Characterization and fermentation of Bamboo leaf litter biomass for ethanol production

A

DISSERTATION REPORT

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CERTIFICATE

This is to certify that the thesis entitled "**Characterization and fermentation of Bamboo leaf litter biomass for ethanol production**'' submitted by Ms. Kanika in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology to Thapar University, Patiala, is a record of students's own work carried out by her under my supervision. The report has not been submitted for award of any other degree or certificate in this or any other University or Institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in dissertation entitled "**Characterization and fermentation of Bamboo leaf litter biomass for ethanol production**" in partial fulfillment of the requirements for award of degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of one year from July 2013 to July 2014, under the supervision of Dr. Dinesh Goyal, Professor and Head, Department of Biotechnology, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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LIST OF ABBREVIATIONS

^{13}C CP/MAS NMR	^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance
ANOVA	Analysis of variance
BLL	Bamboo leaf litter
CMC	Carboxy methyl cellulose
CO_2	Carbon dioxide
CrI	Crystallinity index
Df	Degree of freedom
DNS	3, 5-dinitrosalicylic acid
et al	And others
etc	And other things
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
FTIR	Fourier transform infrared spectroscopy
g	Gram
g/L	Gram per liter
HPLC	High pressure liquid chromatography
H	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
K_2HPO_4	Di potassium hydrogen phosphate

KOH	Potassium hydroxide
L	Litre
M	Molar
mg	Milligram
$MgSO_4.5H_2O$	Magnesium sulphate pentahydrate
MHz	Mega hertz
min	Minute
Mj/kg	Mega joules per kilogram
mL	Milliliter
mm	Millimeter
mM	Millimolar
µg	Microgram
µL	Microliter
MMT	Million metric ton
MTCC	Microbial type culture collection
MT	Metric ton
NCIM	National collection of industrial microorganisms
N	Normal
nm	Nanometer
O_2	Oxygen
OD	Optical Density
Ppm	Parts per million

rpm	Rotation per minute
s	Second
SEM	Scanning electron microscopy
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TGA	Thermo gravimetric analysis
Viz	As follows
W	Watt
XRD	X-ray diffraction

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
A	Alpha
β	Beta
®	Registered
θ	Angle of diffraction

ABSTRACT

Bamboo leaf litter (BLL) biomass was washed to remove adhering debris, dried and powdered (0.5 mm) to investigate its physico-chemical properties. BLL biomass was found to contain (4.3%) moisture, (5.5%) ash, (81.5%) volatile matter, (36%) carbon, (5.3%) hydrogen and (2.1%) nitrogen with calorific value of 15.42 MJ/kg.

Pre-treatment approaches such as fungal solvolysis; fungus alkaline extraction; dilute acid-alkali and microwave-alkali-acid pre-treatment were executed to obtain maximum reducing sugar yield for ethanol production. Microwave-alkali-acid was found to be best pre-treatment strategy with 76% increase in cellulose content while decrease in hemicellulose and lignin was observed to be 34% and 54% respectively. Reducing sugar yield was enhanced from 30 mg/g to 193.7 mg/g along with saccharification efficiency of 25.8% for microwave-alkali-acid pre-treatment.

Scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared analysis (FTIR), Solid state ^{13}C CP/MAS NMR spectroscopy, Thermal gravimetric analysis (TGA) on native and microwave-alkali-acid pre-treated biomass was done to investigate physical and chemical changes.

Further, microwave-alkali-acid pre-treated BLL biomass was used as substrate for ethanol production using partially purified cellulase from *Bacillus subtilis* NA15 followed by ethanol fermentation using *Saccharomyces cerevisiae* (NCIM 3215) via SSF. The statistical optimization of ethanol production using Taguchi orthogonal array design produced 12.84 g/L of ethanol with optimized parameters of 11% substrate loading, 0.5% enzyme loading, 8% yeast inoculum at 4.5 pH and 30°C temperature after 72 h of fermentation. The optimized process parameters may be used for production of ethanol using BLL biomass at pilot scale.

CHAPTER 1
INTRODUCTION

Owing to expansion of human population and industrial development, conventional energy sources such as oil, coal, natural gas, etc. are unable to meet energy gap of industrialized world. The increasing rate of fossil fuels consumption has raised severe concern including the issue of depletion of energy resources in the near future, increase in fuel prices and global climate change. Campbell and Laherrere (1998) predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050 and will last for around 35 years. Environmental, long-term economic and global energy demand is encouraging continuous search for renewable and sustainable sources of energy for development of mankind. Biofuels are gaining acceptance as green technology as they are believed to substantially reduce greenhouse gas emission.

Presently, one of the most encouraging replacements for petro-fuel is bioethanol. Bioethanol is derived from plant sources and used as oxygenated fuel additive. Ethanol production from a variety of feedstock, including sugar substances (sugarcane juice and molasses), as well as starch-based materials such as wheat and corn starch; is not favoured as it possess threat to national food security. Extensive research is being conducted on liquid biofuel production from lignocellulosic biomass such as agricultural residues (wheat straw, rice straw, corn stover and sugarcane trash), forestry waste (wood chips, leaf litter) and municipal solid waste because they are abundant, inexpensive and renewable. Bamboo, a perennial woody grass belonging to the Gramineae family is found to be widely distributed in many countries in Asia, with an annual production of 6–7 million tons (Li et al., 2010). Due to its advantages of short renovation, fast growth and easy propagation, much focus has been directed towards applications of Bamboo in biobased energy field including methanol (Kobayashi et al., 2004) and ethanol production (Shimokawa et al., 2009; Sun and Lin, 2010).

Physico-chemical and compositional analysis of biomass before further processing to ethanol provides a clue for fuel potential of lignocellulosic biomass. Converting lignocellulosic feedstock into ethanol applies a complex and expensive multi-step process that involves three basic steps; pre-treatment of raw feedstock, enzymatic hydrolysis and sugar fermentation using simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF). Lignocellulose exhibit complex and rigid structure composed of cellulose and hemicellulose enclosed by lignin. Cellulose and hemicellulose

represent sugar fraction which can be fermented by microorganisms to yield ethanol. Lignin acts as cushion and obstructs holocellulose saccharification, rendering pre-treatment of lignocellulose an eminent step.

In present study Bamboo leaf litter biomass was characterized for physico-chemical properties and pre-treated to enhance saccharification yield. Pre-treated biomass was subjected to SEM, XRD, FTIR, ^{13}C CP/MAS NMR spectroscopy and TG analysis to see the physical and chemical changes. Further, pre-treated biomass was used as feedstock for ethanol production and different factors for ethanol production were optimized using Taguchi design via SSF.

CHAPTER 2
REVIEW OF LITERATURE

Global attention towards biofuels is prolonged due to fossil fuel depletion, rising fuel prices, and environmental concerns, makes it imperative to scout for alternative renewable and sustainable energy form.

All petroleum-based fuels can be replaced by renewable green fuels such as bioethanol, bio-diesel, bio-hydrogen etc. which provides an alternative source for fuel production, derived from sugarcane, corn, switch grass, algae, wood etc. Ethanol is now the most important renewable fuel in terms of volume and market value (Licht, 2006). Ethanol is widely used as a partial gasoline replacement in the US, where transportation sector consumes about 4540 million liters of ethanol annually, about 1% of the total consumption of gasoline (Wang et al., 1999). Blending ethanol, reduces consumption of fossil fuels, improves engine performance owing to higher octane number and reduces greenhouse gas emissions. The Brazil and United States are the largest bioethanol producers, with annual output of 16.1 billion litres and 5.75 billion litres respectively which accounts for over 90% of the total world production (Balat and Balat, 2009). India stands fourth in the world with a production of about 1.3 billion litres per annum from 105 ethanol plants.

Liquid biofuels are popularly classified as "first-generation" and "second-generation" fuels based on feedstock used. Lignocellulosic biomass is the most abundant feed-stock available on the earth with estimated annual production of 1×10^{10} MT per year (Sanchez and Cardona, 2008). Lignocellulosic biomass having potential for use as a feed-stock for the production of fuel ethanol includes forest residues such as wood; agricultural residues such as sugarcane bagasse, corn cob, corn stover, wheat and rice straw; industrial residue such as pulp and paper processing waste, lignin from pulp and paper mills; municipal solid wastes; and energy crops such as switch grass (Demirbas, 2008; Knauf and Moniruzzamman, 2004).

The composition of lignocellulosic material

Lignocellulosic material consists of mainly three different types of polymers, namely cellulose $(C_6H_{10}O_5)n$, hemicellulose $(C_5H_8O_4)m$, lignin $[C_9H_{10}O_3(OCH_3)_{0.9–1.7}]_x$, together with pectins, extractives, glycosylated proteins and several inorganic materials (Sjostrom, 1993) which are associated which each other to form the structural framework of the plant cell wall. Lignocellulosic composition of various biomasses is presented in Table 1.

Table 1. Cellulose, hemicellulose, and lignin content in waste lignocellulosic residues

Lignocellulose	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grass cuttings	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Swine waste	6.0	28	NA
Hardwoods stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Switch grass	37-45	19-31	12
Poplar hybrid	49	22	23
Silver maple	46	19	21
Weeping love grass	37	22	21

(Source: Sun and Cheng, 2002)

Cellulose

Cellulose, most abundant organic compound on the earth consisting of β, 1-4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose), exists as linear chain of several hundred to 10 thousands of recurring D-glucose subunits, linked by β-1,4 glycosidic bonds (Zhang et al., 2004) as shown in Figure 1. The long-chain cellulose polymers are linked together by hydrogen and vanderwaal bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the cellulosic microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the β-1,4-glycosidic linkages. Lignocellulosic material consists of both crystalline and amorphous forms of cellulose; tightly bounded parallel arranged bundles of cellulosic chains forms crystalline region while this is less ordered and conspicuous in amorphous region.

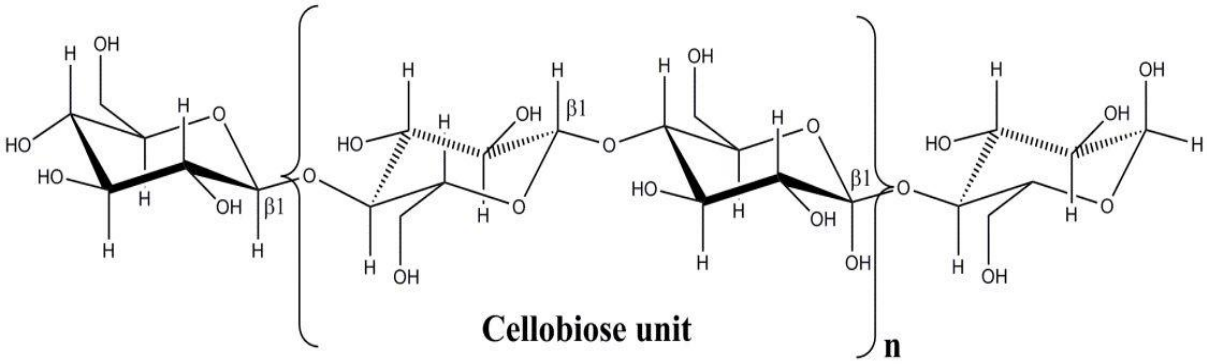

Figure 1. Chemical structure of cellulose *(adapted from Gandarias and Arias, 2013)*

Hemicellulose

Hemicellulose is a second most abundant natural carbohydrate polymer that consists of different monomers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids. The dominant component of hemicellulose from hardwood and agricultural plants, like grasses and straw, is xylan, while this is glucomannan for softwood (Saha, 2003). Hemicelluloses have a random, amorphous, and branched structure with little resistance to hydrolysis (Sjostrom et al., 1993; Ademark et al., 1998). Hemicellulose has a lower molecular weight than cellulose, and branches with short lateral chains that consist of different sugars. Xylan, the most common type of polysaccharide in hemicellulose family consist of D-xylopyranose linked together by β-1,4-linkage with less than 30,000 molecular weight and up to 200 degree of polymerization. The molecular structure of hemicellulose is shown in Figure 2. Hemicellulose serves as a connection between the lignin and the cellulose fibers and gives the whole cellulose hemicellulose–lignin network more rigidity (Laureano-Perez et al., 2005).

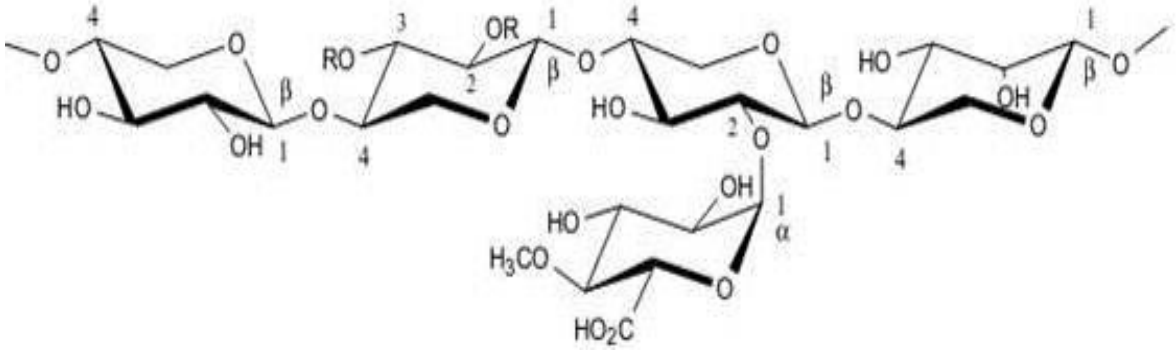

Figure 2. Structure of hemicellulose *(adapted from Nhuchhen et al., 2014)*

Lignin

Lignin, after cellulose and hemicellulose, is one of the most abundant amorphous heteropolymer consisting of three different phenyl propane units (p-coumaryl, coniferyl and

sinapyl alcohol) that are held together by different kind of linkages to form complex with hemicellulose encapsulating cellulose, making it resistant towards chemical and enzymatic hydrolysis. The main purpose of lignin is to give the plant structural support, impermeability, and resistance against microbial attack and oxidative stress. Lignin, with molecular weight of less than 20,000, is found in relatively large amount in hardwood (oak, walnut, maple, poplar, birch) than in soft wood (pine, balsam, spruce, tamarack, fir) and herbaceous plants (grasses, rice and wheat straws). During degradation process, lignin may form furan (furfural and hydroxymethyl-furfural) compounds that could inhibit fermentation (Ran et al., 2014). The macromolecular polymeric structure of lignin is shown in Figure 3.

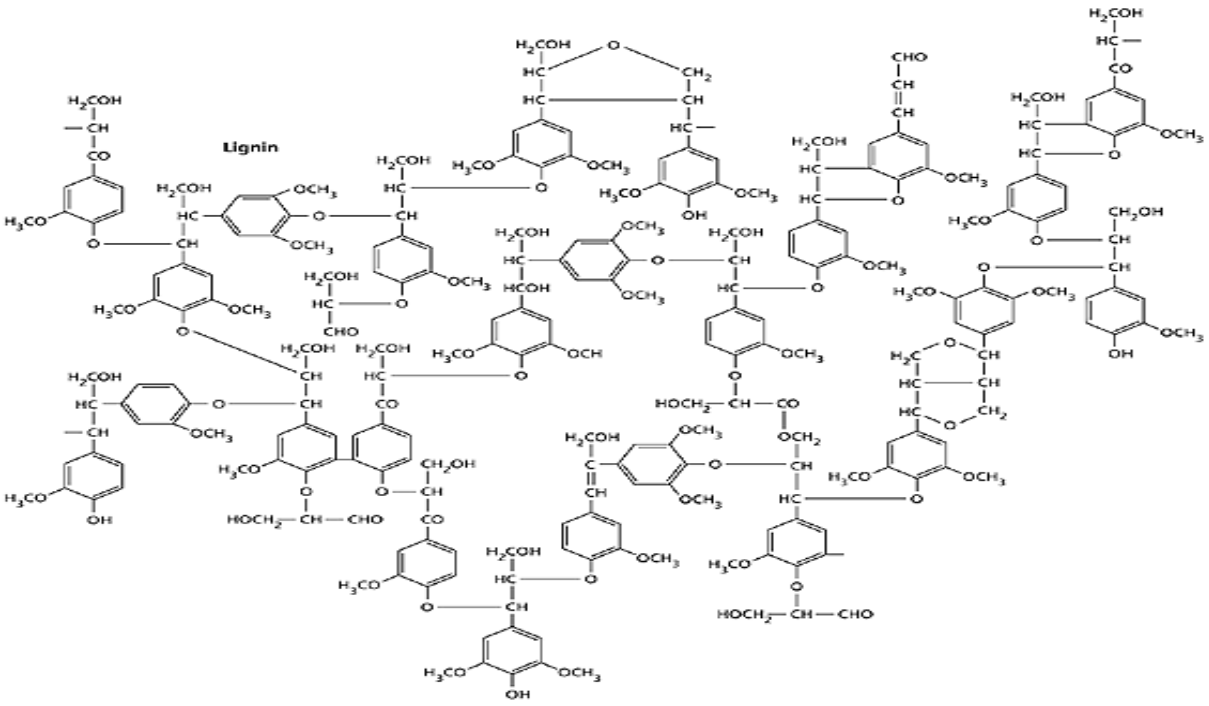

Figure 3. Structure of lignin *(Adapted from Nimz, 1974)*

Large numbers of lignocellulose materials are still untapped, Bamboo being one of them. Bamboo grows naturally in tropical, sub-tropical, and temperate regions around the world (Gratani et al., 2008) encompassing 1250 species within 75 genera. Total growing stock of Bamboo in India is 80.4 MMT with estimated annual harvest of 18.0 MMT. Usually for every 1 MT of Bamboo produced around 0.3 MT residue in the form of top portion, branches, twigs, leaves and roots (Pandey et al., 2009). There are several study on pre-treatment of bamboo using alkali (Wen et al., 2011), organic solvent (Sathitsuksanoh et al., 2010), steam explosion (Asada et al., 2005), acid (Leenakul and Tippayawong, 2010) and biological pre-treatment using white-rot fungi (Zhang et al., 2007). Although Bamboo is recognized as a useful resource but ethanol production from Bamboo leaf litter biomass has

not been reported so far. Relatively few works have been reported for Dendrocalamus, the Indian bamboo variety, which is used in the present study. Leaf litter biomass from bamboo *(Dendrocalamus strictus)* is selected as raw material for ethanol production via SSF.

Pre-treatment of Lignocellulose biomass

A suitable pre-treatment of biomass is necessary to ensure significant yields of sugars from the polysaccharides which can be fermented to ethanol. Adopting random pre-treatment technique for a specific biomass may be expensive, labour intensive, time consuming and may lead to formation of inhibitory by-products. Aim of pre-treatment is to disrupt complex lignocellulose structure, enhancing enzyme accessibility, reduce recalcitrance of biomass by removing lignin and hemicellulose, reduce cellulose crystallinity, and increasing the porosity of the materials.

The pre-treatment step usually represents nearly 20% of the total production costs of the fuel (Chiaramonti et al., 2012). Pre-treatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of by-products inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Pre-treatment methods are classified mainly into "physical", "physico-chemical", "chemical", and "biological" pre-treatment of lignocellulosic materials prior to enzymatic hydrolysis or digestion (Fan et al., 1982; Berlin et al., 2006). Schematic representation of different pre-treatment techniques is depicted in Figure 4.

Physical pre-treatment employs techniques such as mechanical comminution (Silva et al., 2010; Lin et al., 2010), extrusion (Choi et al., 2013) and hydrothermal pre-treatment (Xiao et al., 2013) whereas chemical pre-treatment includes acid (Marzialetti et al., 2008) and alkali (Zhu et al., 2010) hydrolysis, oxidation (Martin et al., 2008), organosolv (Araque et al., 2008), microwave (Ma et al., 2009) treatment are observed for lignocellulose pre-treatment. Physico-chemical pre-treatment involves steam explosion (Ruiz et al., 2008), ammonia fiber explosion (AFEX) (Lau et al., 2010), super critical fluid pre-treatment (Alinia et al., 2010).

Biological pre-treatment of lignocellulose is carried out with white rot fungi, brown rot fungi, some soft rot fungi, and bacteria. White rot fungi like, *Phanerochaete chrysosporium* (Shi et al., 2009), *Trametes versicolor* (Yu et al., 2010b), *Ceriporiopsis subvermispora* (Wan and Li, 2010), *Trichoderma reesei* (Singh et al., 2008), dominates fungal delignification of lignocellulose by removing lignin and hemicellulose, leaving cellulose

intact. The aromatic polymer lignin is well-known for resistance to microbial degradation because of its high molecular weight and presence of various biologically stable carbon-to-carbon and ether linkages (Raj et al., 2007). Fungi degrade plant lignin via oxidative mechanism whereas small number of bacteria such as *Aneurinibacillus aneurinilyticus* (Raj et al., 2007), *Paenibacillus* ITRC S_6 and *Bacillus* ITRC S_8 (Chandra et al., 2008) were observed to degrade kraft lignin.

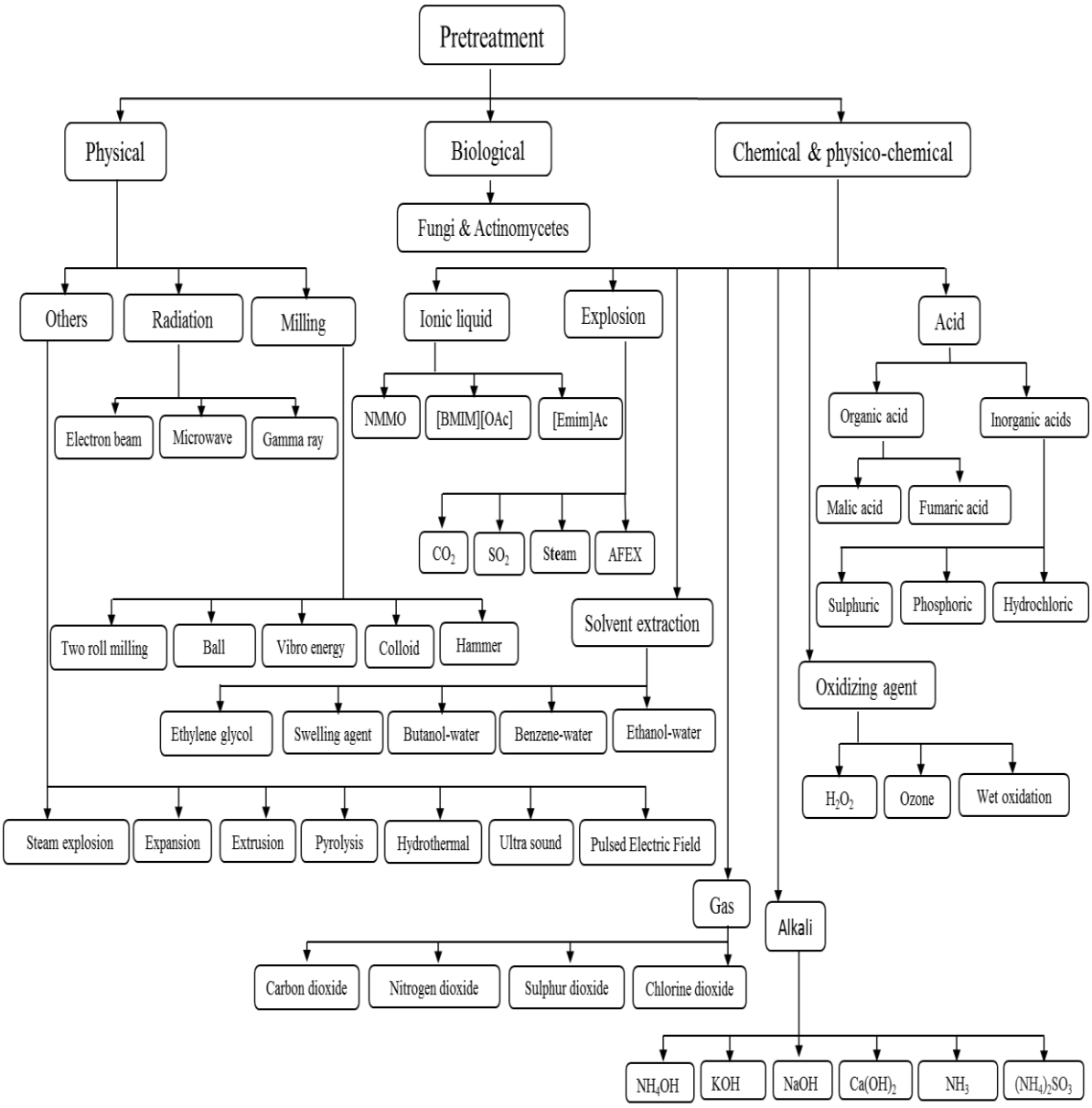

Figure 4. Schematic representation of pre-treatment techniques

As no single organism is reported to produce all the enzymes necessary for bioconversion of lignocellulose to optimum level, synergistic action of consortium of lignocellulolytic microorganism is rapid, with no need of chemical pre-treatment. A fungal consortium of three fungi *Aspergillus nidulans* (Th4), *Scytalidium thermophilum* (Th5), and *Humicola* sp.

(Th10) was used to compost a mixture of silica rich paddy straw and lignin rich soybean trash for a period of three months, converting paddy straw into nutritionally rich compost (Kumar et al., 2007).

With aim of maximizing sugar recovery, researchers have suggested some novel pre-treatment approaches combining physical, chemical and biological methods. Study conducted by Mesa et al. (2011) revealed that dilute acid pre-soaking of sugarcane begasse prior to an organosolv process using NaOH as a catalyst under the optimized conditions, enhances glucose yield.

Biological pre-treatment with white rot fungi is attracting increasing attention due to its particular ability of disrupting the lignin-hemicellulose sheath, low energy input and environmental friendliness (Shi et al., 2009a, b; Yu et al., 2009). Currently, some studies applied microorganisms to enhance the saccharification efficiency of agricultural residues, such as cotton stalks, corn stover, cornstalks, and wheat straw (Shi et al., 2009a, b). However, the dry mass loss and long residence time make the biological pre-treatment uneconomical.

Fungal pre-treatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw was reported by Bak et al. (2009). Simultaneous pre-treatment and sacchariffication of rice husk by *P. chrysosporium* for improved production of reducing sugars was studied by Potumarthi et al. (2013). Conversion of lignocellulosic biomass from grass to bioethanol using alkali and *P. chrysosporium* pre-treatment was reported by Liong et al. (2012).

Alkaline fractionation has been implemented to break the rigid structure of lignocelluloses. It involves many types of reaction between the chemicals and functional groups in the heterogeneous network of lignocelluloses, such as cleaving ether bonds in lignin units and peeling reaction of carbohydrates (Yu et al., 2010a). Alkaline fractionation has been applied to enhance the efficiency of bio-pretreatment in previous studies. Yu et al. (2010a) combined mild alkaline treatment and white rot fungal *(Irpex lacteus* CD2) pre-treatment to improve the digestibility of cornstalks, achieving a maximum glucan bioconversion of 93.86%. The maximum cellulose available of wheat straw reached 66% after combined treatment with white rot fungi *I. lacteus* for 21 days and 0.1% NaOH treatment (Salvachua et al., 2011). Synergistic treatment of poplar with white rot fungus *Trametes velutina* D10149 and alkaline fractionation improved digestibility of cellulose from 4 to 19.5% (Yang et al., 2013).

Ethanolysis, a lignin-degrading solvolysis reaction with ethanol has been studied for pulp and paper production, and bioethanol production from lignocellulosics via enzymatic saccharification and fermentation (Itoh et al., 2003). Ethanolysis solubilizes lignin and is effective to increase susceptibility of hardwood to enzymatic saccharification, but it is known that the reaction is much less effective for softwood unless strong acid such as H_2SO_4 is not added as a catalyst (Pan et al., 2004). Pre-treatment of Japanese cedar wood by white rot fungi and ethanolysis for bioethanol production was studied by Baba et al. (2011).

Dilute acid pre-treatment has the advantage of solubilizing hemicellulose, mainly to xylan, (Alvira et al., 2010). An alkali pre-treatment can solubilize and effectively extract lignin components in a biomass (Fitz-Patrick et al., 2010). To further enhance delignification in the dilute acid-treated biomass, an alkali treatment can be next performed. Sequential acid-alkali pre-treatment of empty palm fruit bunch fiber is reported by Kim et al. (2012). Combined acid-alkali pre-treatment of high dry matter corncob for ethanol production via simultaneous saccharification and fermentation is being carried by Zhang et al. (2010).

Microwave assisted with acid and alkali is considered to be promising pre-treatment technique. It is hypothesized that volumetric and selective heating feature of microwave results in an "explosion" effect among the particles to break down lignin-hemicellulose complex and expose more accessible surface area of cellulose to cellulase for enhanced enzymatic hydrolysis (Ma et al., 2009). Microwave irradiation combined with chemical reaction due to acid and alkali catalysis accelerate the chemical reaction rate. Microwave-acid-alkali treatment of sugarcane bagasse to enhance enzymatic saccharification and fermentable sugar yield is reported by Binod et al. (2012). Advantages and disadvantages of various pre-treatment techniques are summarized in Table 2.

Table 2. Advantages and disadvantages of different pre-treatment techniques

Pre-treatment method	Advantage(s)	Disadvantage(s)
Physical pre-treatment		
Milling	-Increase in accessible surface area and pore size	-Highly energy demanding
Steam explosion	-Cost effective -Lignin transformation and hemicellulose solubilization -High yield of glucose and hemicellulose in two-step process	-Partial hemicellulose degradation -Acid catalyst needed to make process efficient with high lignin content material -Toxic compound generation
Liquid hot-water pre-treatment	-Separation of nearly pure hemicellulose from rest of feedstock -No need for catalyst -Hydrolysis of hemicelluloses	-High energy/water input -Solid mass left over will need to be dealt with
Chemical pre-treatment		
Alkaline hydrolysis	-Efficient removal of lignin -Low inhibitor formation	-High cost of alkaline catalyst -Alter lignin structure
Green Solvents /Ionic liquid	-Lignin and hemicellulose hydrolysis -Ability to dissolve high loadings of different biomass types	-High solvent costs -Need for solvent recovery and Recycle
Microwave pre-treatment	-Accelerate reactions during the pre-treatment process -Improved glucose content in the Hydrolyzate	-Not feasible for large scale operations -Increased cost of pre-treatment -Slow process
Acid hydrolysis	-High glucose yield -Solubilizes hemicellulose	-High costs of acids and need for recovery -High costs of corrosive resistant equipment
Physico-chemical pre-treatment		
Ammonia fiber explosion (AFEX)	-High effectiveness for herbaceous material and low lignin content biomass -Cellulose becomes more accessible -Alters lignin structure -Low formation of inhibitors	-Recycling of ammonia is needed -Causes inactivity between lignin and enzymes -High cost of ammonia
Super critical fluid pre-treatment	-Low degradation of sugars -Cost effective -Increases cellulose accessible area	-High pressure requirements -Lignin and hemicelluloses Unaffected
Biological pre-treatment	-Efficiently degrades cellulose and hemicelluloses -Low energy requirement	- Rate of hydrolysis is low

(Source: Taherzadeh and Karimi, 2008; Brodeur et al., 2011)

Hydrolysis of lignocellulose biomass

Enzymatic saccharification of lignocellulose is the critical step for bioethanol production where complex carbohydrate are converted to simple reducing sugars that can be fermented by yeasts or bacteria to ethanol. The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult; reducing the efficiency of the hydrolysis thus pre-treatment becomes evident. The enzymatic hydrolysis without pre-treatment yields sugars which is < 20% of the theoretical quantity, whereas sugars > 90% of the theoretical quantity are obtained with enzymatic saccharification followed by pre-treatment (Ghosh and Ghose, 2003). Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and mild environment conditions (Ferreira et al., 2009). The optimum conditions for enzymatic hydrolysis via cellulase have been reported as temperature of 40-50°C and pH 4.5 (Neves et al., 2007). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. The enzymatic hydrolysis of lignocellulose is limited by several factors including crystallinity of cellulose, degree of polymerization (DP), moisture content, available surface area and lignin content (Laureano-Perez et al., 2005). Cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively.

Cellulase enzymes involve endo and exoglucanase and β-glucosidases. Endoglucanase (endo 1,4-D glucanhydrolase) attacks the low crystallinity regions of the cellulose fiber, exoglucanase (1,4-β-D glucan cellobiohydrolase) removes the cellobiase units from the free chain ends and finally cellobiose units are hydrolysed to glucose by β-glucosidase (Taherzadeh and Karimi, 2007). Hemicellulolytic enzymes are more complex and are a mixture of at least eight enzymes such as endo-1,4-β-D-xylanases, exo-1,4-β-D xylouronidases, α-L-arabinofuranosidases, endo-1,4-β-D mannanases, β-mannosidases, acetyl xylan esterases, α-glucoronidases and α-galactosidases (Jorgensen et al., 2003). Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses. Various factors influence yields of monomer sugars from lignocellulose. Temperature, pH and mixing rate, substrate concentration, cellulase enzyme loading, and surfactant addition are the main factors that affect yield of enzymatic hydrolysis of lignocellulosic material (Taherzadeh and Karimi, 2007; Sun and Cheng, 2002).

Fermentation

The saccharified biomass is used for fermentation by several microorganisms. Yeasts are especially attractive for cellulosic ethanol production as they are tolerant to high ethanol and inhibitor concentrations, and can grow at low pH values which avoid bacterial

contaminations. But the industrial utilization of lignocelluloses for bioethanol production is hindered by the lack of ideal microorganisms which can efficiently ferment both pentose and hexose sugars (Talebnia et al., 2010). The processes usually employed in the fermentation of lignocellulosic hydrolysate are simultaneous saccharification and fermentation (SSF) separate hydrolysis and fermentation (SHF), consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF). The SSF process has been extensively studied to reduce the inhibition of end products of hydrolysis as cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Fluxogram of SHF and SSF process is depicted in Figure 5 and 6.

Separate hydrolysis and fermentation (SHF)

Conventionally the SHF process has been employed for cellulose hydrolysis, followed by the pre-treatment of the lignocellulose and enzymatic saccharification of biomass where fermentation of simple sugars to ethanol occurs as separate stage. Now a days SSF is not considered suitable due to the low efficiency of the enzymatic hydrolysis of the cellulose, enzymatic inhibition by end products and economic concerns.

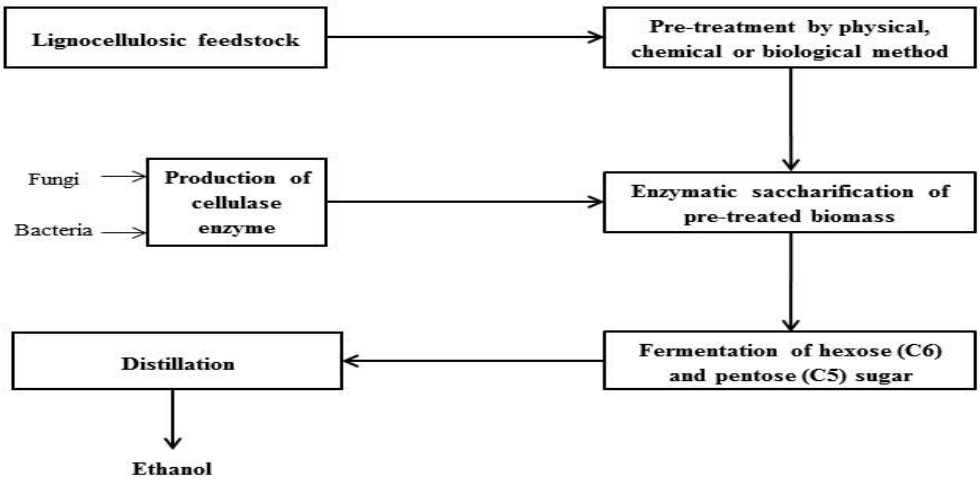

Figure 5. Schematic diagram for separate hydrolysis and fermentation (SHF)

Simultaneous saccharification and fermentation (SSF)

SSF is better alternative to SHF for ethanol production since ethanol yields are enhanced by removing end product inhibition and less reactor volume is needed due to use of single reactor. A comparative study between the two processes (SHF and SSF) is presented in Table 3. It is also cost effective but there exist a difference in optimum enzymatic hydrolysis fermentation posing limitation to use of SSF (Neves et al., 2007). The drawback

of SSF can be overcome by using thermo-tolerant microorganisms which can withstand the higher temperatures needed for enzymatic hydrolysis.

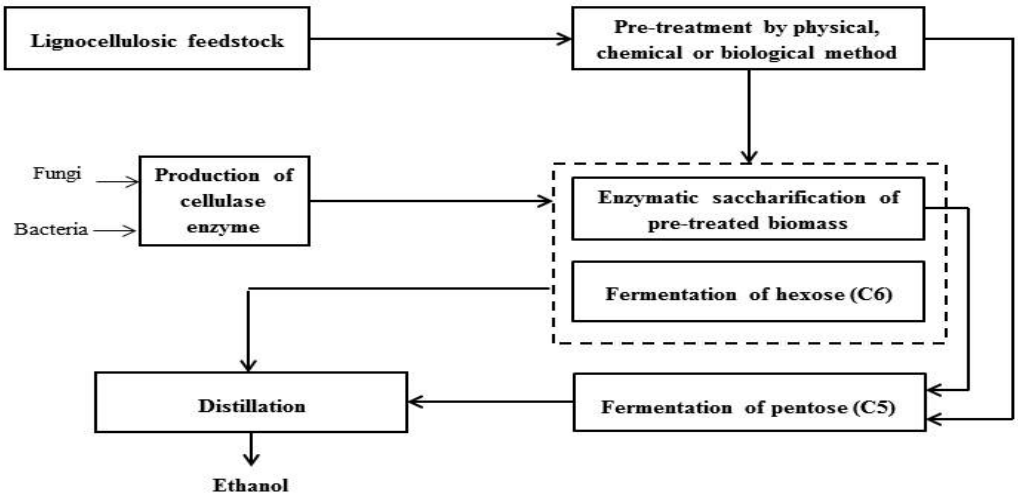

Figure 6. Schematic diagram for simultaneous saccharification and fermentation (SSF)

Table 3. Comparison between SSF and SHF

Fermentation	Features and advantages	Limitations
SSF	-Low costs -Higher ethanol yields -No end product inhibition -Reduces the number of reactors	-Difference in optimum temperature conditions of enzyme for hydrolysis and fermentation
SHF	-Each step can be processed at its optimal operating conditions -Separate steps minimize interaction between the steps	-End product inhibition minimizes the yield of ethanol -Chance of contamination due to long period process

Source: Balat and Balat, 2008; Neves et al., 2007

Taguchi experiment design

Temperature, pH, hydrolytic enzyme volume, and fermentative microbe's inoculum size are the process parameters that play a vital role in lignocellulosic ethanol production (Latifian et al., 2007). The performance of multiple experiments by analyzing one variable at a time (OVAT) approach is time consuming and laborious for identifying various independent variables with their effects (Vishwanathe et al., 2010). Statistically based experimental designs, namely, Plackett-Burman design, Box- Behnken design, and Taguchi orthogonal array design, summarize the collection and sorting of variables to be taken for consideration, determine the variable amount, and analyze the variable at different

parameters and, finally, the effect of variable error. Better quality at low cost is the main aim for generation of Taguchi design of experiment approach to maximize robustness of products and processes. Taguchi experimental design is a fast and considerable way of optimization conferring remarkable outcome in simultaneous study of many factors, making its mark in quality products supplemented with better process performance, and rendering high yield and better stability (Taguchi, 1986). The basic principle involved is the encompassment of large experimental data as orthogonal (unbiased) array in determining the effect of various factors which govern the reaction happening, ensuing in experimental error reduction with improved producibility (efficiency) of experimental outcome.

Taguchi design established the importance of statistically aligned experiments in speculating the settings of product (and/or processes) on various parameters (de-Oliveira and Alves, 2000). Optimization of six SSF parameters (substrate loading, pH, enzyme loading, temperature, time, inoculum size) was done using taguchi orthogonal array design and optimum conditions to achieve maximum ethanol concentration were determined.

CHAPTER 3
MATERIALS AND METHOD

This chapter describes the general materials and methods used throughout work. For the sake of brevity they are described here and not repeated else-where. The methods described were followed for standard procedures, except where specified otherwise.

Lignocellulosic substrate

Leaf litter biomass of bamboo (*Dendrocalamus strictus)* was selected as the representative lignocellulosic feedstock for bioconversion process. Bamboo leaf litter biomass (BLL) was collected from different places of Thapar University, Patiala, Punjab (India), located at 30°19'48"N, 76º24'0"E and 310 m above the sea level.

Processing and pulverization of biomass

BLL biomass was washed thoroughly to remove adhered debris and then dried (Figure 7a) Biomass was grinded using a mechanical blender and sieved to a mesh size of 0.5 mm (Figure 7b). The biomass sample was stored at room temperature in air tight container so as to avoid moisture for further study.

Figure 7. a. Washed and dried Bamboo leaf litter (BLL); b: Pulverised BLL (0.5 mm).

Characterization of BLL biomass

Determination of moisture content

The moisture content of the biomass was determined using the procedure given in ASTM 3173-87. Thoroughly mixed, 5.0 g of BLL biomass was dried at 105°C in a hot air oven till a consistency in weight was observed. Following drying, the sample was moved to a dessicator for cooling, without absorbing moisture. After one hour of cooling, a dry weight

measurement was recorded. The percentage difference of weight is expressed as the moisture content of the sample.

The percent moisture was calculated as follows:

$$\textbf{Moisture Content (\%)} = \frac{(A-B)}{(B-C)} \times 100$$

Where A is the weight of the crucible and sample, and B is the constant weight of crucible and sample after drying, C is the weight of empty crucible.

Determination of ash content

"Ash" is defined as the amount of inorganic material present in the biomass. The ash content in BLL biomass was determined using NREL protocol (LAP NREL/TP-510-42622; 2008). Oven dried biomass sample (2.0 g) was taken in crucible and mass of crucible along with specimen was determined. Crucible was placed in muffle furnace and heated up to 575°C for 4 h. Crucible was removed from the furnace and placed in the dessicator. The above process of heating and cooling was repeated until the specimen is completely ashed (no change of mass occurs after a further period of heating) and after cooling dry weight was recorded. This process allows removal of the volatiles and unburnt carbon.

Ash content was calculated as follows:

$$\textbf{Ash Content (\%)} = \frac{(D-C)}{(B-C)} \times 100$$

Where D is the constant weight of the crucible and the sample after combustion, B is the weight of the crucible and the oven-dried sample, and C is the weight of the silica crucible.

Determination of volatile matter

The volatile matter in the leaf litter biomass was determined by the procedure given in ASTM D 3175-07. Approximately 1.0 g of the BLL sample was taken in crucible with cover and initial weight was determined. The covered crucible was placed in muffle furnace regulated at 950°C for 7 min to obtain rapid heating. Then the covered crucible was cooled to room temperature in a dessicator and weight was recorded. The percentage weight loss was regarded as the percentage of volatile matter.

Volatile matter was calculated as follows:

$$\textbf{Volatile matter (\%)} = \frac{(A-C)}{(B-C)} \times 100$$

Where A is the constant weight of the crucible and the sample after heating, B is the weight of the crucible and the sample, and C is the weight of the silica crucible.

Determination of fixed carbon

Fixed Carbon was the resultant of the summation of percentage moisture, ash, and volatile matter subtracted from 100. All percentages were on the same moisture reference base. Fixed carbon was calculated as follows:

Fixed carbon (%) = 100 - (moisture, % + ash, % + volatile matter, %)

Ultimate analysis of biomass

The basic elements of any biomass are carbon, hydrogen, nitrogen, sulphur and oxygen. These elements were determined by CHNS analyzer (ThermoFinnigan FLASH EA 1112 Series) following standard ASTM protocol (ASTM D5373-08; ASTM D3176-09). About 1.0 mg of BLL sample was used in a tin boat assortment at 900°C in an oxygen atmosphere, so that the carbon is converted to carbon dioxide, hydrogen to H_2O and N to N_2. The percentage of oxygen was determined by means of difference.

Oxygen (%) = 100 – (C, % + H, % + N, % + S, %)

Estimation of gross calorific value

The calorific values of all the BLL biomass samples were determined in a static bomb calorimeter; BCM 211059, using the procedure described in ASTM E711-87. Each biomass sample was mixed thoroughly in the sample bottle and was made into pellet, taking care that the sample is well distributed and then send for analysis. Approximately 1.0 g of sample in the form of pellet was 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 1mL of distilled water was 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 of 10 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. The bomb calorimeter was submerged in a calorimeter cane filled with distilled water. The calorimeter jacket was maintained at constant temperature by circulating water at 25°C.

Determination of biomass extractives

Extractives in BLL biomass was determined using NREL protocol (Sluiter et al., 2008).

Hexane extraction of BLL

BLL Sample (10 g) was added to a tared extraction thimble and weight was recorded to the nearest 0.1 mg. Soxhlet apparatus was assembled and thimble was inserted into the soxhlet

tube. 190 mL of hexane was added to tared receiving flask. Receiving flask was placed on the soxhlet apparatus and heating mantles were adjusted to provide a minimum of 4-5 siphon cycles per hour. Sample was refluxed for 12 h. After refluxing, heating mantles were turned off and glassware was allowed to cool to room temperature. Solid was washed with approximately 100 mL of hexane and allowed to dry.

Ethanol extraction of BLL

BLL Sample (10 g) was added to a tared extraction thimble and weight was recorded to the nearest 0.1 mg. Soxhlet apparatus was assembled and thimble was inserted into the soxhlet tube. 190 mL of ethyl alcohol was added to the tared ethanol receiving flask and receiving flask was placed on the soxhlet apparatus. Heating mantle was adjusted to provide a minimum of 6-10 siphon cycles per hour. Samples were refluxed for 12 hours. After refluxing and cooling, thimble was removed and extracted solid was transferred, onto cellulose filter paper in a Buchner funnel. Solid was washed with approximately 100 mL of fresh ethanol and dried.

Water extraction of BLL

BLL Sample (10 g) was added to a tared extraction thimble and weight was recorded to the nearest 0.1 mg. Soxhlet apparatus was assembled and thimble was inserted into the soxhlet tube. 190 mL of HPLC grade water was added to the tared receiving flask and receiving flask was placed on the soxhlet apparatus. Heating mantle was adjusted to provide a minimum of 6-10 siphon cycles per hour. Samples were refluxed for 12 h similarly as for hexane and ethanol. After refluxing and cooling, solid was washed with 100 mL of HPLC grade water and dried.

Estimation of cellulose

Estimation of cellulose in leaf litter biomass was done by Anthrone Assay (Updegraff, 1969).

About 3 mL acetic acid/nitric acid reagent (15 mL of 80% acetic acid mixed with 1.5 mL of concentrated nitric acid) was added to 0.1 g of BLL biomass and the mixture was kept in a water bath at 100°C for 30 min. After cooling the sample was centrifuged at 8000 rpm for 10 min and supernatant was discarded. The pellet was washed twice with distilled water and 10 mL sulphuric acid (67%, v/v) was added and resulting suspension was allowed to stand for 1 h. The suspension was diluted to 10 times, and 2 mL of anthrone reagent (200 mg of anthrone in 100 mL of concentrated sulfuric acid) was added to 1 mL of diluted mixture. The mixture was incubated in a boiling water bath for 15 min for colour

development. After cooling the tubes, the optical density was read at 620 nm against the reagent blank (1 mL distilled water and 2 mL anthrone reagent). The concentration of cellulose in the sample was calculated using cellulose standard (Appendix III).

Estimation of hemicellulose

Estimation of hemicellulose in leaf litter biomass was done by Georging and van Soest (1979).

NDF (Neutral detergent fiber) solution

BLL biomass (1.0 g) was mixed with 100 mL of cold neutral detergent solution, (neutral detergent solution-18.61 g of disodium ethylene diamine tetra acetate and 6.81 g of sodium borate decahydrate were added to 200 mL of distilled water and dissolved by intermittent heating (solution A). To solution A, 30 g of sodium lauryl sulphate and 10 mL of 2-ethoxy ethanol was added. To this 4.5 g of disodium hydrogen phosphate was added and volume was made up to 1 litre and pH was adjusted to 7.0). To BLL and detergent solution 2 mL of decalin (deca hydro naphthalene) and 0.5 g of sodium sulphite was added. Above Mixture was heated to boiling and refluxed for 1 h. After cooling samples were passed through filter and residue was collected. Residue was washed with hot water and twice with acetone. Residue was collected in pre-weighed crucible and placed in oven at 100°C for 8 h. After drying, crucibles were weighed and percentage weight difference was expressed as NDF.

ADF (Acid detergent fiber) solution

BLL biomass sample (1g) was transferred to refluxing flask and 100 mL of cold acid detergent solution was added (acid detergent solution- 2 g of CTAB in 100 mL of 1N sulphuric acid). Sample was refluxed as detailed for neutral detergent fiber (NDF) and percentage weight difference was expressed as ADF. Hemicellulose concentration in BLL was estimated as difference between NDF and ADF%.

Calculation of NDF:

$$\textbf{NDF (\%)} = \frac{(Y-X)}{Z} \times 100$$

Where Y is Weight of crucible + NDF, X is Weight of empty crucible and Z is Weight of sample.

Calculation of ADF:

$$\textbf{ADF (\%)} = \frac{(Y-X)}{Z} \times 100$$

Where Y is Weight of crucible + NDF, X is Weight of empty crucible and Z is Weight of sample.

Hemicellulose (%) = NDF(%) – ADF(%)

Estimation of lignin

Acid insoluble lignin (AIL) was determined using TAPPI T 222 om-02 and acid soluble lignin (ASL) was estimated using TAPPI Useful method UM 250 (1985). For lignin estimation, about 5 g of extractive free BLL biomass sample was prepared by n-hexane, ethanol and water extraction as discussed earlier. Sample was dried thoroughly in oven at 60°C and 1 g of dried sample was weighed. 15 mL of 72% (v/v) sulphuric acid was added to 1 g of dried biomass sample in a beaker and were thoroughly mixed using glass rod. Beakers were kept in water bath at 30°C for 1 h with stirring after every 5-10 min using stirring rod. Upon completion of the 60 min hydrolysis, beakers were removed from the water bath. Sample was diluted to 4% concentration by adding 420 mL of distilled water. The mass was then autoclaved for 1 h at 121^0C. After cooling, the samples were filtered. The residue and filtrate were both used for AIL and ASL, respectively. The residue after filteration is weighed, to determine AIL. The optical density of the filtrate was measured at 320 nm for determination of ASL. The samples were diluted to bring the absorbance to 0.7–1.0 and dilution factors were recorded. Sulphuric acid (4% v/v) was used to dilute the samples and as blank.

Acid insoluble lignin (AIL) concentration was calculated as follows:

$$\textbf{AIL (\%)} = (A/W) \times 100$$

Where, A is weight of lignin in g; W is oven dried weight of sample

$$\textbf{ASL (\%)} = \frac{\text{UV abs X volume of filterate Xdilution factor}}{\varepsilon \text{ X B X a}} \times 100$$

Where UV abs is the absorbance of sample at 320 nm, volume of filtrate is 440 mL, dilution is the volume of sample of diluting solvent/volume of sample, ε is the absorptivity of biomass at specific wavelength (113 L/g-cm) and B is the oven-dried weight of sample and a corresponds to path length.

Total lignin (%) = AIL (%) + ASL (%)

Estimation of reducing sugar

Reducing sugars in the leaf litter biomass were estimated using DNS (3, 5-dintrosalicyclic acid) method as per Miller (1959). BLL biomass (0.1 gm) was taken in a test tube containing 500 µL potassium buffer (50 mM, pH 7.0) (Appendix II) and volume was made to 1 mL using distilled water. 3 mL of DNS reagent (Appendix III) was added and placed in boiling water bath for 10 min. Solution was cooled down to room temperature and

absorbance was recorded at 540 nm. Reducing sugar concentration was determined using glucose standard curve (Appendix III).

Pre-treatment of BLL biomass

Fungal pre-treatment followed by alkaline fractionation

Inoculum preparation

Phanerochaete chrysosporium is a wood-rot fungus, capable of degrading lignin via its lignolytic system, was used in this study. *P. chrysosporium* MTCC 787, procured form MTCC Chandigarh, India, was grown on PDA (Appendix I) slants at 28°C for 1 week. Sporulated slants were obtained after 4 days of growth and stored at 4°C. 10 mL of sterile distilled water (supplemented with 0.1% (w/v) Tween 80) was aseptically added to each sporulated agar slant and scraped to release the spores. Spore counts were determined with a hemacytometer and an inoculum with a final spore concentration of 2.2×10^6 spores/mL was prepared.

Fungal pre-treatment

The biological treatment was carried out in a 250 mL flask with 20 g of BLL biomass and 100 mL of distilled water. The samples were sterilized in autoclave for 20 min (12℃, 15 lbs) and inoculated with 5 mL inoculum. The samples were incubated at 29°C for 14 days with agitation at 150 rpm. At end of incubation, lignocellulosic substrate was thoroughly washed with distilled water to remove mycelia and dried at 60°C in an oven for 12 h.

Alkaline fractionation

Bio-pretreated samples were further exposed to alkaline fractionation in 250 mL flask, immersing 10 g of biomass sample in 100 mL of 70% (v/v) ethanol aqueous solution containing 1% NaOH (w/v). Biomass samples were incubated at 75°C for 3 h **(**Yang et al., 2013). The insoluble fractions were collected by filtration, thoroughly rinsed to neutral pH and dried overnight.

Procedure for fungal pre-treatment followed by solvolysis

P. chrysosporium MTCC inoculum was prepared as described earlier. Biological pre-treatment of bamboo leaf litter biomass (20 g) was performed in 250 mL flask with 100 mL of distilled water. After autoclave (121°C, 15 lbs, 20 min), biomass samples were inoculated with 5 mL of fungal inoculum (2.2×10^6 spores/mL). Incubation at 29°C for 14

days at 150 rpm is followed by washing with water and drying of pre-treated samples for 12 h.

Solvolysis

Mixed solvent system for solvolysis treatment was prepared using ethanol: acetic acid: water in ratio of 40:10:50. Bio-pretreated bamboo leaf litter biomass was added with mixed solvent system at liquid to solid ratio of 6:1 to ensure biomass is fully submerged in liquid (Baba et al., 2011). Pre-treatment reaction was carried out at 200°C for 60 min in stainless steel autoclave. After cooling samples were subjected to filteration and solid fraction obtained after filteration was washed with 80% acetone three times and then two times with 100 mL distilled water. After washing samples were dried overnight at 60°C.

Procedure for dilute sulphuric acid–sodium hydroxide pre-treatment (H_2SO_4–NaOH)

Bamboo leaf litter biomass (20 g) was treated with 200 mL of 2% (w/v) H_2SO_4 and heated at 121°C for 45 min. After treatment, the solids were separated by filteration and washed with tap water until neutral and then 10 g of biomass was treated with 10 mL of 2% (w/v) NaOH at 80°C. The solids were separated by filteration and washed with tap water until neutral. The solid residue was dried in oven at 60°C.

Procedure for Microwave-alkali-acid pre-treatment

Bamboo leaf litter biomass sample (0.5g) was placed in proprietary express vessel added with 5 mL of 1% (w/v) NaOH as pre-treatment reagent and subjected to microwave pre-treatment using Microwave Accelerated Reaction System (MARS6®, CEM, Buckingham, United Kingdom) with an operating frequency of 2450 MHz and the power range of 100-1800 W. Microwave pre-treatment was carried out for 3 min at 850 W and 150°C (Binod et al., 2012). After treatment, biomass was neutralized using tap water with two consecutive wash and then dried at room temperature. After drying microwave-alkali pre-treated biomass was subjected to 1% (w/v) H_2SO_4 treatment at 10% solid loading for 60 min. Following acid treatment samples were washed thoroughly and air dried.

Percentage recovery of solids was expressed as percentage of total solids recovered after pre-treatment based on initial sample dry weight. Saccharification efficiency was calculated by the following formula as described by NREL (TP-510e42629; 2008).

$$\text{Saccharification (\%)} = \frac{\text{Reducing dugar released (mg)} \times 0.9}{\text{Carbohydrate content in pre-treated biomass}} \times 100$$

After performing above different pre-treatment approaches every sample was analysed for cellulose, hemicellulose, lignin and reducing sugar content to determine pre-treatment efficiency.

Analysis of pre-treated and untreated biomass

SEM analysis

Physical changes in native and microwave-alkali-acid pre-treated BLL biomass were observed by Scanning electron microscope (SEM). Images of the native and treated biomass taken using SEM (Model: JEOL JSM-6510 LV, USA) at magnification 1,000X. The samples were coated with gold using gold sputter at a voltage of 10-15 kV. Gold sputtering provides conductivity to the samples, and then the SEM micrograph was obtained.

XRD analysis

Crystallinity of native and microwave-alkali-acid pre-treated biomass were analysed at room temperature in the scanning angle of 5-50° at the scan speed of 5° min^{-1} using a PANalytical X'Pert PRO diffractometer (Netherlands) with Ni-filter, operated at 45 kV and 40 mA with λ (Cu Kα) = 1.5406 A°. The crystallinity index (*CrI*) of the leaf litter biomass was calculated and determined as described by Segal et al., (1959) as follows:

$$CrI = [(I_{002}-I_{am})/I_{002}] \times 100$$

Where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ of 22.5°, and I_{am} is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2θ of 18° in most literatures (Harris and DeBolt, 2008; Kuila et al., 2011).

TGA

To understand the devolatilization characteristics, TGA of native and microwave-alkali-acid pre-treated biomass were performed using Mettler Toledo instrument (Model: TGA/SDTA 851e). The devolatilization of biomasses were studied the from 20 to 700°C with a heating rate of 10°C min^{-1} with purge gas (Nitrogen) flow rate of 60 ml min^{-1}.

FTIR analysis

The structural changes of native and microwave-alkali-acid pre-treated biomass were obtained using Nicolet Instrument (Model: MAGNA 550, USA). The biomass (10 mg) was well mixed with 200 mg of KBr and the mixture was compressed for preparation of pellets. Each spectrum was the average of 64, co-addition of scans with a total scan time 15 s in the IR range of 400 - 4000 cm^{-1} at 1 cm^{-1}.

Solid state ^{13}C CP/MAS NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectra of native and microwave-alkali-acid pre-treated biomass were obtained at 75.475 MHz using a Bruker AV-300 FT NMR spectrometer (Germany). Dry samples were spun throughout at a rate of 10 kHz using 4 mm zirconia (ZnO_2) rotor with CP pulse programme. Typically 18,000 transients were accumulated in the time domain with contact time of 1 ms and a recycle delay of 3 s. Glycine was used for the Hartman-Hahn matching procedure and as an external standard for the calibration of the chemical shift scale relative to tetramethylsilane (TMS).

Ethanol production using Simultaneous saccharification and fermentation (SSF)

Inoculum preparation

The fermentative microorganism, *Saccharomyces cerevisiae* (NCIM 3215) was maintained independently at 4°C on 5mL of Malt extract glucose yeast extract peptone (MGYP) (Appendix I) slants. After 24 h incubation at 30°C the agar slants were stored at 4°C. Starter culture was prepared by inoculation of one loopful from agar slant cultures into 50 mL of glucose yeast extract (GYE) medium (Appendix I) in two separate 100 mL Erlenmeyer flask. Flasks were incubated at 30°C and 120 rpm for 48 h prior to inoculation into fermentation media. The cell count of actively growing *S. cerevisiae* was measured using haemocytometer, corresponding to 3.2×10^8 cells/mL.

Enzyme preparation

To minimize the cost of hydrolysis, partially purified bacterial cellulase from *Bacillus subtilis* NA15 after ammonium sulphate precipitation and dialysis was lyophilized (SCANVAC, LaboGene, Denmark) to concentrate and then redissolved in sterile potassium phosphate buffer (50 mM, pH 7.0) (Appendix II). The activity of the used enzyme preparation had CMCase, FPase and β-glucosidase activity of 1.46 U/mL, 0.43 U/mL and 0.12 U/mL, respectively (Enzyme solution was prepared previously).

Simultaneous saccharification and fermentation

Optimization of SSF process parameters for ethanol production from pre-treated BLL biomass was done using Taguchi experimental design and experiment layout as suggested by orthogonal array is presented in Table 5.

Pre-treated BLL biomass (w/v) was autoclave-sterilized at 121°C for 15 min in 250 mL Erlenmeyer flask encompassing 100 mL working volume and initial pH was adjusted to a given value with 50 mM citrate buffer (Appendix II). SSF reaction mix was prepared by

adding pre-treated BLL biomass (w/v), enzyme (v/v) and inoculum (v/v), aseptically to ensure final volume of 100 mL supplemented with sterile nutrient solution composed of 50 g/L of yeast extract, $MgSO_4$ and peptone so as to have final concentration of 2 g/L each of yeast extract, $MgSO_4$ and peptone in the fermentation media and maintained at varying pH and temperature as per Table 5. Samples were agitated at 120 rpm and taken at regular time intervals for analysis of ethanol content. Ethanol concentration was estimated using Gas chromatography. The ethanol yield was calculated as follows:

$$Y\ (\%) = [(E \times 0.9)/(S \times 0.51)] \times 100$$

Where E is the ethanol concentration in g/L and S is carbohydrate (cellulose and hemicellulose) concentration in substrate in g/L.

Optimization of process parameters of SSF

Taguchi experimental design matrix, a standard orthogonal array L_{25} (6_5), was used to examine six factors, namely, substrate loading (w/v), enzyme loading (v/v), yeast inoculum (v/v), time (h), pH, and temperature (°C) in five coded levels (Table 4). The L and the subscript (25) represent the latin square and the number of experimental runs, respectively. The layout of the L_{25} Taguchi's orthogonal array is represented in Table 5. The lower and upper levels of optimized factors were selected on the basis of the suitable conditions for the desired growth of the fermentative microbes for efficient bioethanol production. Each of the twenty-five simultaneous saccharification and fermentation (SSF) experiments denoted by "runs" were carried out as per the defined values of six different parameters.

Table 4. Factors and levels in Taguchi experimental design for SSF process

Factors	**Levels**				
	L_1	**L_2**	**L_3**	**L_4**	**L_5**
Substrate loading (w/v%)	3	5	7	9	11
pH	3.5	4	4.5	5	5.5
Temperature (°C)	25	30	35	40	45
Enzyme loading (v/v%)	0.5	1	1.5	2	2.5
Time (h)	24	36	48	60	72
Inoculum size (v/v%)	2	4	6	8	10

Analysis of the Taguchi Orthogonal Array Experiments (Runs)

The results were analysed by experimental design model and three dimensional (3D) contour plot using Design Expert (Version 8.0.7.1, Stat-Ease, Minneapolis, MN), a statistical software package. The controlling factors were identified, with the magnitude of

the effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. The analysis of variance for the response of ethanol production was carried out according to the factors contribution by Taguchi method. The factors in the experimental design considered to be statistically significant at 95% confidence limit were used to determine the ratio (F) and the p-value.

Table 5. Taguchi design layout for optimization of SSF

Runs		Factors					
Std	**Run**	**A: Substrate Loading (w/v)**	**B: pH**	**C: Temperature (°C)**	**D: Enzyme Loading (v/v)**	**E: Time (hours)**	**F:Inoculum Size (v/v)**
2	1	3	4	30	1	36	4
23	2	11	4.5	30	0.5	72	8
6	3	5	3.5	30	1.5	60	10
22	4	11	4	25	2.5	60	6
1	5	3	3.5	25	0.5	24	2
24	6	11	5	35	1	24	10
8	7	5	4.5	40	2.5	24	4
7	8	5	4	35	2	72	2
17	9	9	4	45	1.5	24	8
20	10	9	5.5	35	0.5	60	4
16	11	9	3.5	40	1	72	6
19	12	9	5	30	2.5	48	2
4	13	3	5	40	2	60	8
10	14	5	5.5	25	1	48	8
9	15	5	5	45	0.5	36	6
25	16	11	5.5	40	1.5	36	2
15	17	7	5.5	30	2	24	6
5	18	3	5.5	45	2.5	72	10
11	19	7	3.5	35	2.5	36	8
3	20	3	4.5	35	1.5	48	6
18	21	9	4.5	25	2	36	10
13	22	7	4.5	45	1	60	2
21	23	11	3.5	45	2	48	4
12	24	7	4	40	0.5	48	10
14	25	7	5	25	1.5	72	4

Validation of the Experimental Model

To confirm predicted optimum conditions, SSF was performed using theoretical optimum conditions. The model was validated by performing the SSF trial employing Taguchi optimized fermentation process parameters on BLL pretreated 11% (w/v) biomass in 100 mL of fermentation medium. The best fermentation process parameters comprised 11% (v/v) substrate loading, 0.5% (v/v) enzyme loading, 8% (v/v) inoculum size, pH of 4.5, and

temperature of 30°C. The fermentation was carried out at 120 rpm for 60 h with 12 h sample collection interval. The validation of the experimental model was executed in triplicates by determining the ethanol concentration (g/L) and reducing sugar concentration (mg/g of biomass).

Procedure for determining ethanol concentration

Gas chromatography was used to measure the yield of ethanol and to determine the efficiency of the SSF. The gas chromatograph used was NUCON SERIES 5700 utilizing Porapak-Q column as the stationary phase with a flame ionization detector using N_2 at flow rate of 30 mL/min as a carrier gas. Fermentation sample was centrifuged and filtered using a 0.45 μm filter. Hydrogen (30 mL/min) and compressed air (300 mL/min) were used as fuel gas and the oven temperature was held at 155°C. Sample (1 μL) was injected where injector and detector temperature were maintained at 175°C and 250°C respectively. 100% (v/v) ethanol was used as standard (Appendix III). The peak eluted was noted (using NUCHROM software) and by knowing the area of peak, the concentration of ethanol was calculated.

Statistical analysis

All the experiments were performed in triplicate and the data were analyzed by analysis of variance (ANOVA) using COSTAT software followed by the Tukey-kramer honestly significant difference test. Simple univariate statistics (means, standard deviations, minimums and maximums) were computed for each parameter. Results were expressed as mean ± S.D. (n=3).

CHAPTER 4
RESULT AND DISCUSSION

Bamboo leaf litter (BLL) was subjected to physico-chemical characterization to determine feasibility for use as feedstock to obtain ethanol. Further, pre-treatment of BLL biomass using various approaches has been employed to enhance reducing sugar yield, compositional and structural characteristics of revealed using SEM, XRD, FTIR, TGA, Solid state ^{13}C CP/MAS NMR spectroscopy analysis. Pre-treated biomass was subjected to SSF for ethanol production, using Taguchi experiment design for modelling and optimization of different factors.

Physico-chemical characterization of BLL biomass

Physico-chemical characterization of biomass feedstock becomes imperative to evaluate potential biomass for biofuel production before further processing.

Proximate analysis

Proximate analysis is the most often employed practice for biofuel characterization. Proximate analysis is used to determine the weight percent of moisture, volatile matter (VM), fixed carbon (FC) and ash content of a biomass. The percentage of moisture, ash, volatile matter and fixed carbon in BLL biomass was found to be 4.25, 5.45, 81.50, and 8.87% respectively (Table 6). Comparative low moisture content feedstock (<15%) is preferred by thermal conversion process, while bio-conversion can utilize biomass with high moisture content (Nanda et al., 2013). In addition, biochemical conversion of lignocellulosic substrate is favored by high moisture content for submerged and simultaneous saccharification and fermentation as well, whereas comparatively lower moisture (40-50%) of biomass is suitable for solid state fermentation. Greater ash content is believed to hamper enzymatic saccharification of lignocellulosic biomass (Bin and Hongzang, 2010). Utilization of biomasses as bio-fuel feedstock with higher ash content requires washing to remove water soluble and loosely adhered inorganic materials and appropriate pre-treatment approach to minimize ash content. Biomass possessing high volatile matter and low ash is considered to be a promising substrate for ethanol production, as higher volatile matter content of the biomass causes an improved combustion, resulting in a better burn out and lower unburned carbon in the ash.

The significance of the volatile matter and fixed carbon contents is that they provide a measure of the ease with which the biomass can be ignited and subsequently gasified, or

oxidised, depending on how the biomass is to be utilized as an energy source. Fixed carbon and volatile matter provides fuel properties which renders comparison of fuel after biochemical production from lignocellulose biomass exhibiting positive effects on the calorific value.

Table 6. Proximate analysis and calorific value of BLL biomass

*Proximate analysis (wt.%)				Calorific value
Moisture	**Ash**	**Volatile matter**	**Fixed carbon[a]**	**(MJ/kg)**
4.250 ± 0.282	5.45 ± 0.233	81.50 ± 4.344	8.87 ± 0.468	15.42

**Values are mean ± SD (n =3); [a] % of fixed carbon calculated from difference of moisture, ash and volatile matter content.*

Areca nut husk, Bonbogori and Moj biomass showed volatile matter of 82, 78 and 75%, respectively and fixed carbon content of 8, 15 and 17%, respectively (Sasmal et al., 2012). Naik et al. (2010) reported proximate analysis of different lignocellulosic biomass, namely, wheat straw, flax straw, trimothy grass, pinewood and barley straw with moisture, ash, fixed carbon and volatile matter in range of 5.8-7.9, 1.3-9.8, 4.8-16 and 77.9-82.4% respectively.

Calorific value

The gross calorific value (GCV) of lignocellulosic feedstock is foremost aspect which signifies energy or amount of heat released when ignited in presence of excess air. BLL biomass was observed to have calorific value of 15.42 MJ/kg (Table 6). Result was observed to be in good agreement with calorific value of 15.9, 15.6 and 18.1 MJ/kg for Trimothy grass, wheat straw and pinewood respectively, studied by Nanda et al. (2013). High calorific value of wheat straw (20.3 MJ/kg), pinewood (19.6 MJ/kg) (Naik et al., 2012), biomass of Bonbogori (21.24 MJ/kg) and Moj (20.3 MJ/kg) (Sasmal et al., 2012) makes the biomass more efficient for the production of bio-energy.

Ultimate analysis

The percentage of C, H, N, S, and O in BLL biomass was observed to be 36.08, 5.28, 2.10, 0.050 and 56.38% respectively (Table 7). Ultimate analysis is particularly important in evaluating fuel feedstock in terms of it pollution potential, calculating higher heating value and also to determine theoretical air-fuel ratio. Lignin, hemicellulose and cellulose content of lignocellulosic biomass correspond to fuel carbon content whereas lower nitrogen and sulphur content in energy fuels is crucial for environment protection due to their gaseous

emission. The higher amount of oxygen is responsible for the higher volatile matter content but it lowers the calorific value (Sasmal et al., 2012).

Table 7. Ultimate analysis of BLL biomass

*Ultimate analysis (wt.%)				
C	**H**	**N**	**S**	**O[b]**
36.083 ± 2.91	5.284 ± 0.220	2.10 ± 0.085	0.050 ± 0.002	56.38 ± 3.00

**Values are mean ± SD (n =3); [b] % of O calculated from the difference of C, H, N and S.*

Similar studies were carried out to determine the CHNSO analysis of Canadian biomass (Naik et al., 2010), biomass of North-East Indian region (Sasmal et al., 2012), biomass and biochars of North America (Nanda et al., 2013) for assessing potential of lignocellulosic feedstocks.

Extractive determination of BLL biomass

The yield of hexane, alcohol and water extracts for BLL biomass samples are presented in Table 8. Polar and non-polar compounds need to be extracted before processing of lignocellulosic biomass for ethanol production due to their use as other value added chemicals. Extraction process was performed to separate the class of compounds such as non-polar lipids, hydrocarbon compounds and terpenoids, etc. using hexane extraction and ethanol extraction process to separate polar compounds such as chlorophyll, polar waxes, sterol and other minor constituents. Water extraction was carried out to separate inorganic materials and non-structural sugars, etc. The total amount of extractive content was detected to be around 35 wt.% of BLL biomass constituting of 9.95 wt.% hexane extract, 10.47 wt.% ethanol extract and 14.39 wt.% water extract. 10.3 wt.%, 2.1 wt.% and 1.7 wt.% water, ethanol and hexane extract respectively was found for wheat straw was studied by Naik et al. (2010).

Table 8. Extractive and lignocellulose composition of BLL biomass (g/100 g biomass)

Extractives	***Weight (%)**
Hexane extract	9.95 ± 0.656
Ethanol extract	10.47 ± 0.412
Water extract	14.39 ± 0.772
Cellulose	26.06 ± 1.64
Hemicellulose	23.81 ± 1.12
Lignin	14.45 ± 0.776

**Values are mean ± SD (n =3)*

Cellulose, hemicellulose and lignin composition of BLL biomass is described in Table 8. Cellulose component in lignocellulosic BLL biomass makes 26.06% of the dry sample. Hemicellulose and lignin accounted for 23.81 and 14.45% of BLL biomass respectively and are observed to be in suitable agreement with composition of flax straw with 28.7% cellulose, 26.8% hemicellulose (Naik et al., 2010). Cellulose and hemicellulose represent potentially fermentable carbohydrate fraction in form of pentose and hexoses for bio fuel generation but lignin retards conversion process owing to its recalcitrance. Naik et al. (2010) showed cellulose content of 34.6, 39.0 and 32.5% for wheat straw, pine wood and barley straw, respectively. North-East Indian biomass such as Moj, Banbogori and Areca nut husk also showed cellulose content of 53.46, 51.56 and 44.46%, which may be potential feedstock for renewable fuel (Sasmal et al., 2012).

Pre-treatment of BLL biomass

In present work four different pre-treatment strategies, fungus ethanolysis; fungus-alkaline fractionation; dilute-acid-alkali and microwave -alkali-acid pre-treatment were employed to enhance reducing sugar yield from BLL biomass. To obtain maximum yield, combination of physical, chemical and biological methods was tested and best pre-treatment approach was selected based on cellulose, hemicellulose, lignin and reducing sugar content, as summarized in Table 9.

Fungus-solvolysis

Pre-treatment of BLL biomass was performed with fungus and solvolysis to break rigid structure of lignocellulose. The process consists of biological treatments with selective lignin-degrading fungi (*P. chrysosporium* MTCC 787*)* and subsequent solvolysis. Employing this particular pre-treatment, resulted in 54.5% increase in cellulose content along with 9.11% and 41% reduction in hemicellulose and lignin content respectively (Table 9), compared to native BLL biomass. Reducing sugar content of BLL biomass increased from 30 to 107 mg/g (Table 9) when treated with *P. chrysosporium* (MTCC 787) followed by ethanolysis. The recovery of solids after fungal solvolysis pre-treatment was 72%, due to removal of hemicellulose and lignin and saccharification efficiency of 11.3% was observed. The combined process enabled the separation of lignin, cellulose and hemicelluloses. Many white rot fungi have been studied for their lignin-biodegradation activity, out of which *Phanerochaete chrysosporium* brings about a gradual depolymerization of lignin and produces many types of lignolytic enzymes viz. lignin peroxidase, Mn-peroxidase and laccase (Mishra et al., 2007). Ethanolysis reactions are

effective in lignin solublization to enhance lignocellulose enzymatic saccharification, which advocated lignin reduction and cellulose increase in current study. Pre-treatment of cotton stalks with *P. chrysosporium* led to a 35.5% reduction in the initial lignin content; however, the enzymatic digestibility of the pretreated cotton stalks was lower than that of untreated stalks (Shi et al., 2009). Baba et al. (2011) reported pre-treatment of Japanese cedar wood by white rot fungi and ethanolysis for ethanol production with increase in sugar production by 7 times. Pre-treatment of beech wood by ethanolysis and white rot fungi (*Ceriporiopsis subvermispora*) for ethanol production by SSF was observed by Itoh et al. (2003).

Table 9. Cellulose, hemicellulose, lignin and reducing sugar content of native and pre-treated Bamboo leaf litter

Pre-treatment	***Cellulose (%)**	***Hemicellulose (%)**	***Lignin (%)**	***Reducing sugar (mg/g)**
Untreated BLL	26.06 ± 2.0d	23.81 ± 1.37a	14.45 ± 0.95a	30.42 ± 1.67d
Fungus-solvolysis	40.28 ± 1.81bc	21.64 ± 1.51a	8.53 ± 0.42b	107.1 ± 5.25c
Fungus-alkaline fractionation	37.24 ± 2.23c	20.59 ± 0.93a	7.23 ± 0.25bc	94.3 ± 5.19c
Dilute acid-alkali	42.53 ± 1.75ab	16.22 ± 0.89b	8.27 ± 0.59bc	169.4 ± 10.5b
Microwave-acid-alkali	45.89 ± 1.70a	15.70 ± 1.40b	6.66 ± 0.59c	193.7 ± 9.69a

**Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.*

Fungus-alkaline fractionation

In fungus alkaline pre-treatment method white rot fungus pre-treatment, followed by ethanol-alkaline extraction was adopted to deconstruct complex structure of Bamboo leaf litter biomass. After 14 days of biological pre-treatment with *P. chrysosporium* (MTCC 787) and alkaline extraction, content of cellulose was increased by 43% whereas hemicellulose and lignin was decreased by 13.5% and 41% (Table 9). An increase in reducing sugar concentration to 94 mg/g was also observed with fungus alkaline pre-treatment. Solid recovery of 78% and saccharification efficiency of 11.06% was found for fungal-alkaline fractionation. Fungus solvolysis and fungus alkaline extraction were found to exhibit approximately similar saccharification efficiency of 11.3 and 11.06%

respectively. White rot fungus (*P. chrysosporium)* is reported to be a promising microorganism for bio-pre-treatment. Combination of biological pre-treatment with physical or chemical processes improves treatment efficiency (Rabelo et al., 2008). Enhanced delignification of Bamboo leaf litter biomass observed during study was probably due to lignin depolymerisation and modification of lignin macromolecules. Results obtained were in fair agreement with study carried by Yang et al. (2013) for improved bioconversion of poplar wood powder by white rot fungus and alkaline fractionation. Yang et al. (2013) presented total lignin reduction from 24.3 to 10.6 % after 16 weeks of pre-treatment. Yu et al. (2009b) reported the total sugar yield of rice hull after the combination pre-treatment of hydrogen peroxide treatment and fungal treatment was great higher than that after the sole pre-treatment. 15 day bio-treatment of cornstalk with mild alkaline pre-treatment was observed to enhance delignification and xylan loss to reduce recalcitrance thus improving enzymatic digestibility (Yu et al., 2010).

Dilute acid-alkali pre-treatment

To improve pre-treatment process efficiency, a combination of acid-alkali pre-treatment was used for fractionating biomass, providing substrate with high cellulose content. Pre-treating Bamboo leaf litter biomass with dilute-acid-alkali resulted in 63% increase in cellulose content and 43% decrease in lignin amount. Hemicellulose content was decreased by 32% (Table 9). Considerable enhancement in reducing sugar yield (169 mg/g) was observed for dilute-acid-alkali pre-treatment (Table 9). 63% solid recovery was observed for microwave-alkali-acid pre-treatment of BLL biomass along with 23.65% saccharification efficiency. High ethanol concentration can be achieved by increasing the concentration of fermentable sugar by employing pre-treatment which improves cellulose availability by removing non-cellulosic material. During study it was observed that acid treatment prior to alkali pre-treatment decreased non cellulosic, hemicellulose and lignin components of BLL biomass which in consequence improved release of reducing sugar. Alkali treatment breaks the ester bonds between lignin, hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers (Gaspar et al., 2007) whereas acid disrupt lignin and increase cellulose accessibility to enzyme hydrolysis. However acid treatment might result in formation of fermentation inhibitors such as furfural and HMF (Taherzadeh and Karimi, 2008). Results presented by Zhang et al. (2010) for dilute-acid alkali pre-treatment of corn cob were observed in reasonable degree of agreement with current investigation, showing 81% reduction in lignin content and 53.8% increase in

cellulose. Consecutive acid and alkali pre-treatment of sugarcane begasse was carried by Giese et al. (2013) and reported increase in the activity of cellulase enzymes towards cellulose yielding significant amount of reducing sugars.

Microwave-acid-alkali pre-treatment

Microwave-alkali-acid treatment yield increase in cellulose content (76%), and decrease in hemicellulose (34%) and lignin content (54%) which was found to be maximum among all pre-treatments under study. Reducing sugar release from BLL biomass was observed to be around 194 mg/g of biomass (Table 9) and 69% solid recovery after microwave-alkali-acid pre-treatment. 25.81% saccharification efficiency was exhibited by microwave-alkali-acid pre-treatment. Use of microwave is one of promising pre-treatment method as it can reduce the pre-treatment time at higher temperature. Microwave based pre-treatment approach utilizes both thermal and non-thermal effects due to intermolecular collision as a result of realignment of polar molecules with microwave oscillations and thus accelerates physical, chemical and biological processes (Ma et al., 2009). Binod et al. (2011) have reported reducing sugar yield of 0.665 g/g dry biomass with microwave treatment of sugarcane bagasse with 1 % NaOH at 600 W for 4 min followed by enzymatic hydrolysis. Combining microwave-alkali-acid treatments with 1% NaOH followed by 1% sulphuric acid resulted in an increase in reducing sugar yield to 0.83 g/g dry biomass. The application of microwave irradiation for 10 min at 250 W to switch grass immersed in 3% NaOH (w/v) produced the highest yields of reducing sugars. Balcu et al. (2011) investigated the use of acid in combination with microwave. They reported maximum reducing sugar yield at 140°C with 0.82% aqueous solution of sulphuric acid.

Among all four pre-treatments, that is, microwave-alkali-acid was observed to be best pre-treatment approach which significantly enhances cellulose and reducing sugar content with improved delignification. Microwave-alkali-acid pre-treatment was observed with maximum saccharification efficiency among all four pre-treatment. Table 10 compares results obtained from several combinatorial pre-treatment methods performed by researchers over the years with the observations obtained in present work.

Present work suggests that microwave-acid-alkali pre-treatment is an effective and promising combinatorial pre-treatment approach for lignocellulose pre-treatment to meet biofuel demand. Microwave-acid-alkali pretreated biomass was selected for ethanol production via simultaneous saccharification and fermentation.

Table 10. Combined pre-treatment approaches for lignocellulose

Raw material	Pre-treatment I	Pre-treatment II	Achievement (s)	Reference
Bamboo leaf litter biomass	Microwave (3min, 850 W,150 °C) - alkali (1 % (w/v) NaOH)	Dilute acid (1 % w/v H_2SO_4)	76% increase in cellulose, 54% decrease in lignin content, 194 mg/g of reducing sugar	Present study
Corn cob	Dilute acid (2 % v/v H_2SO_4)	Alkali treatment (2% w/v NaOH)	Lignin content was reduced by 81.0%	Zhang et al., 2010
Sugarcane bagasse	Microwave treatment (850 W, 3 min)	Alkali-acid (1% NaOH, 1% H_2SO_4)	83% reducing sugar yield	Binod et al., 2011
Sugarcane bagasse	Ultrasound treatment (24 kHz, 400 W, 30 °C, 20 min)	Alkali treatment (2.89% w/v NaOH)	92% reducing sugar yield	Velmurugan & Muthukumar, 2012
Sugarcane bagasse	Biological treatment with *Ceriporiopsis subvermispora* (28 °C, 2-10 weeks)	Microwave hydro- thermolysis (200 °C**,** 20 min)	32% sugar recovery in insoluble fraction and 15% sugar recovery in soluble fraction	Sasaki et al., 2011
Water hyacinth	Biological treatment with *Echinodontium taxodii* (28 °C, 10 days)	Acid treatment (0.25% v/v H_2SO_4)	46% reducing sugar yield	Ma et al., 2010
Corn stalks	Biological pre-treatment with *Irpex lacteus* (28 °C,15 days)	Alkaline treatment (0.25 M NaOH)	Lignin loss increased from 75.67% to 80%	Yu et al., 2010
Rice hull	H_2O_2 (2% v/v, 48 h) treatment	*Pleurotus ostreatus* treatment for 18 days	50% reducing sugar yield	Yu et al., 2009
Rice straw	10% Ammonia solution	Ionic liquid ([Emim]Ac)	97% of the glucose conversion	Nguyen et al., 2010
Wheat straw	Steam explosion (1.5 MPa at 198°C for 10 min)	alkaline peroxidase pre-treatment (4.0% v/v H_2O_2) along with (1% w/v NaOH)	Hemicellulose and lignin reduction by 5 and 6% , respectively	Chen et al., 2008
Poplar wood chips	Biological pretreatment (*Lenzites betulina) at* 28 °C for 4 weeks	Liquid hot water pretreatment *at* 200°C for 30 min.	92.33 % hemicellulose removal, 2.66-fold increase of glucose yield	Wang et al., 2012

Analysis of pre-treated and untreated biomass

In order to investigate effect of pre-treatment, features of native and microwave-alkali-acid pre-treated BLL biomass was characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared analysis (FTIR), Solid state ^{13}C CP/MAS NMR spectroscopy, and Thermal gravimetric analysis (TGA).

Scanning electron microscopy

Physical changes in native and microwave-alkali-acid pre-treated BLL biomass were observed by SEM. SEM observation of untreated (Figure 8a) and pre-treated biomass (Figure 8 b) showed microwave-alkali-acid pre-treatment induced structural change in the biomass.

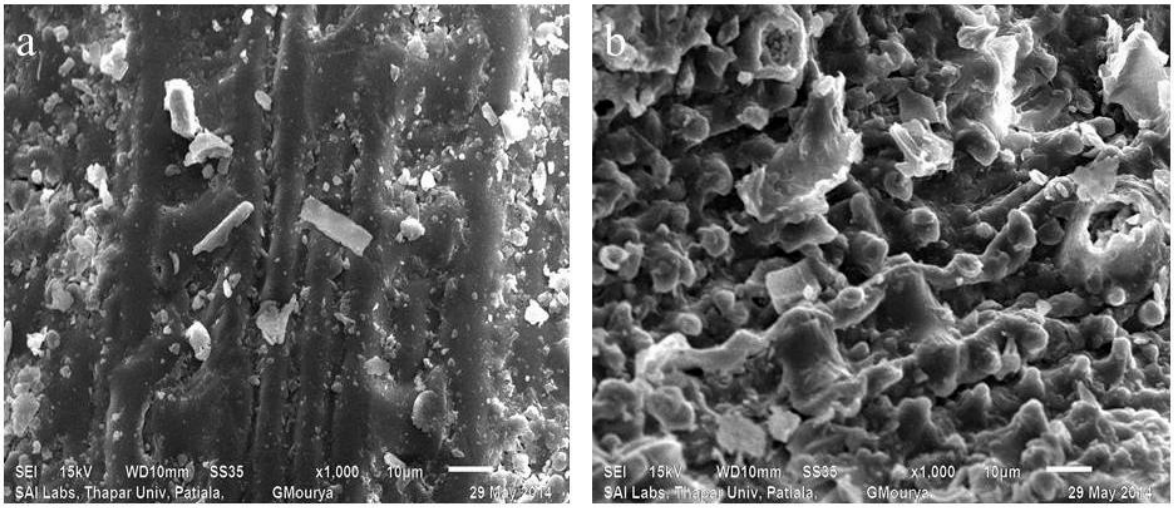

Figure 8. Scanning electron micrograph of (a) native and (b) Microwave-acid-alkali pre-treated BLL biomass (magnification: 1,000X).

SEM revealed that untreated BLL sample exhibit ordered and compact surface structure and a fibrillar, intact morphology which was destroyed in pretreated biomass. The surface of pre-treated BLL biomass has numerous uneven folds and spherical and globular shape, which were attributed to deposited lignin as reported in literature (Xiao et al., 2011). Similar structural changes were observed for sugarcane bagasse pretreated with microwave alkali pretreatment (Binod et al., 2012), sugarcane top pretreated with ultrasound assisted with surfactant (Sindhu et al., 2013), hot compressed water treatment of *Tamarix* biomass (Xiao et al., 2011).

X-ray diffraction

The XRD profile of native and microwave-alkali-acid pre-treated BLL biomass is presented in Figure 9. *CrI* in native and microwave-acid-alkali pre-treated biomass were 33.74 and

42.57%, respectively. A significant increase (8.83%) in *CrI* was recorded in pre-treated biomass as compared to native biomass. Overall biomass crystallinity depends on cellulose, hemicellulose and lignin content. Highly crystalline biomass hinders enzymatic hydrolysis whereas biomass with low crystallinity exhibits improved lignocellulose digestibility (Fan et al., 1980).

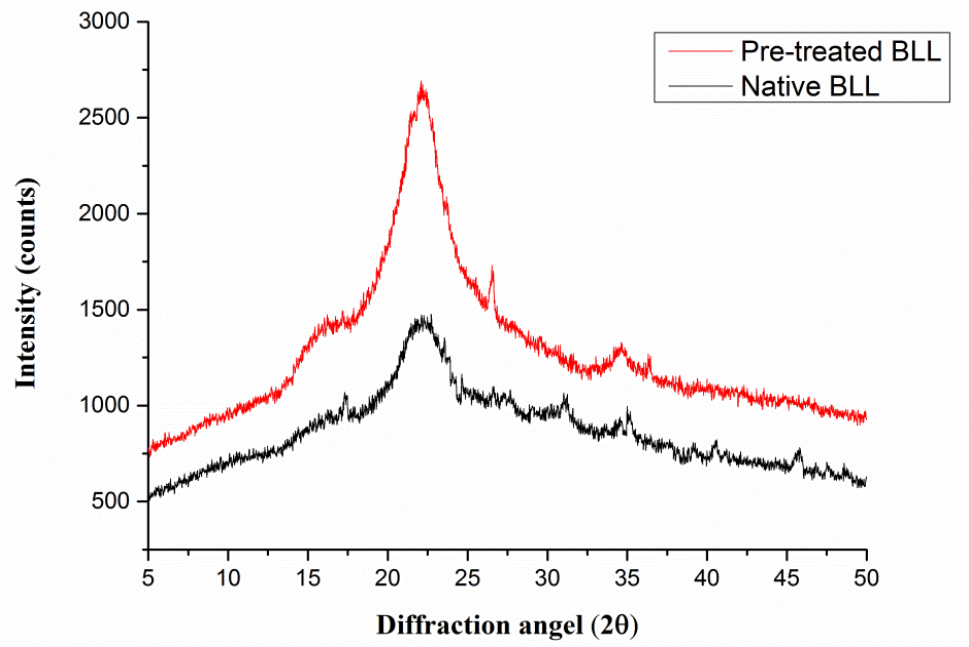

Figure 9. XRD profile of native and microwave-alkali-acid pre-treated BLL biomass.

The result was in close proximity with the earlier report of surfactant-assisted ultrasound pretreatment of sugarcane tops (Sindhu et al., 2013) with 48.85 and 65% *CrI* of pretreated biomass. Treatment of microwave-acid, microwave-alkali and microwave-alkali followed by acid pretreatment of sugarcane bagasse also showed 5.35, 11.85 and 12.11% increase in *CrI* (Binod et al., 2012). Hot compressed water pre-treated *Tamarix ramosissima* demonstrated significant increase in *CrI* when compared to that if untreated *Tamarix* (Xiao et al., 2011). Increase in *CrI* by 6% was also reported in enzyme pretreated *Lantana camara* biomass (Kuila et al., 2011). Pre-treatment is believed to eliminate hemicellulose from hydrolysis surface and changes cellulose crystallinity (Xiao et al., 2011). Probably, crystallinity has been damaged owing to the synergistic effect of alkali, microwave and acid treatment, leading to the removal of amorphous cellulose and enhanced saccharification of crystalline cellulose fibers of bamboo leaf litter biomass. Hemicellulose and lignin solubilisation together with less ordered cellulose is probably the main reason for increase in cellulose crystallinity.

Fourier transform infrared spectroscopy

FTIR spectroscopy analysis was carried out to detect changes in functional groups of biomass and FTIR spectrum is shown in Figure 10. The main characteristics were attributed to different chemical groups of cellulose, hemicellulose and lignin. Most prominent bands in biomass sample were 3450, 2927, 1741, 1631, 1535, 1045 and 856 cm^{-1} (Figure 9). The peaks in fingerprint based on literature values are summarised in Table 11 (Adel et al., 2011; Meng et al., 2012; Xiao et al., 2011).

The band in the range of 1500-1700 cm^{-1} represents the aromatic ring stretch of lignin, 3200-3600 is related to O-H stretching vibrations of alcohol and phenol, 1400 to 900 cm^{-1} is designated to vibrations of variety of bonds such as C-H, C-O, C-N, P-O, O-H and C-H symmetric and asymmetric stretching bands appears in the region of 2900 cm^{-1}. Deep band at 3450 cm^{-1} is related to O-H stretching vibrations caused by presence of alcoholic and phenolic hydroxyl group involved in hydrogen bond. The O-H stretching band was changed to higher wave number and somewhat broadened as a result of pre-treatment which is an indication of weaker intra and intermolecular hydrogen bonding and thereby lower crystallinity (Jeihanipour et al., 2009). The band position at 2927 cm^{-1} is attributed to C-H stretching vibration and showed enhancement in intensity of peak, indicating that the methyl and methylene portions of cellulose were ruptured (Xiao et al., 2011).

Reduction in peak intensity was observed in the 1741 cm^{-1} band, attributed to hemicellulose acetyl and uronic ester groups or linkages in lignin and/or from ester linkage of carboxylic group of the ferulic and *p*-coumaric acids of lignin and/or hemicellulose (Sindhu et al., 2012) indicating lignin and hemicellulose removal.

Bands in the range of 1000-1200 cm^{-1} were related to structural features of cellulose and hemicelluloses. The enhancement of absorption peaks at 1045 cm^{-1} after pre-treatment indicated the relative increase in cellulose content in the solid residue (Binod et al., 2012) due to the release of hemicellulose fraction in raw materials by acid hydrolytic reaction. Similar results were presented by Pang et al. (2012) for study of effects of microwave power and irradiation time on pre-treatment efficiency and characteristics of corn stover using combination of steam explosion and microwave irradiation pre-treatment.

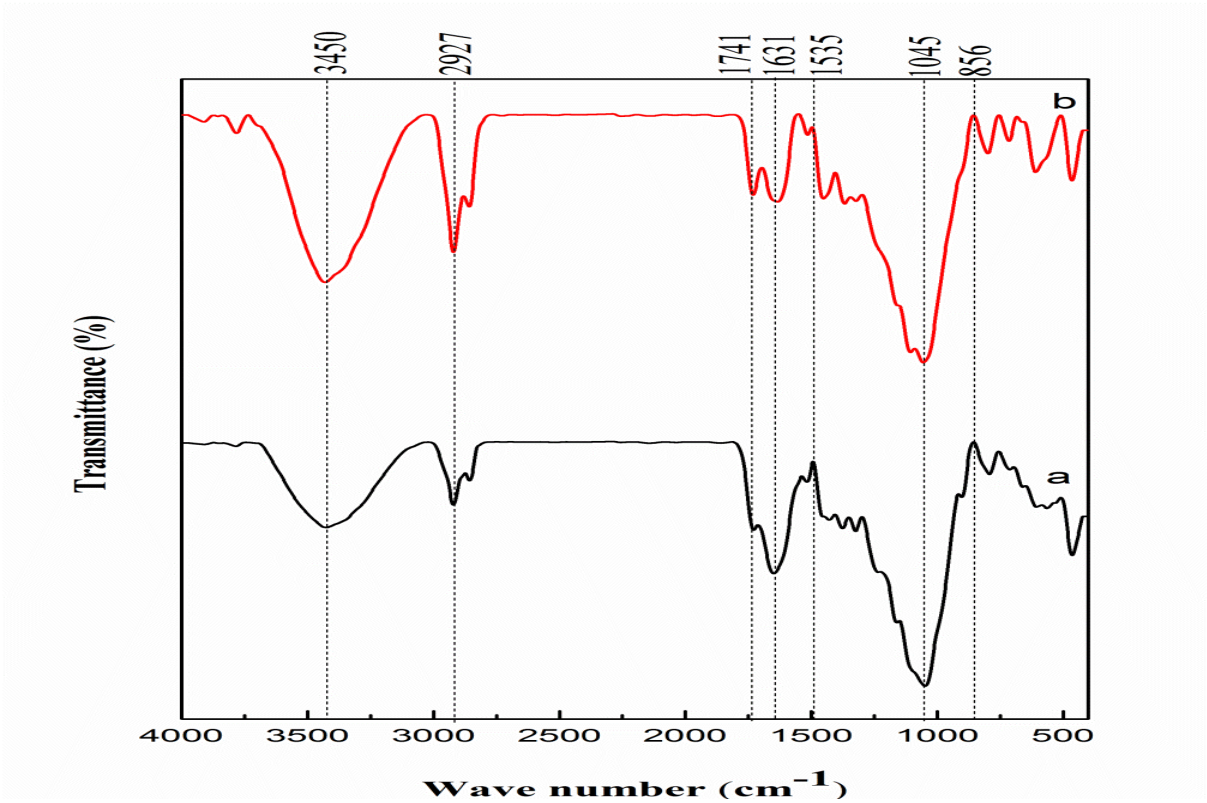

Figure 10. FTIR spectra of native and microwave-alkali-acid pre-treated biomass (a: Native BLL; b: Microwave-alkali-acid pretreated BLL)

Table 11. Assignment of FTIR bands of functional groups in biomass sample

Name of characteristic group	**Wavenumber (cm^{-1})**
O-H stretching vibration (hydrogen bonded)	3456
Stretching of $–CH_2$ and $–CH_3$	2927
C=O stretching of unconjugated ketone, carbonyls, and ester groups; C=O in xylan acetates (hemicelluloses)	1741
Bending mode of the absorbed water and the stretching of C=O in lignin	1631
Aromatic C=C stretching of lignin	1535
C–O stretching vibration in cellulose and hemicelluloses	1045
Glycosidic linkage of hemicelluloses	856

Solid state CP/MAS ^{13}C NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectroscopy is an important tool to analyze the structural features of lignocellulosic biomass. Solid state CP/MAS ^{13}C NMR spectra of the native and microwave-alkali-acid pre-treated biomass are presented in Figure 11. Signal assignment for various biomasses has been assigned based on the literatures (Rezende et al., 2011; Shi et al., 2011; Kohn et al., 2011) and summarised in Table 12. Solid state ^{13}C CP/MAS NMR provides characteristic chemical shift values ascribed to cellulose, hemicellulose and lignin. The region between 60 and 110 ppm was dominated by prominent signals, which were assigned mostly to cellulosic carbons. The sharp signal at 104 ppm corresponds to ordered cellulose (C1, C4, C6) while signal at 83 and 63 ppm are due to disordered cellulose carbons. Difference in relative signal intensities between untreated and pre-treated BLL biomass reflects the changes owing to pre-treatment. The higher cellulose content of the pre-treated biomass was seen as distinct peak (72 ppm) assigned for C2, C3 and C5 of cellulose. Less prominent peak at 172.2 ppm in pre-treated biomass corresponds to acetyl groups of hemicellulose, ascribed to hemicellulose removal. It can be concluded that main changes occurring in structure of microwave-alkali-acid pretreated BLL biomass were due to degradation of hemicelluloses with striking changes in lignin structure.

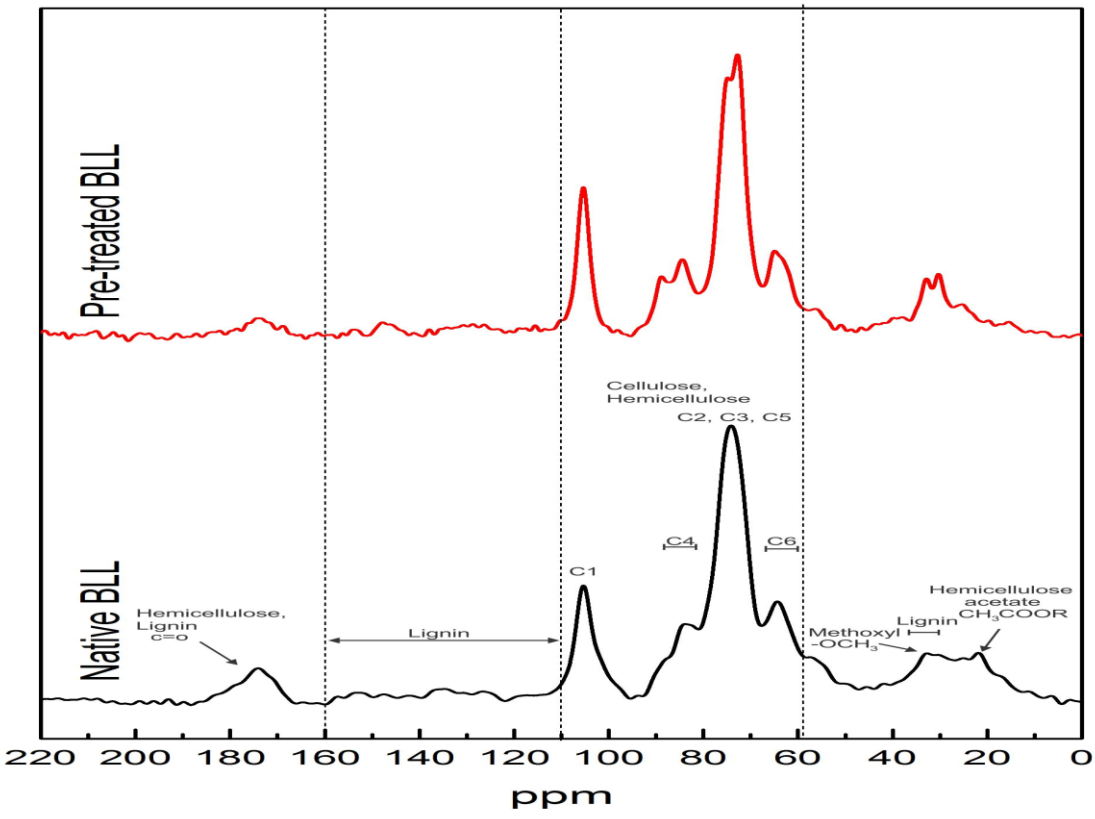

Figure 11. Solid state CP/MAS ^{13}C NMR spectra of native and microwave-alkali-acid pre-treated biomass.

Table 12. Signal assignments for chemical shifts obtained from solid state ^{13}CCP/MAS NMR spectroscopy of biomass

Chemical shift (ppm)	Assignments
173	Carboxyl group of hemicelluloses
104	Anomeric carbon (C1) of cellulose
72	C2, C3, C5 of cellulose, $OC_{\alpha}H_2$ carbon of lignin
32	Methoxyl (OCH_3) and methylene (CH_2) groups in lignin
63	C6 of crystalline cellulose
83	C4 of amorphous cellulose and hemicelluloses, $OC_{\beta}H_2$ carbon of lignin
23	CH_3 in acetyl group in hemicelluloses

The results were in good agreement with hot water pretreated biomass of *Tamarix ramosissima* (Xiao et al., 2011) and biological pretreated lime wood (Delmotte et al., 2008). Absence of peak at 23 ppm in pre-treated biomass, ascribed to acetyl group in lignin indicates considerable reduction on lignin levels of microwave-alkali-acid pre-treated BLL biomass.

Thermal gravimetric analysis

To understand the changes in thermal properties of biomass in native and pre-treated BLL, TGA of native and pre-treated biomass was carried out and presented in Figure 12. The behaviour of biomass materials during devolatilization was related to the presence of chemical constituents such as cellulose, hemicellulose and lignin (Raveendran et al., 1996). Overall in regard to TGA distribution of untreated and pre-treated biomass (Figure 12), their profiles were found to be distinct from each other. Curve for pre-treated biomass dropped significantly, compared to native, when TGA heating temperature increased from 200 to 350°C. The TGA of bamboo leaf litter biomass showed onset temperature of devolatilization in the range of 200-250°C, which corresponded to 10% weight loss with respect to the final weight loss. The main weight loss ends at 350-390°C followed by a slow and continuous weight loss. The former step was due to the primary devolatilization, whereas the later was attributed to the degradation of heavier chemical structures in the solid matrix (Fisher et al., 2002). The hemicellulose and cellulose part of biomass usually

start to decompose at around 206°C and 315°C while the decomposition of lignin start above 330°C respectively (Ledakowicz and Stolerak, 2002).

The thermal decomposition study was conducted on rapeseed (*Brassica napus L*), sponge ground fibers, sugarcane bagasse, banana fibers by several researchers and their study revealed that the thermal decomposition of hemicelluloses occurred at temperatures ranging from 150°C to 350°C, cellulose was decomposed at temperature range of 275-350°C, and lignin was gradually decomposed between the temperatures 250°C and 500°C (Chen et al., 2010; Guimaraes et al., 2009). Similar devolatilization properties were also found with hot water pretreated biomass of *Tamarix ramosissima* (Xiao et al., 2011) and heat pretreated sugarcane bagasse (Chen et al., 2010). Flattening of tail beyond 500°C was observed mainly due to removal of lignin in pre-treated biomass, as long tail was observed due to high percentage of mannose or lignin content (Naik et al., 2010).

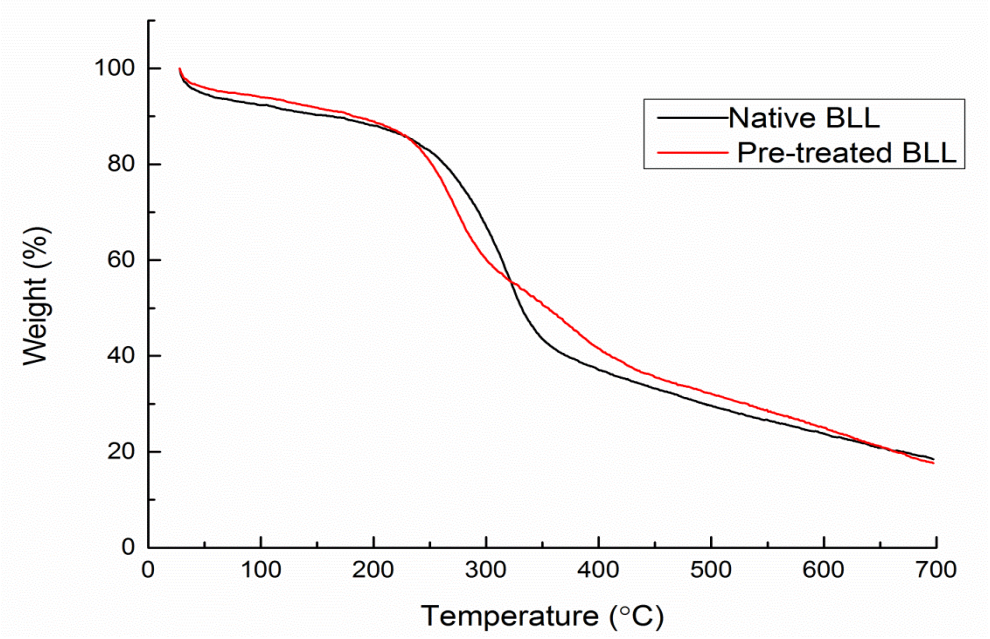

Figure 12. TGA of native and microwave-alkali-acid pre-treated biomass

Analysis of native and microwave-alkali-acid pre-treated BLL biomass with above mentioned analytical techniques revealed hemicellulose and lignin removal and degradation, hence substantially altering lignocellulosic structure which improves enzymatic hydrolysis and facilitate enhanced ethanol production using SSF.

Ethanol production using Simultaneous saccharification and fermentation

Taguchi design is a powerful and efficient option to determine optimal combination of factor levels to facilitate analysis of design. In present study, the effect of 6 factors on SSF process of pre-treated BLL biomass were investigated using Taguchi experimental design in 25 runs (Table 5). Response was obtained in terms of ethanol concentration (g/L), as function of six factors, viz, substrate loading (w/v)%, temperature (°C), pH, enzyme loading (v/v)%, yeast inoculum size (v/v)%, time (h).

Table 13 provides ethanol concentration which falls in range of 1.2 to 12.4 g/L as combined effect of six factors in their specified range. In run 2 (std. 23), with a combination of 11% (w/v) substrate loading, 0.5% (v/v) enzyme loading, 8% (v/v) yeast inoculum at 4.5 pH and 30°C temperature, maximum ethanol concentration (12.4 g/L) was observed after 72 h of incubation.

Table 13. Response values of L_{25} Taguchi orthogonal array design for SSF optimization

Runs		**Response**	
Std	**Run**	**Ethanol Concentration (g/L)**	**Ethanol yield (%)**
2	1	5.2	14.89
23	2	12.4	35.55
6	3	8.4	24.06
22	4	10.9	31.22
1	5	1.2	3.44
24	6	6.8	19.48
8	7	4.4	12.66
7	8	2.5	7.16
17	9	5.8	16.81
20	10	6.3	18.04
16	11	7.4	21.19
19	12	5.2	14.89
4	13	4.9	14.03
10	14	7.3	20.91
9	15	5.4	15.46
25	16	5.8	16.61
15	17	5.7	16.32
5	18	3.8	10.88
11	19	8.2	23.49
3	20	4.3	12.31
18	21	9.9	28.36
13	22	4.6	13.18
21	23	8.5	24.35
12	24	7.1	20.34
14	25	6.8	19.48

Analysis of the screened variables from a five-level orthogonal array design led to identification of the key factors affecting ethanol production. The results obtained were fed into the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, Minn. USA) and analyzed using ANOVA. The actual value range of the screened variables was presented in Table 14.

Table 14. ANOVA for selected factorial mode of Taguchi design

Source	Sum of Squares	Df	Mean Square	F Value	Probability > F
Model	150.388	20	7.5194	89.94498	0.000267
A-Substrate Loading	67.0304	4	16.7576	200.4498	0.001507
B-pH	6.7664	4	1.6916	20.23445	0.006444
C-Temperature	15.3424	4	3.8356	45.88038	0.001346
E-Time	16.4304	4	4.1076	49.13397	0.001178
F-Inoculum Size	44.8184	4	11.2046	134.0263	0.000164

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The predicted R^2 (0.91) was in close proximity with adjusted R^2 (0.98), advocated for greater significance of model. The statistical analysis allowed us to determine the contribution of experimental factors (signals) in comparison to noise, where the signal should be fairly large in comparison to noise. Thus, the estimated adequate precision of 42.26 for ethanol production, represented the signal to noise ratio, is an adequate signal. A lower value for the coefficient of variation (CV) suggests higher reliability of the experiment and obtained CV value of 4.55% demonstrates a greater reliability of the trials. The model *F* value of 89.94 as well as values of $P > F$ (<0·0001) indicated that the model terms *(A: substrate loading, B: pH, C: Temperature, E: Time, F: Inoculum size)* were significant (Table 14).

Results showed that at higher substrate amount of 9% (run 21) and 11% (run 2), enhanced ethanol production of 9.9 and 12.4 g/L respectively was obtained (Table 13). Results presented by Shengdong et al. (2007) to optimize ethanol production from pre-treated rice straw at 10% loading using SSF with 54% ethanol yield was also found to be in reasonable agreement.

Present study reveals that ethanol concentration increase from 8.4 to 12.4 g/L as pH shift from 3.5 to 4.5 at an optimum temperature of 30°C. Increase in pH and temperature beyond 4.5 and 30°C respectively exerts a negative impact on overall ethanol production due to stress on yeast cells owing to combination of previously described factors (Table 13). pH

optimization for ethanol production from wild grass using Taguchi design shows an optimum pH of 4.3 as reported by Das et al. (2014). Similar result of ethanol concentration (17.4 g/L) at pH 4.5 from bagasse pith hydrolysate was reported by Dasgupta et al. (2013). Findings were supported by Wan et al. (2012) for softwood conversion to ethanol at 30°C and 5 pH resulting in ethanol yield of 85.1% and 51% yield from Topioca stem at 30°C and 5.5 pH (Magesh et al., 2011). A maximum ethanol titer of 32.6 g/L was obtained from sugarcane bagasse at optimized process conditions of 35°C, 5.5 pH and 72 h fermentation time (Sasikumar and Viruthagiri, 2008).

A number of numerical solutions were suggested by the Design-Expert software (Version 8.0.7.1, Stat-Ease, Minneapolis, MN), within the experimental range of parameters for maximum ethanol production. The model predict, response based on RSM and estimates ethanol concentration of 12.7 g/L at substrate loading (11% w/v), pH (4.5), temperature (30°C), Enzyme loading (0.5% v/v), inoculum size (8% v/v) and time (60 h) (Figure 13). Figure 13 is a 3D Contour plot showing interaction between pH and substrate loading for ethanol. In order to validate, the predicted model experiment was conducted in triplicates at model predicted conditions. At regular intervals (12 h), samples were withdrawn to estimate ethanol and reducing sugar concentration. The maximum experimental value for ethanol production at model predicted experiment condition was 12.8 g/L. The excellent correlation between predicted (12.7 g/L) and experimental values (12.8 g/L) of experiment conducted at combination of parameters predicted by model, justified the validity of the regression model obtained.

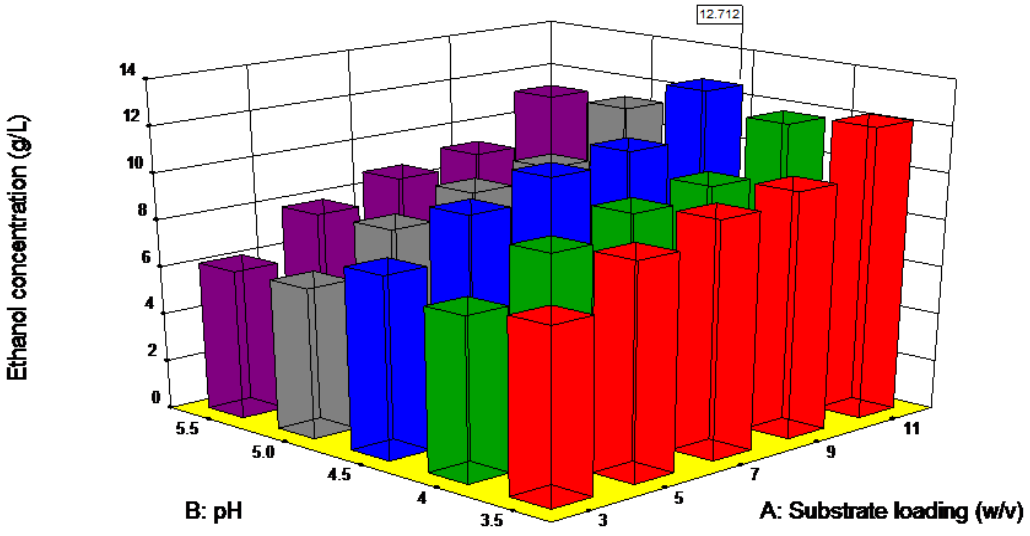

Figure 13. 3D contour plot showing interaction between substrate loading and pH

During saccharification and fermentation process, reducing sugar release from lignocellulose was enhanced owing to enzyme action and was consumed to ethanol. Figure 14 shows time course for reducing sugar and ethanol concentration during SSF of pre-treated and native BLL biomass where reducing sugar was converted to ethanol in time dependent manner at model predicted fermentation parameters.

Native BLL biomass exhibits reducing sugar concentration of 3.2 g/L at start of SSF experiment and reaches to maximum (5.6 g/L) after 36 h of incubation without any pre-treatment. Highest ethanol concentration (1.2 g/L) was obtained at 48 h of fermentation of native untreated BLL biomass. For pre-treated BLL biomass an increase in reducing sugar concentration (48.7 g/L) was observed around 36 h with production of 8.97 g/L ethanol. The 12 h fermentation of pre-treated BLL during SSF resulted in 29.33 g/L of reducing sugar and only 4.2 g/L ethanol concentration. Ethanol concentration does show significant increase up to 48 h and levelled off after 60 h.

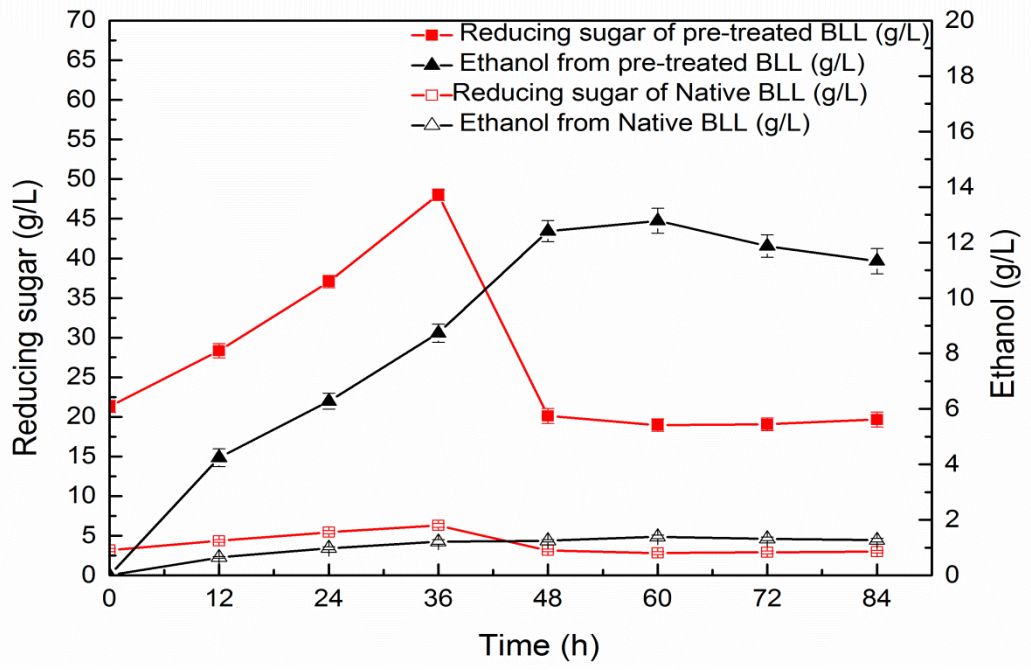

Figure 14. Ethanol production and reducing sugar consumption during SSF of pre-treated and native BLL biomass over time period of 84 h

*The vertical bars designate the standard errors for the mean of three replicative tests

Highest ethanol concentration for pre-treated BLL biomass was achieved after 60 h of SSF was 12.84 g/L which corresponds to ethanol yield of 35.5%. In the first 36 h, a higher reducing sugar concentration appeared in reaction mixture because the rate of yeast fermentation could not keep up with that of enzyme hydrolysis. Reducing sugar concentration reached peak at about 36 h and decreased gradually for native and pre-treated

biomass because the fermentation rate reached the rate of enzyme hydrolysis. Hydrolytic efficiency of cellulase is negatively affected by catabolite repression and increase in lignin concentration (Mussatto et al., 2008). After 36 h of incubation, enzyme accessibility to cellulose and hemicellulose was reduced due to concentration of lignin and ash (Oberoi et al., 2012).

Reducing sugar left after 48 h were mainly xylose and arabinose which could not by fermented by yeast to ethanol. An enhancement of 11.5 g/L in ethanol concentration was observed for SSF of native (1.2 g/L) and pre-treated (12.8 g/L) BLL biomass. Increase in reducing sugar production till 12 h (40 g/L) and maximum ethanol concentration of 24.25 g/L at end of 24 h was reported by Oberoi et al. (2012).

The ethanol yield, productivity and fermentation conditions for ethanol production from BLL biomass via SSF was compared with other lignocellulosic bioethanol processes (Table 15) with different fermenting strains.

Ethanol concentration of 11.2 g/L with wheat straw (Qi et al., 2010), 17.40 g/L with begasse pith sample (Dasgupta et al., 2013) is being reported via simultaneous saccharification and fermentation. This study reported for first time the statistical optimization and validation of different fermentation process parameters for bioethanol production from microwave-alkali-acid pretreated bamboo leaf litter biomass using Taguchi orthogonal array design, namely, substrate loading (w/v)%, temperature (°C), pH, enzyme loading (v/v)%, yeast inoculum size (v/v)%, time (h). Final ethanol concentration and yield attained under optimum fermentation conditions was 12.8 g/L and 35.5% of theoretical value which was identical to model predicted response. The model was successfully validated to achieve maximum ethanol production and significant factors were determined. The ethanol yield obtained with current process is found to be in reasonable agreement in comparison to other processes utilizing different lignocellulosic waste feedstocks for bioethanol production.

Table 15. SSF of different lignocellulose biomass for ethanol production

Feedstock	Type of Hydrolysis	Microorganisms	Fermentation conditions	Ethanol (g/L)	References
Bamboo leaf litter biomass	Microwave- acid-alkali pre treatment	*Saccharomyces cerevisiae* NCIM 3215	11% substrate loading, pH 4.5, 30ºC, 0.5% enzyme loading, 8% inoculum, 60h.	12.8 g/L	Present study
Corn stover	SO_2 catalyzed steam explosion	Baker's yeast	30 ºC, pH 5.5, 96 h	26.4 g/L	Ohgren et al., 2007
Orange peels	Two-stage sulfuric acid hydrolysis	*S. cerevisiae*	32.5ºC, pH 5.0, 15 h	12.97 g/L	Oberoi et al., 2010
Wheat straw	Pre-treatment with 3 % H_2SO_4 and 0.5% Tween 20	*S. cerevisiae*	40ºC, pH 4.8, SSF	11.2 g/L	Qi et al., 2010
Bagasse pith hydrolysate	Steam and acid (8% w/w H_2SO_4) pre-treatment	*Kluyveromyces* sp. IIPE453 MTCC 5314	45°C, pH 4.5, 5% inoculum, 4% substrate loading, 48h	17.40 g/L	Dasgupta et al., 2013.
Softwood	2% dilute (HCl) acid pre-treatment	*Pichia stipites CBS 6054* *S. cerevisiae Y5*	30°C, pH5, 96h	27.40 g/L	Wan et al., 2012
Tapioca stem	Dilute acid (1.25% H_2SO_4) and dilute alkali (NaOH) treatment	*Fusarium oxysporum*	30°C, pH 5.5, 2% inoculums	8.64 g/L	Magesh et al., 2011
Kinnow waste and banana peels	Steam explosion	*P. tannophilus* MTCC 1077 *S. cerevisiae G*	30°C, 48h	26.84 g/L	Sharma et al., 2007
Sugarcane Bagasse	Sodium chlorite and acetic acid pre-treatment	*Kluyveromyces fragilis*	35°C, 3% substrate loading, pH 6, 72h	32.6 g/L	Sasikumar & Viruthagiri 2008

SUMMARY

1. Physico-chemical characterization of Bamboo leaf litter biomass was done and volatile matter, ash content, carbon, oxygen, cellulose, hemicellulose, lignin content was found to be 81.5, 5.4, 36, 56, 26, 23.8, 14.4% respectively. These characteristics, signifies potential of Bamboo leaf litter biomass as feedstock and high energy value for ethanol production.
2. Among all four pre-treatment employed under study, microwave-alkali-acid showed highest delignification, increasing cellulose (76%) and reducing sugar with reduction in hemicellulose (34%), lignin (54%) content and 25.8% saccharification efficiency. Microwave-alkali-acid was observed as most efficient pre-treatment approach to reduce recalcitrance and improving enzymatic hydrolysis of lignocellulose biomass.
3. Structural and chemical changes in native and microwave-alkali-acid pre-treated biomass were investigated. Microscopy studies revealed that pre-treatment caused breakage of complex matrix and partial defibrillation of pre-treated Bamboo-leaf litter biomass.
4. XRD analysis of native and microwave-acid pretreated biomass showed increase of 8.8% in *CrI*. Destruction of biomass crystallinity was observed along with lignin and hemicellulose solubilization. Results were also supported by FTIR and Solid state ^{13}C CP/MAS NMR. Thermal analysis of native and pre-treated biomass showed that thermal stability increased in biomass, which was attributed to removal of hemicelluloses and increase in crystallinity after pre-treatment processing.
5. The statistical optimization and validation of different fermentation process parameters for ethanol production from Bamboo leaf litter biomass using Taguchi orthogonal array design was reported and maximum ethanol concentration of 12.8 g/L was found which was corresponded to 35.5% productivity was observed.
6. The ethanol yield obtained with current process was in reasonable agreement to other processes utilizing different feedstock for bioethanol production. In essence, the pre-treatment and statistical optimization of fermentation process parameters can transform, Bamboo leaf litter biomass, bioethanol.

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APPENDIX I

(A) MGYP MEDIA

(B) GYE MEDIA

Ingredients	Quantity (g/L)
Glucose	10
Yeast extract	1
$(NH_4)_2SO_4$	5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.5

(C) PDA

Ingredients	Quantity (g/L)
Potato extract	4
Dextrose	20
Agar	15

APPENDIX II

(A) Sodium Citrate buffer (pH 3.5, 4.0, 4.5, 5.0 and 5.5) (50mM)

Stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate

Use x mL A+ y mL B and dilute to 100 mL with 50 mL distilled water

A (mL)	B (mL)	pH
37.0	13.0	3.5
33.0	17.0	4
25.5	24.5	4.5
20.5	29.5	5
13.7	36.3	5.5

(B) Potassium phosphate buffer (pH 7.0 and 6.0) (50mM)

Stock solutions: A: 1M K_2HPO_4; B: 1 M KH_2PO_4

Use x mL A+ y mL B and dilute to 1000 mL with 995 mL distilled water

A (mL)	B (mL)	pH
3.075	1.925	7
0.660	4.340	6

APPENDIX III

(A) Standard curve of Glucose

Reducing sugar concentration of pre-treated and untreated biomass was measured by modified Dinitrosalicylic acid (DNS) method (Miller, 1959) using dextrose as the standard.

Materials:

a. Stock: 2 mg/mL glucose

b. DNS reagent

Distilled Water	1415 mL
3, 5-Dinitrosalicyclic acid	10.5 g
NaOH	19.8 g
Dissolve the above and then add:	
Rochelle salts (Na-K tartarate)	306 g
Phenol (melt at 50°C)	7.6 mL
Sodium meta-bisulphite	8.3 g

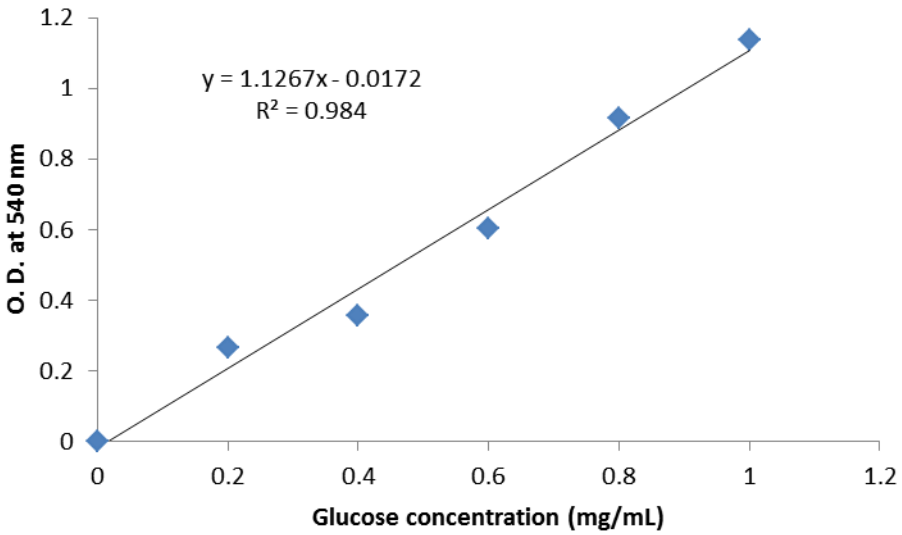

Figure 14. Standard curve of glucose

(B) Standard curve of Cellulose

Cellulose concentration of pre-treated and untreated bamboo leaf litter biomass was measured by Anthrone Assay (Updegraff, 1969) using CMC as standard.

Materials:

a. Stock: 10 mg/mL CMC

b. Anthrone reagent (200 mg of anthrone in 100 mL of concentrated H_2SO_4)

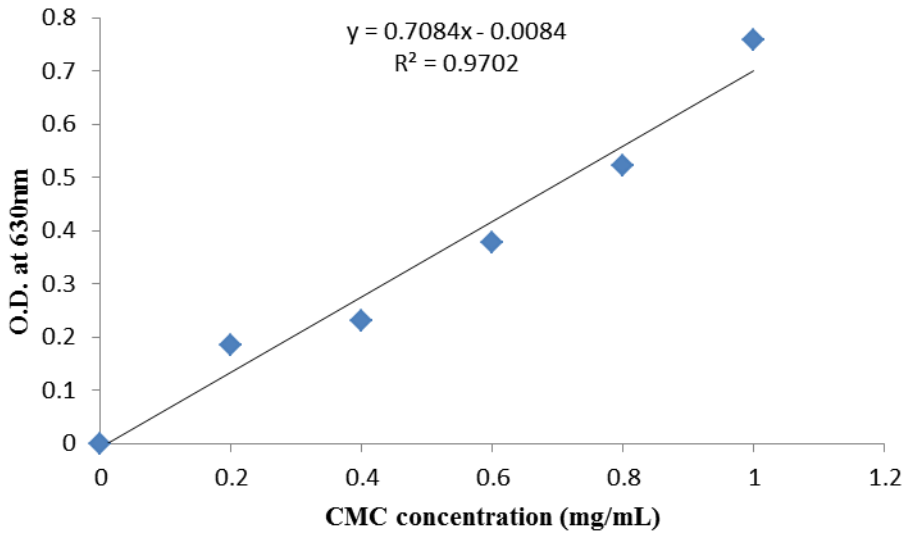

Figure 15. Standard curve of cellulose

(C) Standard for ethanol

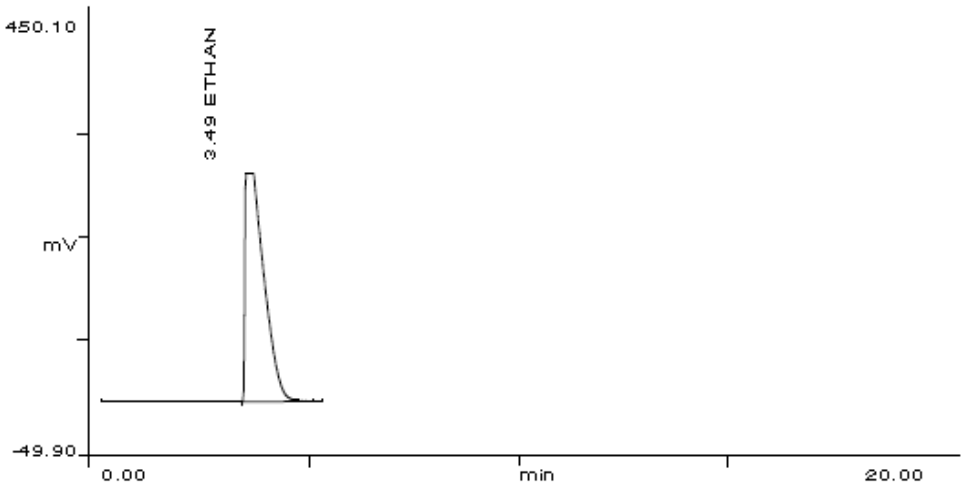

Figure 16. Ethanol standard graph (100% v/v)