced in the USA during the early 1980s as a means to design robust products and processes. Taguchi method has been widely used in manufacturing industry for decades to optimize design of a product or process with a single quality characteristic (QC).Taguchi method focuses on improving the design of manufacturing processes and products before the production begins. This statistical process control tool has wide usage in several industries and R&D sectors to keep the quality of production on target [1]. The beauty of Taguchi methodology in experimental design and analysis is that it can provide optimum solution of a problem instantaneously by conducting less number of experiments. The application of taguchi method lies in quality control orthogonal array (OA), ANOVA (analysis of variance) and signal to noise ratio (S/N ratio) which are the three corner stones of this robust technique for optimizing process parameters. Taguchi method for optimization is schematically represented in the Fig. A1 [2].

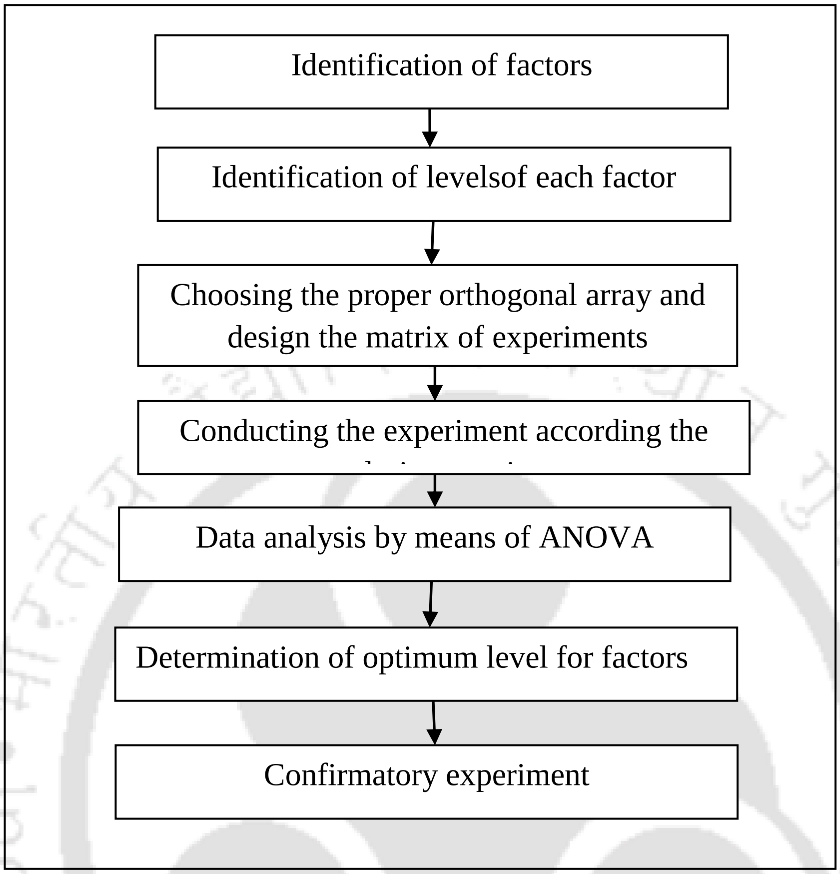

Fig A.1. Schematic Representation of Taguchi method of Design approach

Quality Characteristics

Orthogonal array

The word orthogonal (Greek word *orthos* meaning straight and *gonia* meaning angle) array means a balanced set of tables in which each column is balanced within itself and any two columns in the arrays are balanced. Orthogonal arrays have been created to facilitate the experiment designs. These arrays are set of tables, which provide the desired information with least possible number of experimental run and yield reproducible results with adequate precision. Each array can be used to suit a number of experimental situations. A comparative chart was given (Table A1) to show that the number of experiments conducted using Taguchi method of design approach is minimal.

Table A.1 Comparison of number of experiments between full factorial and orthogonal designs

Factors (number)	Level (number)	Experiment (number)	
		Full Factorial	Taguchi
3	2	8	4
3	3	27	9
4	2	16	8
4	3	81	9

Orthogonal arrays are designated by the notion “L” with a subscript. The subscript designates the number of runs.

S/N ratio

The traditional method of handling the results of multiple samples per unit trial condition is to use the mean of the trial results to calculate the factor effects. However, this is a simple method but it does not capture the variability of data within the group. A better way to compare the population behavior is to use the mean square deviation (MSD) of the results. For convenience of linearity and to accommodate wide ranging data a log transformation of MSD, known as signal to noise ratio (S/N) is recommended for analysis of results [3]. The term signal represents desirable value (mean) and noise represents the undesirable value (standard deviation from mean). Three types of S/N ratio based on calculation of MSD (Table A-2) are robustly used to indicate the quality of optimization process [4].

Table A.2. Different types of S/N ratio and their applications

Name	Mathematical Formula	Application
Larger the better	S/N = -10 $[MSD_1]$= Mean of sum squares of reciprocal of	It is applied when the goal is to maximize the response
Nominal the best	S/N= -10 Log_{10} $[MSD_2]$ $[MSD_2]$=Ratio of square of mean to the	It is applied when the goal is to target the pre determined S/N ratio
Smaller the better	S/N = -10 MSD_3= mean of sum of squares of	It is applied when the goal is to minimize the response

Analysis of variance (ANOVA)

The analysis of variance is a statistical technique of analyzing the experimental data as well as to decide which kinds of effects are important and to estimate the effect too.

$$f(x) = f_0 + \sum_{i=1}^{n} f_i\,(x_i) + \sum_{i=1}^{n}\sum_{j=i+1}^{n} f_{ij}\,(x_i, x_j) + \ldots\ldots f_{1,2\ldots n}(x) \qquad \text{(A-1)}$$

The above stated equation represents functional combinations of the input parameters, where, *n* represents the number of inputs,f_o is a constant (bias term) and the other terms on the right hand side represent the univariate, bivariate, trivariate etc..

For a set of data (results) $y_i \ldots y_n$ the total variation can be calculated by adding deviations of the individual data from the mean value. Thus, the total variation of the system, defined by the total sum of squares term.

$$SS_T = \sum (y-\dot{y})^2 \text{ for } i=1,2,\ldots\ldots n \qquad \text{(A-2)}$$

The percent of influence of a particular factor (say A) can be represented as,

$$P_A = \frac{(S_A-(f_A V_e)}{S_T} \qquad \text{(A-3)}$$

where,

$f_A =$ The degrees of freedom of factor A

V_e= variance for the error terms

Prediction of the optimum performance

The conclusive step of optimization is to compare the predicted or calculated value with experimental value of the quality characteristics (e.g., delignification ratio). The predicted value was calculated by using the following Eq A- 4 [5].

$$\frac{S}{N}_p = \frac{S}{N}_m + \sum_{i=1}^{n} \left(\frac{S}{N}_i - \frac{S}{N}_m\right) \qquad \text{(A-4)}$$

where,

$\frac{S}{N}_p$ = PredictedS/Nratio

$\frac{S}{N}_m$ = Total mean S/N ratio

$\frac{S}{N}_i$ = Mean S/N ratio at the optimal level

"n" is the number of the main design parameters that affect the quality characteristics.

Multi response

Taguchi method of optimizing process parameters cannot be used directly for solving multi response problems therefore the present study used "desirability function" to combine two or more factors into a single response and then determine the optimal levels. The goal of the study was to maximize both the production of ethanol and hydrogen therefore the present study used "larger the better characteristics" for optimization the entire process. The desirability function can be written as (Eq.A-5)

[6-7].

$$d = \begin{cases} & y \le y_{mi} \\ \frac{y - y_{mi}}{y_{ma} - y_{mi}}, & y_{mi} \le y \le y_{ma} \\ & y \ge y_{ma} \end{cases} \qquad \text{(A-5)}$$

The value of "d" lies within 0 to 1. The y_{ma} represents the ideal value of the response and y_{mi} represents the minimum value of response. The overall desirability can be defined as the geometric mean of the individual desirability (Eq. A-6).

$$\hat{D} = (d_E \times d_p)^{0.5} \qquad \text{(A-6)}$$

The experimental results were analyzed statistically to determine the individual effect of each parameter. The optimum conditions were determined theoretically and validate the same with experimental results.

References

[1] Bagchi, TP. Taguchi Methods Explained. Prentice-Hall, India. 1993

[2] Dobrzañski L, Domagala J, Silva J. Application of Taguchi method in the optimisation of filament winding of thermoplastic composites. Archives of Materials Science. 2007;134.

[3] Roy RK. Design of experiments using the Taguchi approach: 16 steps to product and process improvement. John Wiley & Sons. 2001.

[4] Gopalsamy BM, Mondal B, Ghosh S. Taguchi method and ANOVA. An approach for process parameters optimization of hard machining while machining hardened steel. Industrial Research 2009; 68: 686–695.

[5] Kim H.R, Lee K.Y. Application of Taguchi method to determine hybrid welding conditions of aluminum alloy. Industrial Research. 2009;68: 296–300.

[6] Chang MY, Tsai GJ, Houng JY. Optimization of the medium composition for the submerged culture of Ganoderma lucidum by Taguchi array design and steepest ascent method. Enzyme and Microbial Technology. 2006; 38: 407-414.

[7] Ramanujam R, Raju, R Muthukrishnan N.Taguchi Multi-machining Characteristics Optimization in Turning of Al-15 % SiC p Composites using Desirability Function Analysis. 2010; 1: 120-125.

Appendix-B

Error Analysis

Error in a scientific measurement usually does not mean a mistake or blunder. Instead, the terms "error" and "uncertainty" both refer to unavoidable imprecision in measurements. When an experiment is repeated for more than once, then there is an obvious observation of the fluctuation in the results. Therefore, in the laboratory, more often data were combined to obtain the final quantity. Whereas, ‘error analysis’ determines the accuracy of calculations and reliability of the reported experimental data.

Especially, two types of errors were considered during this study viz.

Systematic error:

Systematic errors are biases in measurement which lead to the situation where the mean of many separate measurements differs significantly from the actual value of the measured attribute. All measurements are prone to systematic errors, often of several different types. Sources of systematic error may be imperfect calibration of measurement instruments (zero error), changes in the environment which interfere with the measurement process and sometimes imperfect methods of observation can be either zero error or percentage error.

Random error:

Random errors are errors in measurement that lead to measurable values being inconsistent when repeated measures of a constant attribute or quantity are taken. The word random indicates that they are inherently unpredictable, and have null expected value, namely, they are scattered about the true value, and tend to have null arithmetic mean when a measurement is repeated several times with the same instrument. All measurements are prone to random error. The concept of random error is closely related to the concept of precision. The higher the precision of a measurement instrument, the smaller the variability (standard deviation) of the fluctuations in its readings.

Error during the measurement of concentration

Throughout the study, different concentrations were measured with the help of UV-vis spectrophotometer and High Performance Liquid Chromatography (HPLC). For that, calibration curves were drawn from the known concentrations of the specific species. Both the instrument provide measurement with three-digit precession after the decimal and during repetition, only fourth digit variation was observed. The coefficient of determination (R^2) for the calibration curves were varied between 0.9891 (calibration in HPLC) to 0.9994. Thus during the measurement of concentrations, maximum error occurred was 1.08 %.

Research Outputs

Peer- reviewed journals

Sasmal S, Goud VV and Mohanty K. Determination of salutary parameters to facilitate bio-energy production from three uncommon biomasses using thermogravimetric analysis. ***Journal of Thermal Analysis and Calorimetry*** (2013), 111(3),1649-1655.

Sasmal S, Goud VV and Mohanty K. Optimisation of the acid catalysed pretreatment of areca nut husk fibre using the Taguchi design method. ***Biosystems Engineering*** (2011), 110 (4), 465-472.

Sasmal S, Goud VV and Mohanty K. Characterization of biomasses available in the region of North-East India for production of biofuels**. *Biomass and Bioenergy*** (2012), 45, 212-220.

Sasmal S, Goud VV and Mohanty K. Ultrasound Assisted Lime Pretreatment of Lignocellulosic Biomass toward Bioethanol Production. ***Energy & Fuels*** (2012), 26 (6), 3777–3784.

Sasmal S, Goud VV and Mohanty K. Delignification Kinetics of lime pretreatment – An ineluctable tread for augmenting enzymatic hydrolysis, **Journal of Biobased Materials and Bioenergy** (2013),7,pp 660-664.

Sasmal S, Goud VV and Mohanty K. Dilute sulfuric acid pretreatment of Bon bogori (*Ziziphus rugosus*): proselyte to amorphous biomass for biofuel production, **Journal of Bioprocess Engineering and Biorefinery** (2013), DOI:10.1166/jbeb.2013.1052.

National/International Conference Proceedings

Sasmal S, Goud VV and Mohanty K., Optimization of dilute acid pretreatment of *Zizupus rugosus* applying Taguchi robust design method., International congress on Renewable Energy, 2-4 November, 2011, Tezpur University, Assam., pp. 81-87.

Sasmal S, Goud VV and Mohanty K., Dilute Acid Pretreatment of Albizzia Lucida towards Biofuel Production: Process Optimization Using Taguchi Robust Design Method., Renewable Energy Technologies: Issues & Prospects (ISBN: 978-93-80697-95-6) Edts: G. Sankar, B. Das, R. Blange, 2011, pp 75-78.

Sasmal S, Goud VV and Mohanty K., Determination of kinetic parameters of Areca nut husk fiber using thermo-gravimetric analysis, International Conference on Renewable Energy., 17-21 Jan 2011, Jaipur.

Sasmal S, Goud VV and Mohanty K., Enzymatic digestibility of Arecanut husk fiber by optimization of the acid catalyzed pretreatment process., 60th Canadian Chemical engineering conference 24-27 Oct 2010, Saskatoon, Canada.