Essential oil extraction from Java Citronella (*Cymbopogon winterianus* Jowitt) and valorisation of the spent biomass to value added chemicals

A

Thesis

Submitted in partial fulfilment of the
Requirements for the degree of

Doctor of Philosophy

by

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DECLARATION

This is to certify that the thesis entitled "**Essential oil extraction from Java Citronella (*Cymbopogon winterianus* Jowitt) and valorisation of the spent biomass to value added chemicals**", submitted by me to the *Indian Institute of Technology Guwahati*, for the award of the degree of **Doctor of Philosophy**, is a bonafide work carried out by me under the supervision of Prof. Vaibhav V. Goud. The content of this thesis, in full or in parts, have not been submitted to any other University or Institute for the award of any degree or diploma. I also wish to state that to the best of my knowledge and understanding nothing in this report amounts to plagiarism.

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This is to certify that the thesis entitled "**Essential oil extraction from Java Citronella (*Cymbopogon winterianus* Jowitt) and valorisation of the spent biomass to value added chemicals**", submitted by **Robinson Timung** (136107028), a PhD student in the *Department of Chemical Engineering, Indian Institute of Technology Guwahati*, for the award of the degree of **Doctor of Philosophy**, is a record of an original research work carried out by him under my supervision and guidance. The thesis has fulfilled all requirements as per the regulations of the institute and in my opinion has reached the standard needed for submission. The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

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Dedicated

With love

To my Wife, Parents and families for their continuous Support, Encouragement and love

To all my teachers, my well wishers

&

To all those who are backbone for me who helped me and encouraged me to reach this position in my life

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ABSTRACT

The present study mainly emphasises on investigating a suitable technique for the extraction of essential oil from Java citronella (*Cymbopogon winterianus* Jowitt). Distillation methods which are relatively simple, cheap, environment friendly, produce good quality oil are preferred for essential oil extraction from grasses. Thus, in this thesis, hydro distillation technique was used to estimate the quantity of essential oil present in different parts of Java citronella plant. As the hydro distillation process has wide range of parameters hence optimisation of hydro distillation of Java citronella plant parts was carried out with central composite design of experiments for three level - three factors (like time, solute to solvent ratio and plant parts) using response surface methodology (RSM). Comparison of the oil yield from different parts of the plants revealed that leaves contained about 40 % and 17 % more essential oil (yield of oil from leaves: 2.38 % vw^{-1}) than the stems and whole aerial parts, respectively. Higher essential oil contained in the fresh leaf samples (2.43% on a dry weight basis) compared to dried leaves (2.12% on a dry weight basis) indicates that the moisture content has a significant impact on the oil yield. The oil obtained from the leaves found to contain higher percentage (about 95 %) of commercially important compounds such as citronellal (55.23 %), geraniol (26.29 %) and citronellol (13.41 %) which has significant anti-bacterial effect. Hence, the extracted citronella oil was tested with six bacteria strains namely *Bacillus subtilis, Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Escherichia coli,* and was found to be effective against all the tested strains, which revealed that citronella oil with selection of specific concentration could act as an anti-bacterial supplement for the treatment of various bacterial infections. After distillation, the left-over aqueous liquid, also called as hydrosol was analysed using HPLC and found to contain sugar alcohols such as erythritol (0.07 $mg.ml^{-1}$) and mannitol (0.03 $mg.ml^{-1}$), which could be utilised in nutraceutical and pharmaceutical industries. Further, hydro distillation process was compared with Steam distillation (SD), Ultrasonic assisted HD (UA-HD) and Ultrasonic assisted SD (UA-SD) on the basis of essential oil yield and its composition. Although UA-SD process gave highest citronella oil yield, but the relative constituent's percentage of the oil extracted using different processes are almost same among all the studied processes in this thesis.

After essential oil extraction, the spent biomass (biomass after essential oil extraction) does not serve the purpose of cattle feed and usually discarded as waste or burn haphazardly leading to environmental problems. Hence, in this thesis, attempt has been made to utilise the spent biomass of citronella for the production of value-added chemicals viz. total reducing sugars (TRS) and levulinic acid. Further the results obtained from spent biomass of citronella was compared with bagasse, which is a standard lignocellulosic biomass mostly used for bioenergy and biochemical production.

Pretreatment of biomass to alter their recalcitrant structures is an essential step to obtain high yield of products via bioconversion processes. In this study, main emphasis was to compare the results evaluated in terms of total reducing sugars (TRS) yield after acid, alkali, and subcritical water pretreatment process performed using laboratory scale equipment using different lignocellulosic biomass. The chemical pretreatment (i.e. dilute acid and alkali pretreatment) process followed by enzymatic hydrolysis (using *Trichoderma reesei* 26291) was compared on the basis of TRS yield. The acid pretreatment of bagasse and citronella biomass performed at optimised condition (0.1 M H_2SO_4, 120 °C and 120 min) showed maximum TRS yield of 452.27 $mg.g^{-1}$ and 487.50 $mg.g^{-1}$, respectively. Subsequent enzymatic hydrolysis of acid pretreated biomass showed maximum TRS released of 226.99 $mg.g^{-1}$for citronella and 282.85 $mg.g^{-1}$ for bagasse at 10 FPU, 50 °C and 48 h. On the other hand, alkali pretreated biomass showed maximum TRS yield of 45.22 $mg.g^{-1}$ for bagasse and 73.88 $mg.g^{-1}$ for citronella biomass. Later the enzymatic hydrolysis (at 10 FPU, 50 °C and 48 h) of alkali pretreated biomass showed maximum TRS of 396.70 $mg.g^{-1}$ and 345.49 $mg.g^{-1}$ for bagasse and citronella, respectively. Considering high TRS yield, dilute acid pretreatment process using sulfuric acid could be considered as a good choice for the hydrolysis of sugarcane bagasse and spent citronella biomass. Although, chemical pretreatment process results in a rapid and good yield of fermentable sugars after enzymatic hydrolysis but also suffers from additional cost of solvent recovery, toxicity, and hydrolysate neutralisation prior to fermentation. Thus, the results of chemical pretreatment processes have been compared with subcritical water (SCW) hydrolysis process which holds significant promise as a 'green' alternative to the chemical pretreatment techniques. The SCW hydrolysis process was optimised with response surface methodology using three level-three factors central composite design model. The high regression coefficients of the response (R^2 = 0.99) indicates that model fits very well to the data. At optimum condition, TRS yield obtained was 320.50 $mg.g^{-1}$ (50.93 %) for bagasse (at temperature (T) = 170.3 °C, time (t) = 30.7 min, and biomass loading (BL) = 4.18 wt%), and 279.64 $mg.g^{-1}$ (46.32 %) for citronella biomass (at T

= 164.38 °C, t = 22.46 min, and BL = 5.00 wt%). The concentration of degraded products (5-HMF and furfural) increased with an increase in the temperature and attained maximum 46.3 $mg.g^{-1}$ of 5-HMF and 4.5 $mg.g^{-1}$ of furfural at 220 °C and 40 min for bagasse. Similarly in case of citronella maximum 5-HMF (5.77 $mg.g^{-1}$) and furfural (10.93 $mg.g^{-1}$) was obtained at 220 °C. Results of SCW hydrolysis of bagasse and citronella revealed that maximum TRS can be achieved at lower temperatures but requires longer reaction time. For that reason, reaction kinetics was estimated at two different reaction time, (1) total reaction time (i.e., 40 min) and (2) reaction time at which maximum TRS yield achieved (i.e., 30 min); see Fig. 6.8-6.11. This reaction kinetics is important for better understanding of hydrolysis of biomass under SCW condition.

Further, the subcritical hydrolysate sample with maximum total reducing sugars (TRS) obtained at optimum condition was processed to produce levulinic acid (LA) using homogeneous biodegradable methane sulfonic acid (MSA) as a catalysts. A second order polynomial model was developed for the acid-catalysed hydrolysis of biomass to LA yield. A linear regression model was capable of predicting the Levulinic acid yield with respect to the effect of reaction temperature, time and acid concentration. The optimal LA yield of 22.73 wt% (at T = 203 °C, t = 44.55 min, and MSA concentration = 0.66 M) for bagasse and 11.17 wt% (at T = 231.86 °C, t = 38.44 min, and MSA concentration = 0.66 M) for citronella was achieved. A good fit between the experimental and model predicted data (R^2 = 0.99) indicates that the applied model was highly significant. Comparative results with the literature depicted that sulfonic acid catalysts showed similar selectivity to mineral acids for the conversion of sugars to levulinic acid. Hence, MSA considered as a more potent catalysts for the conversion of biomass to LA compared to mineral acids.

The work presented in this thesis is focused mainly on the extraction of essential oil using distillation techniques; transforming all non-valorised solid and liquid wastes into bio-sourced co-products such as total reducing sugars and levulinic acid. This work could give important inputs on effective utilisation of spent aromatic biomass for synthesis of value added products using a greener technique. The methodology adopted in this work can form a framework for investigations in other aromatic plant species employing suitable process model.

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Nomenclatures

AGP III	Angiosperm Phylogeny Group III
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
BBD	Box Behnken Design
BL	Biomass Loading
BSS	British Standard Society
CI	Crystallinity Index
$Ca(OH)_2$	Calcium Hydroxide
CCD	Central Composite Design
CO	Citronella Oil
DA	Dilute Acid
DF	Degree of Freedom
DNS	Dinitrosalicylic Acid
DOE	Design of Experiment
DP	Degree of Polymerization
DTG	Differential Thermo Gravimetric
EDX	Energy Dispersive X-ray spectroscopic
EH	Enzymatic Hydrolysis
EO	Essential Oil
FPU	Filter Paper Unit
FTIR	Fourier Transforms Infrared Spectroscopy
GCMS	Gas Chromatography Mass Spectroscopy
GVA	γ-valerolactone
Ha	Hectare
HBr	Hydrobromic Acid
HCl	Hydrochloric Acid
HD	Hydro Distillation
HMF	Hydroxy Methyl Furfural
HPLC	High Performance Liquid Chromatography
H_3PO_4	Phosphoric Acid
H_2SO_4	Sulfuric Acid

IU	International Units
KBr	Potassium Bromide
KOH	Potassium Hydroxide
LA	Levulinic Acid
MAHD	Microwave Assisted Hydro Distillation
MHG	Microwave Hydro Diffusion and Gravity
MSA	Methane Sulfonic Acid
MSDf	Microwave Steam Diffusion
MSM	Mineral Salt Media
MTHF	Methyl Tetrahydrofuran
NaOH	Sodium Hydroxide
NCI	Network Chromatography Interface
NREL	National Renewable Energy Laboratory
OAHD	Ohmic Assisted Hydro Distillation
PDA	Potato Dextrose Agar
PT	Pretreatment
RI	Refractive Index
RSM	Response Surface Methodology
SCW	Subcritical Water
SD	Steam Distillation
SEM	Scanning Electron Microscopy
SFME	Solvent Free Microwave Extraction
TGA	Thermo Gravimetric Analysis
TRS	Total Reducing Sugars
UAHD	Ultrasonic Assisted Hydro Distillation
UASD	Ultrasonic Assisted Steam Distillation
UV-Vis	Ultra Violet Visible Spectrophotometer
XRD	X-ray Diffraction

Notations

R^2	Square of correlation coefficient (dimensionless)
°C	Degree Celsius
%	Percentage
nm	Nanometer

µm	Micrometer
mm	Millimetre
cm	Centimetre
cm^{-1}	Per centimetre
µl	Microliter
ml	Mililitre
L	Litre
sec	Second
min	Minute
h	Hour
min^{-1}	Per minute
µg	Microgram
mg	Milligram
g	Gram
kg	Kilogram
cP	Centipoise
MPa	Mega Pascal
psi	Pounds per square inch
kHz	Kilo Hertz
rpm	Revolution per minute
$kg.cm^{-2}$	Kilogram per square centimetre
$mg.g^{-1}$	Milligram per gram
$mg.kg^{-1}$	Milligram per kilogram
$g.g^{-1}$	Gram per gram
$mg.ml^{-1}$	Milligram per millilitre
$g.lit^{-1}$	Gram per litre
$g.lit^{-1}.min^{-1}$	Gram per litre per minute
$lit.g^{-1}.min^{-1}$	Litre per gram per minute
$mol.lit^{-1}$	Mole per litre
kJ. Mol^{-1}	Kilojoule per mole
$J.mol^{-1}.K^{-1}$	kilojoule per mole per kelvin
$MJ.Kg^{-1}$	Mega joule per kilogram
vol%	Volume percentage
wt%	Weight percentage

T_{50}	Temperature of 50% weight loss
R_{50}	Rate of weight loss at T_{50}
°C.min^{-1}	Degree Celsius per minute
v.v^{-1}	Volume by volume (concentration)
w.w^{-1}	Weight by weight (concentration)
w.v^{-1}	Weight by volume (concentration)
E_a	Activation energy (kJ.mol^{-1})
R	Universal gas constant (J.mol^{-1}.K^{-1})
T	Temperature of reaction (K)
A	Pre-exponential factor (min^{-1})
k	Rate constant (min^{-1})
t	Time (min)
M	Molarity
W	Watt
C_B	Concentration of TRS
C_{A0}	Initial mass of the biomass
Y	Predicted response
P	Probability value
β_o	Intercept
β_i, β_{ii}, β_{ij}	Coefficients for linear, quadratic and interaction effect respectively
x_i, x_j	Independent variables
k_1	First order reaction rate constants of saccharides formation
k_2	First order reaction rate constants of saccharides degradation

Chapter 1

Introduction and review of literature

In this chapter, after a brief orientation towards the basics of aromatic plants, state of the art with respect to the extraction of essential oil (EO) from aromatic plants, characterisation, utilisation of spent biomass for the production of reducing sugars using various pretreatment techniques followed by its use in the levulinic acid production has been presented. Among these various processing steps, focus has been towards essential oil extraction using hydro distillation technique (HD), pretreatment of spent biomass using acid, alkali, and subcritical water (SCW) hydrolysis to produce reducing sugars which is the major source or feedstock for bioenergy production and conversion of total reducing sugars (TRS) to levulinic acid. Subsequently, the possible scope for further research has been discussed. Finally, the objectives of the thesis and its organisation are summarised.

1.1 General background

Aromatic plants comprise of those plants that possess volatile fragrance substance, which occurs as an essential oil, oleoresin, balsam and gum exudate either in the bark, stem, flower, fruit, root and wood. They are an important component of agriculture and have grown in India for their fragrance and therapeutic use for decades (Djilani et al., 2012). Aromatic plants are one of the major natural resources of our country (India) occurring in diverse ecosystems. In India, the area under aromatic plants is more than 20,000 ha with an annual production of about 1500 tonnes. Northeast India is one of the richest repositories of Medicinal and Aromatic plants. These aromatic crops contain 1-8 wt% of essential oil. India is the 3rd largest producer of essential oil after China and Brazil. The demand and price of essential oils and herbal products are consistently increasing in global markets due to high prosumer movement. Essential oils are volatile liquid essences concentrated from certain parts of aromatic plant species (Mu'azu et al., 2012). Leafy plant materials (e.g. lemongrass, citronella, ocimum spp., jamrosa mint) contain larger essential oil volumes as compared to fruits (bergamot, orange, lemon, juniper), bark (e.g. cassia, cinnamon, canella), seeds (e.g. fennel, coriander, caraway, dill, nutmeg), wood (e.g. sandal, cedar, pine) and rhizomes (e.g. ginger, calamus, curcuma, orris). Much lesser quantity of oil is available in flower part (e.g. clove, rose, mimosa, lavender, carnation, rosemary, jasmine,) of the plant. The major

chemical constituents of essential oils mainly consist of 90-95 wt% volatile fractions such as aldehydes, alcohols, and esters, whereas 1-10 wt% contains carotenoids, flavonoids, sterols, etc. They are usually secondary metabolites of plants, which they use for protecting themselves against insects, infections and also to attract pollinators. The constituents of essential oils provide their economic value and intrinsical properties (Wei et al., 2013). The quality, quantity, and composition of essential oil depends on the species of the extracted plant, place of origin, geographical locations, environmental conditions (normally warm climates have more essential oils), ecological (habitat), genetic variability (chemotype), time of harvest and extraction process. Therefore, its characterisation is important as the chemical composition varies with the plant species. These variations occur mainly due to variation of chemotypes, adaptation of plant to the encircling environment, and its developmental conditions. The extraction methods, preservation techniques, and conditioning process of essential oil are thus very necessary so that its composition does not alter and the quality of the oil be maintained in its originality. The deterioration of essential oil may occur due to oxidation, polymerisation, resinification, interaction of functional groups, and the hydrolysis of esters (Turek and Stintzing, 2013). These processes may occur due to heat, presence of air (oxygen), moisture, trace metallic particles and catalyst. Moisture plays a major factor in deterioration than light, thus essential oil should first be treated to remove moisture, metallic impurities, and then clarified before it is stored in well-filled, tightly closed containers at low temperature. These oils form indispensable ingredients of the necessities in many spheres of human activity. It constitutes a natural source of high value industrial raw material for agriculture, pharmaceutical, food, cosmetic industries and other aroma related products. It also opens up new opportunities for farmers with significant scope for rural development (Nakahara et al., 2003). The most-traded essential oils are mints, citronella, basil, orange, lemongrass, sandalwood, eucalyptus, geranium, lavender, jasmine, and tuberose. Some of the common aromatic plants with its major components of essential oil are tabulated in **Table. 1.1**.

Table 1.1: Aromatic plants with major components of its essential oil.

Common name	Scientific name	Main components of essential oil
Bergamot orange	*Citrus aurantium*	linalyl acetate, linalool, d-limonene
Basil	*Ocimum basilicum*	Citronellol, linalool, camphor, eugenol
Camphor	*Cinnamomum camphora*	camphor, safrole, cineol
Caraway	*Carum carvi*	d-carvone, limonene, dihydrocarvone
Cinnamon	*Cinnamomum verum*	eugenol, β-caryophyllene,
Citronella	*Cymbopogon spp.*	citronellal, citronellol, geraniol
Clove	*Syzygium aromaticum*	eugenol, β-caryophyllene
Coriander	*Coriandrum sativum*	α-linalool, geraniol, geranyl-acetate
Eucalyptus	*Eucalyptus spp.*	cineole, phellandrene, piperitone
Geranium	*Pelargonium spp.*	citronellol, geraniol, citronellylformate
Ginger	*Zingiber officinale*	zingiberene, geraniol, nerol
Jasmine	*Jasminum spp.*	benzyl acetate, phytol, isophytol
Lavender	*Lavandula spp.*	linalyl acetate, borneol, geraniol
Lemon	*Citrus limon*	limonene, β-pinene, γ- terpinene
Lemongrass	*Cymbopogon citratus*	citronellal, citronellol, neral, geranial
Nutmeg	*Myristica fragrans*	α-pinene, β-pinene, sabinene
Palmarosa	*Cymbopogon martinii*	linalool, geranyl acetate, geraniol
Patchouli	*Pogostemon cabin*	patchoulol, norpatchoulenol, azulene
Pepper	*Piper nigrum*	limonene, sabinene, pinene, caryophyllene
Pepermint	*Mentha piperita*	menthol, menthone, pinene, menthyl acetate
Rose	*Rosa damascena*	stearoptene, nerol, geraniol, citronellol
Sandalwood	*Santalum album*	santalol, santalyl acetate, santalene
Spearmint	*Mentha spicata*	menthol, menthone, Carvone
Sweet orange	*Citrus sinensis*	limonene, myrcene
Vetiver	*Vetiveria zizamoides*	vetiverol
ylang-ylang	*Cananga odorata*	linalool, β-caryophyllene, farnesene

Several extraction methods like solvent extraction, distillation (hydro and steam) and supercritical fluid extraction (SFE) are usually used to extract essential oil, but the quantity and quality of the oil depends on the techniques employed (Chanthai et al., 2012). The selection of the extraction method depends on the lineaments of the plant from where oil is

extracted, as the oil can exist in various parts of the plants like seeds, stem, leaves, fruits, flowers, and roots (Man et al., 2012). Thus, essential oil extraction technique is important factor to achieve good yield and quality. Since the essential oil yield is very low, it is crucial to optimise the parameters of the extraction process to achieve good quality oil with high yield (Luthria, 2009). The established extraction techniques such as solvent extraction and maceration, generally use organic solvents and involves large volume of solvents, more extraction time and has reproducibility issues. Moreover, the essential oil obtained in the process might contain tracings of the solvent hampering the quality of the oil (Manaf et al., 2013). And, although supercritical fluid extraction is a green and efficient technique to produce high quality oil with good yield but has a high processing cost. Hence hydro and steam distillation technique, which are environment-friendly, relatively cheaper, simple, and produce high quality oil, are usually chosen to extract the essential oil, especially from grasses (Muhammad et al., 2012).

1.1.1 Problems and scope associated with aromatic plants

Aromatic spent biomass represents one of the amplest and underutilised biological resources on Earth. More than 6.0 million tons per annum of aromatic spent biomass are produced in India alone (Rout et al., 2013). After the extraction of valuable essential oil from aromatic plants by hydro or steam distillation, the spent biomasses are non-cattle feed and hence discarded as waste or burn haphazardly leading to environmental problems (Timung et al., 2016). There is an increasing trend towards the conversion of such spent lignocellulosic wastes into energy or value added products. The sustainability of the human race hinges on possibility of providing enough food, energy, carbon-based products and chemicals to our increasing population, considering the long-term health of the environment (Zheng et al., 2009). The consumption of energy is increasing at a faster rate due to urbanisation leading to the depletion of fossil fuels. Lignocellulosic biomass such as aromatic spent biomass, bagasse, etc. are clean and renewable sources of energy, which remains an alternative to fossil fuels due to its high abundance as well as long term economic and environmental concerns (Uihlein et al., 2009, Tsao et al., 1982). About 220 billion oven-dry tons per year of biomass is available in the world, which is considered as the largest source of sustainable energy (Anon, 2004). Besides producing bio-ethanol, biofuels, and biogas, lignocellulosic biomass can be used for value added chemicals production like levulinic acid (Yan et al., 2008). In view of the above, extensive research has been carried out in this thesis on the utilisation of one such un-noticed or underutilised energy crop, i.e. citronella biomass. The

selection of citronella biomass for the present work is mainly based on the following facts and reasons: high abundance of this biomass in the Northeast part of India; minuscule or no report on conversion of spent citronella biomass to value added chemicals; and enough scope still lies on the utilisation of citronella biomass for production of essential oil using newer or advanced techniques.

The production of value added chemicals from relatively low-cost carbon resources is also a key issue for sustainability (Lynd et al., 2003). Lignocellulosic biomass constitutes different makeup of cellulose (30-50 wt%), hemicellulose (20-35 wt%) and lignin (10-25 wt%), associated together in a different degree of hetero-matrix depending on the source, species and type of biomass (Dorrestijn et al., 2000; Carere et al., 2008; Chandra et al., 2007). The conversion of such complex feedstock to chemicals is beneficial for the development of a sustainable society (Agbor et al., 2011). The platform chemicals produced from cellulosic and hemicellulosic fractions of lignocellulosic biomass are 5-hydroxy methyl furfural (5-HMF) and furfural respectively. Further, these chemicals can be utilised as a raw material to produce other value added product like levulinic acid (**Fig. 1.1**). The value added chemicals which could be deduced from lignocellulosic biomass by integrated physical, chemical, and biochemical processing are succinic acid, 3-hydroxypropionic acid, glutamic acid, aspartic acid, levulinic acid (LA), glycerol, 4-hyroxybutyrolactone, itaconic acid, 2-5, furan dicarboxylic acid, xylitol, sorbitol, and glucaric acid. Among those biochemicals, levulinic acid is distinguished as promising biomass-derived platform chemical. It is a chemical precursor, which can be prepared from HMF, glucose, fructose, sucrose, cellulose and lignocellulosic biomass. It is also a precursor to pharmaceuticals, pesticides, cosmetics, and food additives. Potential biofuels, biopolymer, bio-pesticide can also be prepared from levulinic acid. Till recently, H_2SO_4 has been the most commonly used mineral acid for the production of levulinic acid, even if HCl, HBr, and H_3PO_4 also have been frequently reported as applicable homogeneous catalysts. However, those mineral acids are highly corrosive and harmful to the environment. On the other hand, Sulfonic acids are environmentally friendly, biodegradable, non-oxidising catalysts that are much less corrosive than sulphuric acid.

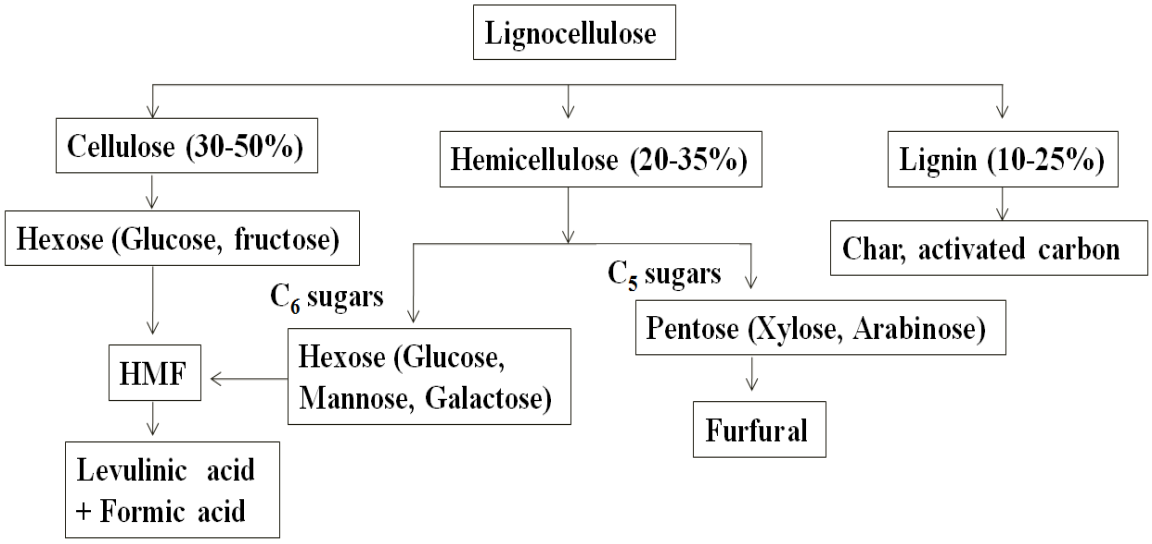


Fig. 1.1: Schematic representation for conversion of lignocellulosic biomass.

Apart from cellulose and hemicellulose, lignin is an assuring raw material for production of various biochemicals. The possible chemicals that can be obtained from lignin are phenols, vanillin catechols and complex mixtures of quinines (Suhas et al., 2007). However, the complex structure of lignin constitutes the most recalcitrant component, which makes it the greatest barrier to achieve an economic separation. This inherent property of lignocellulosic materials makes them resistant to enzymatic and chemical degradation. Therefore, for efficient conversion of cellulose into reducing sugars, it is essential to modify the physical and chemical properties of the plant cell wall through the pretreatment step. Furthermore, appropriate pretreatment is a key step for the effective utilisation of cellulosic biomass due to its fractious nature. The efficiency of biomass conversion to fermentable sugars hinges on biomass type and technique utilised in pretreatment. Owing to these differences, one method available for the deconstruction of one biomass may not be applicable for another biomass. Moreover, the same biomass responds differently to different pretreatments, and the optimised conditions need to be derived separately for the individual biomass.

Hence, the work in this thesis is focused mainly on citronella oil extraction and production of levulinic acid from spent citronella biomass. After essential oil extraction from citronella applying distillation techniques, the spent biomass would be pretreated using dilute acid and alkali, followed by enzymatic hydrolysis to determine and compare the yield of total reducing sugars. Also subcritical water hydrolysis, which is greener technique, would be employed to alter the structure of the biomass and subsequently produce total reducing

sugars. The obtained reducing sugars in the subcritical water hydrolysis process would then be used as a raw material for the production of levulinic acid. A less corrosive, homogeneous environmentally friendly biodegradable sulfonic acid catalyst, would be used as a catalyst, an alternative catalyst to corrosive mineral acids for levulinic acid production.

1.1.2 Java citronella (*Cymbopogon winterianus* Jowitt)

Java citronella is an aromatic plant, belonging to Kingdom- Plantae, Clades- Angiosperms, Monocots and Commelinids, Order- Poales, Family- Poaceae, Subfamily- Panicoideae, Tribe- Andropogoneae, Subtribe- Andropogoninae and Genus – Cymbopogon Spreng., according to APG III system (Angiosperm Phylogeny Group III system, 2009) of flowering plant classification. Citronella plant is commercially categorised into two species: (1) Ceylon citronella - obtained from *Cymbopogon nardus* Rendle, and (2) Java citronella - obtained from *Cymbopogon winterianus* Jowitt (Wijesekera, 1973). Ceylon type is a perennial, robust, stoloniferous grass of about 1 m height having broad leaves and large compound panicles. The spikelets are awnless, lanceolate, and flattened on the back. On the other hand, Java citronella is a tufted perennial grass of about 2 m height with stout, erect, terete, leafy node clumps, and fibrous roots. Leaf-blades are linear, having serrate margins with a long acuminate tip. The compound panicle is very large, with 20 mm long racemes. The two lower spikelets (one sessile and the other pedicelled) are homogeneous, male or neuter, and the remaining pairs are heterogeneous in both racemes. The ovary is superior, monocarpellary, unilocular, and stigma is bifid and leathery (Joy et al., 2001). The region experiencing well-distributed rainfall all over the year is considered favourable for the cultivation of citronella plants. Sandy loamy soil with high moisture content, without waterlogging is the appropriate soil condition for the plant growth. The best planting period is the rainy season, and the ideal season of harvesting is summer and the early winter. The first harvest starts six months after plantation. Harvesting can be done 3 - 4 times annually with an interval of 2.5 - 3 months, but harvesting during late winter significantly reduces the oil yield (Blank et al., 2007). To extend the shelf life of the plant material, drying is important after post-harvest to decrease the moisture content in order to prevent the microbial and enzymatic activity (Rocha et al., 2011).

1.1.3 Citronella oil

Citronella oil, which is an essential oil known for its natural insect repellent, is of great interest to the industries, especially the pharmaceutical and fragrance industry. Java type

citronella oil containing about 85 wt% of commercially important compounds like citronellal, geraniol, and citronellol are considered to be more superior to that of citronella oil obtained from Ceylon type, which contains only 55-65 wt% of these three major compounds (Wany et al., 2014; Wijesekara, 1973). Hence, Java type is more widely cultivated in tropical and subtropical countries like India, Sri Lanka, Malaysia, Taiwan, Ecuador, Madagascar, Guatemala, Honduras, Brazil, Argentina, Mexico, and West Indies (Jayasinha et al., 2003). The Northeast part of India is one of the important biodiversity hotspots having diverse agro-climatic zones, which provides ample conditions for the cultivation of Java citronella. Major Java citronella oil producing states of India are Assam, Karnataka, Uttar Pradesh, Madhya Pradesh, Maharashtra, Tamil Nadu and West Bengal (Wany et al., 2013). Java citronella is grown abundantly in Assam and widely available in the Northeast part of India. Citronella plant grown in the Northeastern region of India is rich in citronellal content, which signifies good quality citronella oil (Ahmed, 2005). Citronella oil can be used in fragrance industries such as in perfumery, cosmetics, soap, confectionery and flavouring industry (Wany et al., 2013). The oil has its medicinal applications and has been widely used in the pharmaceutical industry and for aromatherapy. It has its therapeutic uses as an analgesic, anticonvulsant, anxiolytic, etc. and is a favourable agent towards anti-fungal, anti-bacterial, anti-parasitic, nematicidal, and insect repellent (Almeida et al., 2001, 2003, 2004; Wany et al., 2013). In traditional practices, citronella oil has been used as an antipyretic, aromatic tea, vermifuge, diuretic and in mental illness (Wany et al., 2013). Citronella grass has been traditionally used in Thailand to treat stomach ache, indigestion, intestinal cramps, irritable bowel, flatulence, gastritis and as a blood tonic and diuretic (Salguero, 2003). The demand for citronella oil and other essential oils has increased significantly due to its wide applications in industries.

1.1.4 Hypothesis

This thesis proposes a pilot biorefinery concept considering aromatic biomass, specifically Java citronella. As the extraction yield of essential oil in aromatic plants is generally limited to 1-8 wt% and therefore, approximately 90-98 wt% of non-valorised solid and liquid wastes are generated. Such problems can be reduced or eliminated by developing new innovative scheme allowing a global valorisation of wastes with the goal of a sustainable and environmentally friendly process. This work is an endeavour to establish the possibilities of transforming all non-valorised solid and liquid wastes into bio-sourced co-products such as value added anti-bacterial molecules, biochemicals or bioenergy and hence depict the importance of spent biomass of aromatic plants. Citronella biomass has been chosen due to

its abundance as a raw material in the Northeastern part of India, as well as it has a high growth rate per hectare per year and can be harvested 4 - 5 times in a year. Aromatic spent biomass which is generally left out for disposal or burnt haphazardly creating pollution, can be converted into reducing sugars, which is a raw material for the production of bioenergy such as bioethanol, biomethanol, etc. and various value added chemicals (One such most important chemical is levulinic acid, a chemical precursor, which can be prepared from HMF, glucose, sucrose, cellulose, residues of crops and forestry biomass). Hence, the principal focus of this thesis is the efficient extraction of essential oil from citronella biomass and thereafter production of reducing sugars from the spent biomass and its conversion to levulinic acid.

1.1.5 Scope of the work

A noble prize laureate Mr. Richard Axel, in the field of aroma science for his contribution towards medicine, establishes that in future essential oils are the best source for medicines as it would be based on mostly Terpenes and Sesquiterpenes. Prices of turpentine are going up gradually because of their wide applications in different industries. The demand for essential oil is on the rise in both developing and developed countries due to their growing recognition in the pharmaceutical, cosmetic, agricultural, and food industries. Hence, extensive research is needed to evaluate the best conditions for the production process so that the production and the quality of essential oil can be increased to meet the growing demand. However, more study is required to optimise the conditions of operational parameters of the extraction process. Essential oil being volatile, are very sensitive to heat, oxygen, and moisture; hence, more research is needed to study the quality control of the oil so that the quality of fragrance and flavours can be maintained as per the internationally accepted standards. On the other hand, the growing application of levulinic acid has been expected to drive higher demand due to its wide utilisation in pharmaceuticals, pesticides, cosmetics, and as a solvent. Minimising the cost of production with new and advanced technology considering the positive impact on environment will also increase the growth of levulinic acid market. Hence, in this thesis, after the extraction of essential oil from Java citronella, the spent biomass would be used for the production of levulinic acid. The efficiency of levulinic acid production from spent citronella biomass would be compared with sugarcane bagasse, which is a standard lignocellulosic biomass most widely used for bioenergy and biochemical production.

1.2 Literature Review

In the following section, the detailed literature review on the essential oil extraction followed by chemical pretreatment (dilute acid and alkali), subcritical water hydrolysis and levulinic acid production have been reported.

1.2.1 Essential oil extraction

Essential oils can be extracted using various methods. Essential oil extraction techniques play an important role in order to preserve or retain their unique and original constituents. Numerous reports are available in the literature on the extraction of essential oil; some of them are summarised in the following paragraph.

Okoh et al. (2010) evaluated the effect of different extraction processes on the properties and yield of essential oil from rosemary (*Rosmarinus officinalis* L.) such as solvent-free microwave extraction (SFME) and hydro distillation (HD). The yield of essential oil they had obtained through SFME and HD were 0.39 wt% and 0.31 wt%, respectively. SFME oil contains fewer monoterpene hydrocarbons (25.77 wt%) than HD extracted oil (32.95 wt%), whereas the higher quantity of oxygenated monoterpenes (28.60 wt%) was present in SFME oil than HD oil (26.98 wt%). Golmakani and Rezaei (2008) have examined an advanced HD technique utilising a microwave oven known as microwave-assisted hydro distillation (MAHD). They observed that MAHD requires lesser extraction time (75 min) compared to HD (4 h). Ohmic assisted hydro distillation (OAHD) is another advanced HD technique, which took 24.75 min for extraction of essential oil from thyme, while HD needed 1 h (Gavahian et al., 2012). Bousbia et al. (2009) have compared the microwave hydro diffusion and gravity (MHG) and HD process for the extraction of essential oil from rosemary leaves (*R. officinalis*). As per their results, the MHG process required 15 min to complete the extraction while it took 3 h for HD. Also, MHG does not use water or solvent and no residue is generated in the process. The energy requirement is higher for HD compared to rapid MHG, and it also increased the antioxidant and antimicrobial activities. Farhat et al. (2011) have evaluated the advanced steam diffusion technique using microwave heating known as microwave steam diffusion (MSDf) for the extraction of essential oil from orange peel. They found that MSDf required lesser extraction time (only 12 min compared to 40 min for SDf) for complete extraction of essential oil which also has a similar aromatic profile and yield. Reis et al. (2006) have obtained citronella oil yield of 9.40 wt% using Clevenger apparatus with heptane as a solvent. Man et al. (2012) have extracted citronella oil using ohmic heating hydro distillation with eight extraction cycles from 15 min to 180 min

and obtained the maximum oil yield of 0.37 wt%. As per their reports, kinetics followed 2nd order model with $R^2 = 0.99$, initial extraction rate of 0.49 $g.lit^{-1}.min^{-1}$, extraction capacity of 3.74 $g.lit^{-1}$ and 2nd order constant of 0.04 $lit.g^{-1}.min^{-1}$. Cassel et al. (2006) have evaluated the steam distillation of natural citronella and found an essential oil yield of 0.94 wt%. Wany et al. (2013) have studied the extraction of essential oil from the citronella plant using HD, steam distillation, and solvent extraction process and observed the citronella oil yield of 1.00 wt%, 0.7 wt%, and 0.8 wt%, respectively.

From the above literature, it can be observed that distillation is the most preferred method for production of essential oil from citronella plant material. Although several researchers have explored various distillation process, however still there is enough scope for improvement of the essential oil distillation process. Thus, in this thesis, extraction of essential oil using hydro distillation, steam distillation as well as ultrasonic assisted hydro and steam distillation technique has been investigated. The details of each of the process are discussed in the subsequent chapters.

1.2.2 Pretreatment process

The primary aim of lignocellulosic biomass pretreatment is to alter the cellulose structure and make the cellulose structure more accessible to enzymatic hydrolysis and chemical production. Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass to fermentable sugars conversion (Jacobsen et al., 1999). Several principles characterise an effective pretreatment technique: avoiding size reduction, preserving hemicellulose fractions, limiting the formation of inhibitors due to degradation products, minimising energy input, and being cost-effective. Although extensive research has been reported towards the development of numerous effective pretreatment techniques on various lignocellulosic biomass, but only a few of the reported pretreatment methods have been reached to a demonstration-scale. Therefore, research is still underway to investigate an effective pretreatment technique that should be able to maximise the recovery of available carbohydrates such as cellulose and hemicellulose while minimising the degradation of sugars and the generation of possible inhibitors. Different pretreatment methods such as chemical (acid, alkali, organosolv, etc.), physical (ultrasound, milling, comminution, etc.), Physico-chemical (steam explosion, AFEX, liquid hot water, etc.) and biological (using microorganisms such as fungi and bacteria) are practiced, and others are still in development stage (Sathitsuksanoh et al., 2010; Shevchenko et al., 2000). Among all the pretreatment processes, the chemical pretreatment process results in a rapid and good yield of reducing

sugars after enzymatic hydrolysis. But chemical pretreatment requires recovery of corrosive chemicals and hydrolysate neutralisation before fermentation, whereas physical treatments require high energy leading to high cost. The biological pretreatment is effective, but the slower rate of conversion and cost of enzymes does not make the process popular. With the advancement of technologies, pretreatment is believed to have great potential for improvement of efficiency causing less effect to the environment and lowering cost through research and development. Thus, in this thesis, dilute acid and alkali pretreatment process has been employed for the hydrolysis of spent citronella biomass.

1.2.2.1 Dilute acid pretreatment

Acid pretreatment includes the application of dilute or concentrated acids on lignocellulosic biomass material to disrupt the rigid lignocellulosic matrix. Generally, acid pretreatment is practiced either at a low acid concentration at high temperature or high acid concentration at low temperatures (Kosaric et al., 2001). At high acid concentration, the hydrogen bonding of the cellulose chains gets disrupted, reducing the crystallinity of the cellulose to a relatively amorphous state and releasing very little glucose (Orozco et al., 2007). Concentrated acid hydrolysis is not preferred due to its high corrosiveness hence not environmentally and economically feasible (Sivers and Zacchi, 1995). Dilute acid (DA) hydrolysis is a more established method and provides less probability of sugar degrading to inhibitors such as 5-HMF or furfural, but requires more time and higher temperature to increase the reaction rate (Luo et al., 2002). DA has received extensive interest due to the possibility of replacing cellulose enzymatic hydrolysis and pretreatment step for lignocellulosic biomass to enhance digestibility (Mcmillan, 1994; Lee et al., 1999; Nguyen, 2000). Different acids such as sulfuric acid, hydrochloric acid, nitric acid, phosphoric acid, etc. have been evaluated, but sulfuric acid has been commonly used (Mosier et al., 2005; Torget et al., 1992). Dilute sulphuric acid pretreatment process facilitates enzymatic hydrolysis of lignocellulosic biomass (Himmel et al., 2007; Kumar et al., 2009). A Glucose yield of greater than 80 wt% at sulfuric acid equivalent of 0.05 vol%, was reported (Mok and AntalJr, 1992). Dilute sulfuric acid pretreatment process has been successfully applied with different types of lignocellulosic biomasses such as bagasse, switchgrass, corn stover, etc. (Digman et al., 2010; Xu et al., 2009; Wyman et al., 2009). Olive tree biomass was pretreated with 1.4 vol% H_2SO_4 at 210 °C, resulting in 76.5 wt% yield and cashew apple bagasse pretreated with diluted H_2SO_4 at 121 °C for 15 min yielded 0.47 $g.g^{-1}$ glucose (Cara et al., 2008; Rocha et al., 2009). Lu et al. (2009) studied the H_2SO_4 catalysed hydrothermal pretreatment of rapeseed straw at

180 °C using acid concentration (0.5–2 vol%), treatment time (5–20 min) and solid content (10–20 wt%), and further optimise the process using central composite design of response surface methodology. They demonstrated that hemicellulose in the rapeseed straw could be removed efficiently by H_2SO_4-catalysed hydrothermal pretreatment at high solid loading (20 wt%). Noureddini et al. (2010) evaluated the dilute sulfuric acid hydrolysis for the conversion of distillers' grains and corn fiber to monomer sugars and furfural formation at the acid concentrations of 0.5-1.5 vol%, temperatures of 120-140 °C and biomass loadings of 5-20 wt%. They reported an increase in the formation of monomeric sugars with increasing reaction time. The highest yield observed was at 140 °C with lower substrate loadings, higher acid concentrations within 20 - 30 min. The formation of furfural was lower for the distillers' grains samples compared to corn fiber at 120 °C, and furfural formation increases with increasing temperature. Shi et al. (2012) studied the dilute sulfuric acid pretreatment of corn straw and rice straw at 121 °C with different acid concentrations (1, 2, 3, 4, and 5 vol%) and residence time (30, 60 and 90 min). The pretreated feedstock was further enzymatic hydrolysed using cellulase from *Trichoderma viride* and obtained a yield of 72.38 wt% and 82.84 wt% from com straw and rice straw, respectively. Chen et al. (2012) studied the dilute acid pretreatment of corn stover and observed a 30 % improvement in cellulose digestibility as well as improved hydrolysis of xylan and glucan hydrolysis by 15 % and 30 %, respectively.

1.2.2.2 Alkali pretreatment

Alkali pretreatment has proven a useful method among different existing pretreatment technologies because pretreated alkali feedstocks yield a good amount of fermentable sugar after enzymatic hydrolysis (Xu et al., 2011). On the other hand, it is a non-hazardous chemical agent, less expensive, and requires less energy (Chang et al., 1998). The major effect of alkali pretreatment is delignification. Delignification reactions involve the formation of several different acids produced during the degradation of carbohydrates that introduce hydrophilic groups into the lignin structure (Klinke et al., 2002). Several researchers explain the phenomena of delignification of lignocellulosic biomass materials. Kim et al. (2006) classified three different stages: initial, bulk and residual. Alkali pulping with sodium hydroxide, phenolic α-O-4-linkages in lignin, and some non-phenolic β-O-4-linkages are separated in the initial stage. The separation of non-phenolic β-O-4-linkages is the main reaction in the bulk phase, whereas carbon-carbon linkages in lignin are separated, and carbohydrates are degraded in the residual delignification stage. In the process of alkali

pretreatment, lignin fractions get removed without loss of structural carbohydrates such as glucose, xylose, arabinose, etc. It is also reported that alkali pretreatments remove acetyl groups from hemicellulose, which improves hydrolysis by lowering the steric hindrance of enzymes (Falls et al., 2011). The sole disadvantage of this process is longer reaction time, but researchers have overcome this drawback by elevating the reaction temperature to restrict the reaction time (Chang et al., 2001). The alkali material reacts with biomass in three modes namely: reaction with lignin, neutralisation of organic acids, reaction with resins, and waxes of biomass materials. Alkali pretreatments successfully increase the lignocelluloses digestibility without the production of furfural and methyl furfural (Harmsen et al., 2010). Rabelo et al. (2008) evaluated the pretreatment of sugarcane bagasse (size range from 0.25 - 1.39 mm) with calcium hydroxide and observed that the particle size influences the release of fermentable sugars in the enzymatic hydrolysis using low cellulase loading (3.5 FPU g^{-1} dry pretreated biomass) and low β-glucosidase (1.0 IU g^{-1} dry pretreated biomass). Chang et al. (1998) studied alkali pretreatment of bagasse and wheat straw using calcium hydroxide and found that less pretreatment time (1-3 h) requires high temperature (85-135 °C), whereas longer pretreatment time (e.g., 24 h) requires low temperatures (50-65 °C) to achieve high sugar yield. A material balance on bagasse showed that the biomass yield after pretreatment was 93.6 wt%. Cheng et al. (2010) compared alkali pretreatment of rice straw using sodium hydroxide (NaOH) and calcium hydroxide at 55 °C and 95 °C, respectively. In the case of NaOH, delignification was around 8.6 - 23.1 % and for calcium hydroxide 13.1 - 27.0 %. While at higher temperature delignification achieved was 9.9 - 14.5 % with water alone at 95 °C, but the effect was minimal at 55 °C. The maximum glucose yield obtained was 142.3 $mg.g^{-1}$ and 176.3 $mg.g^{-1}$ dried biomass in NaOH pretreated, and lime pretreated biomass, respectively.

The above literature revealed that extensive research on chemical pretreatments (dilute acid and alkali) has been practiced for decades. To give insights into the chemical pretreatment process, dilute acid pretreatment using sulphuric acid and alkali treatment using NaOH has been examined for the production of total reducing sugars from spent citronella biomass and bagasse. The detailed discussions on the above-mentioned pretreatment processes are presented in Chapter 5.

Chemical pretreatment process, which gives a rapid and good yield of sugar after enzymatic hydrolysis, has certain disadvantages. It requires recovery of corrosive chemicals and neutralisation of hydrolysate before fermentation, whereas conventional physical

treatments require high energy, leading to high cost. However, a greener approach for utilising the lignocellulosic biomass in the production of different value added products is an emerging field for researchers. Hence, the subcritical water hydrolysis process, a possible greener technology to produce total reducing sugar from the biomass, has been investigated in the subsequent chapter (Chapter 6).

1.2.3 Subcritical water (SCW) hydrolysis

Subcritical water defined as water which maintains in its liquid state between its boiling point (100 °C) and critical temperature (374 °C) under adequate pressure (Abdelmoez et al., 2007; Yoshida et al., 2014). Subcritical water technology is considered a green technique and provides extensive advantages over conventional methods. It is a one-step autohydrolysis process, requires less reaction time, cheap, non-toxic, non-corrosive, etc. and provides a wide range of dielectric constant, density, and viscosity, which can be obtained by tuning the operational temperature and pressure. Subcritical water has high ion products and possesses the characteristics of a catalyst (Adachi, 2015). Water at subcritical condition targets the cellulose and hemicellulose fractions of the biomass to solubilise and liberate pentose and hexose sugars while disrupting structure of lignin and cellulose crystallinity (Khajenoori et al., 2009; Prado et al., 2014; Rostagno et al., 2014; Zhu et al., 2013; Zhao et al., 2009; Zhao et al., 2012). However, the hydrolysis rate and sugar yield depend on the biomass cell structure and composition. Subcritical water extraction has gained significant interest towards extraction of saccharides from different feedstocks such as cellulose, oil palm fronds hemicellulose, coconut meal, sugarcane bagasse, cotton stalk, tobacco stalk, wheat stalk, and sunflower stalk, etc. (Akpinar et al., 2009; Sasaki et al., 2000; Norsyabilah et al., 2013; Khuwijitjaru et al., 2014). In the subcritical treatment of biomass, polysaccharides degrade to oligosaccharides (Degree of polymerisation (DP) <10) or monosaccharides, and monosaccharides decompose to degradation products depending on the operational temperature and reaction time (Cardenas-Toro et al., 2014). Moeller et al. (2013) evaluated the saccharide conversion on the degree of cellulose crystallinity (38 – 74 % crystallinity index) at 205 °C and obtained approximately 40 wt% conversion with low crystallinity values (33 - 42 % crystallinity index). The effect of crystallinity on the formation of glucose and 5-HMF was insignificant. Tolonen et al. (2013) evaluated the cellulose degradation using subcritical and supercritical water at 280-380 °C and obtained water-soluble oligosaccharides, insoluble oligosaccharides, glucose, fructose, 5-HMF, and furfural.

Maximum oligosaccharide (25 wt%) obtained was at 380 °C and 0.25 sec. Other researchers like Ehara et al. (2005); Sasaki et al. (2000); Adschiri et al. (1993) and Sasaki et al. (2004) reported that dissolution of cellulose occurs at temperatures >250 °C. Nabarlatz et al. (2007) examined the hydrolysis of hemicellulose using six lignocellulosic biomass, namely almond shells, barley straw, corn cob, olive stone rice husk, and wheat straw. They reported that it formed Xylo-oligomers with mostly 4-O-methylgucuronoxylan with different degrees of substitution of acetyl groups. The highest and lowest yield obtained was with corn cob (60 wt% initial xylan) and rice husk (30 wt% initial xylan), respectively, which was due to the proportional acetyl content; maximum in a corn cob and least in rice husk. Polymerisation of lignin was observed in the substrate with high lignin content (olive stone and almond shell) but with a low yield of oligosaccharides. Garrote et al. (2004) evaluated the autohydrolysis of barley husks at 190 – 220 °C. At 200 °C, the process gives the highest amount of xylo-oligosaccharides (18 wt%) and arabino-saccharides (2.2 wt%). While maximum xylose (3 wt%) and arabinose (2.2 wt%) attained was at 220 °C. The formation of oligosaccharides was favoured up to 200 °C, and a further increase in the temperature results in the formation of more non-saccharide compounds (oleic acid, hexanoic acid, dehydroabietic acid, octadecanoic acid, and tetradecanoic acid) and degraded products (furfural). Wiboonsirikul et al. (2007) obtained 30 wt% of reducing sugars from rice bran, whereas Zhu et al. (2013) obtained 61.5 wt% reducing sugars from sugarcane bagasse. Vegas et al. (2008) evaluated the hydrolysis of rice husks using subcritical water at 180 °C and different severity coefficient (3.5 - 4.0). They obtained oligomers with a degree of polymerisation less than 25 with 90 - 94 % of total xylo-oligosaccharides at low severities. Xylo-oligosaccharides were initially formed but later degraded, with maximum xylo-oligosaccharide produced at a severity coefficient of 3.9. Prado et al. (2014) examined the subcritical water hydrolysis of pressed palm fiber, grape seed, and coconut husk to produce fermentable sugars using a 50 ml semi-batch reactor at 208 - 257 °C for 30 min. They observed increasing yield with an increase in the reaction temperature in all the feedstocks and obtained the maximum sugars of 11.9 wt%, 6.4 wt%, and 11.7 wt% from pressed palm fiber, grape seed, and coconut husk, respectively.

1.2.4 Levulinic acid production

Levulinic acid is a sugar derived building blocks, produced from the degradation of hexose sugars and is a chemical bridge to connect biomass and petroleum. It is a non-toxic (LD_{50} = 1850 $mg.kg^{-1}$) white crystalline solid organic compound derived from HMF of hexose sugar. It is a precursor for ethyl levulinate and methyl tetrahydrofuran, which can be blended with

gasoline or diesel to produce cleaner fuel. Further, it can be converted to fuel additives such as valeric esters and gamma-valerolactone (GVL). The most widely used approach to produce bio-based levulinic acid is the dehydrative treatment of carbohydrates or lignocellulosic biomass with acid. Levulinic acid is the common product of acid-catalysed dehydration and hydrolysis of hexose sugars. The acid-catalysed decomposition of C_6-sugar fragments (e.g., glucose) leads to 5- hydroxymethyl-2-furaldehyde as the intermediate product, which is subsequently rehydrated to give levulinic and formic acids as the products (Zeng et al., 2012; Nazlina et al., 2012). Other technologies include acid hydrolysis of furfuryl alcohol, oxidation of ketones with ozone, and hydrolysis of acetyl succinate esters. However, intensive research is still required to develop processing schemes for viable chemical production from lignocellulosic biomass (Ramos-Rodriguez et al., 1968). Hayes et al. (2006) studied a two-stage mineral acid-catalysed reaction process with Biofine™ technology and obtained a yield of 70 - 80 wt% levulinic acid from cellulose and 70 wt% yield of furfural from hemicellulose. The feed is treated at 210 – 230 °C (>3 MPa) for less than 30 sec in a plug flow reactor to depolymerised the feed to sugars in the first stage. In the second stage, the substrate was treated at 195 – 215 °C (~1.5 MPa) for 15 to 30 min to produce levulinic acid. The highest levulinic acid yield was achieved at a higher reaction temperature, higher acid concentration, and longer reaction time. Even, a similar trend was reported by Girisuta (2007) and Yan et al. (2008) during their study on levulinic acid. Zeng et al. (2012) produced levulinic acid from glucose and sucrose using solid catalyst $Ag_3PW_{12}O_{40}$ salt and SO_4^{2-}/TiO_2-ZrO_2, respectively. The highest yield obtained for glucose was 81.61 wt% under the optimal conditions of 200 ℃, 2 h, 0.9 g catalyst dosage and 40 $g.lit^{-1}$ glucose concentration. Whereas maximum yield of 50 wt% was obtained for sucrose at the optimal conditions of 180 ℃, 1 h and 1 g catalyst dosage. Ramos-Rodriguez et al. (1968) obtained the maximum yield of levulinic acid: 64.4 wt% from hexose sugars, 71.6 wt% from cellulose and 67.8 wt% from sucrose. Heeres et al. (2009) prepared the levulinic acid using ruthenium catalysts from C_6 sugar and obtain a yield of above 60 wt% at 140 ℃ and 2 h. Jiang et al. (2010) studied the catalytic hydrolysis of sucrose with $SO_4^{2-/}Fe_2O_3$-Al_2O_3-SiO_2 to produce levulinic acid and obtain a yield of 33.05 wt% at 200 ℃, 24 h and 3 wt% catalyst concentrations. Li et al. (2012) produced levulinic acid from sucrose using H_2SO_4 as a catalyst and obtain a yield of 43.31 wt% at 110 ℃, 60 min, 3.5 $mol.lit^{-1}$ catalyst concentrations, and 0.4 $mol.lit^{-1}$ sucrose concentrations. The above literature revealed that researchers have been exploring different minerals acids as well as a solid catalyst for

levulinic acid production. But again, the usage of greener catalysts always has a better chance for sustainability considering the continuous negative impact on the environment due to the application of different chemicals. Hence, in this thesis, a biodegradable, non-toxic sulfonic acid catalyst has been investigated for the production of levulinic acid in Chapter 7.

From the above literature, it has been observed that there is still enough scope to research on production of value added products from aromatic lignocellulosic materials. Therefore, the thesis entitled "**Essential oil extraction from Java Citronella (*Cymbopogon winterianus* Jowitt) and valorisation of the spent biomass to value added chemicals**" is focused on the extraction of essential oil using distillation techniques; utilisation of spent biomass for the production of aforesaid total reducing sugars; and its subsequent conversion to levulinic acid. Throughout the thesis, the objectives as mentioned in section 1.3 have been established.

1.3 Objectives of the present study

The objective of the present study is to compare different citronella oil extraction techniques and evaluate the quality of the oil by estimating their physico-chemical properties using different analytical techniques. Efforts are made to improve the extraction efficiency by introducing a newer technology such as ultrasonic-assisted distillation. After essential oil extraction, the spent biomass has been pretreated, hydrolysed, and further utilised to induce the value added product. The major objectives of the thesis are as follows:

1. Optimisation of essential oil extraction from Java citronella using hydro distillation and its comparison with steam distillation, ultrasonic assisted hydro distillation, and ultrasonic assisted steam distillation
2. Physico-chemical characterisation of fresh and spent citronella biomass
3. Pretreatment of the spent citronella biomass using dilute acid and alkali pretreatment techniques and its comparison with bagasse (standard lignocellulosic biomass)
4. Subcritical water hydrolysis of spent citronella biomass and its comparison with bagasse
5. Levulinic acid production from sugarcane bagasse and spent citronella biomass

In the present study, the extraction of essential oil (EO) was studied using the hydro distillation technique (HD) and compared with other distillation techniques. After the extraction of essential oil, the spent biomass was pretreated using dilute acid, alkali, and subcritical water technique for the production of reducing sugars, which is the major source

or feedstock for bioenergy production. The hydrolysate in the subcritical water hydrolysis process with maximum total reducing sugars (TRS) obtained was subsequently used for levulinic acid production. All the processes mentioned above have been thoroughly evaluated and optimised using response surface methodology and described as chapter wise in the thesis. The schematic flowchart of the work carried out in the thesis is represented in **Fig. 1.2**.

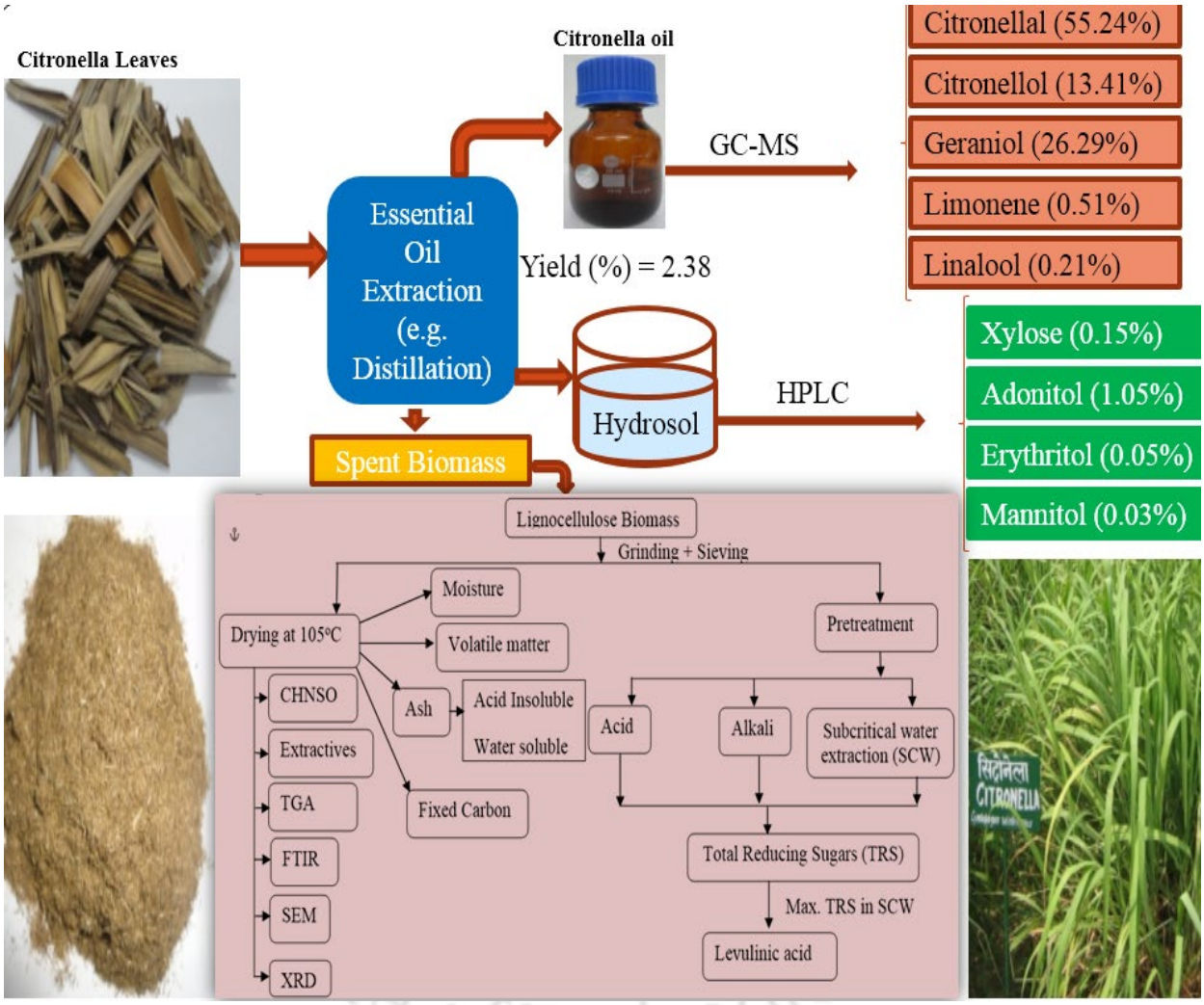


Fig. 1.2: Schematic representation of the Thesis work.

1.4 Organisation of the thesis

The PhD thesis is organised in eight chapters.

Chapter 1: Introduction and literature review

This chapter addresses the state of the art, the possible scope for further research, objectives and organisation of the thesis.

Chapter 2: Materials and methodology

This chapter addresses all the materials and process methodology, including analytical techniques adapted for the complete study. The schematic flow chart of the work is designed in order to initiate the work systematically and organised.

Chapter 3: Optimisation of essential oil extraction from Java citronella using hydro distillation and its comparison with steam distillation, ultrasonic assisted hydro distillation, and ultrasonic assisted steam distillation

This chapter describes the essential oil extraction from Java citronella. The hydro distillation (HD) process was optimised using response surface methodology. The effect of moisture content on the citronella oil yield was evaluated. The components of the extracted oil were identified and quantified using gas chromatography mass spectroscopy (GCMS). Further, the quality of citronella oil was determined from the physico-chemical characterisation of the extracted oil. HPLC analysis of the hydrosol samples was performed to determine its composition. The later part of this chapter describes the comparison of essential oil extraction processes such as HD using the Clevenger apparatus, Steam distillation (SD), Ultrasonic assisted HD (UA-HD) and Ultrasonic assisted steam distillation (UA-SD) from fresh Java citronella. The comparison was made based on citronella oil yield and composition.

Chapter 4: Physico-chemical characterisation of citronella and bagasse biomass

The main emphasis of this chapter is to characterise Java citronella biomass (fresh and spent), which is widely available in the region of Northeast India, to find its potentiality as a feedstock for the production of bioenergy and biochemical. Besides proximate and ultimate analysis, physico-chemical characterisation of the extracted oil and biomass were carried out using bomb calorimeter, EDX, TGA, XRD, and CHNS, and their respective outcomes are explained vividly. The results obtained in citronella biomass were further compared with bagasse.

Chapter 5: Pretreatment of spent citronella biomass using alkali and dilute acid pretreatment techniques and its comparison with bagasse

The first step in biochemical conversion of lignocellulosic biomass is pretreatment. The purpose of this step is to enhance the rate of subsequent enzymatic hydrolysis and fermentation. Pretreatment is the most energy-intensive step for biofuel production. This chapter describes the evaluation of citronella biomass as a feasible feedstock towards bioenergy production by evaluating the release of total reducing sugars (TRS) from the biomass through pretreatment. Further, the release of TRS was compared with sugarcane bagasse. Besides dilute acid and alkali pretreatment, the biomass were hydrolysed using cellulase enzyme (Trichoderma reesei 26291) to obtain maximum reducing sugars. The pretreated biomass was also subjected to cellulase production using Phanerochaete chrysosporium NCIM 1106. Later the residual biomass left after pretreatment and enzymatic hydrolysis was characterised using XRD and SEM for evaluation of morphological and structural changes. Experiments were designed with three factors viz. sulfuric acid concentration (molarity), temperature (°C) and reaction time (min) for dilute acid pretreatment process; whereas NaOH concentration (molarity), temperature (°C) and time (min) were chosen for alkali pretreatment, for the selected biomass, respectively. Efforts have been made to optimise the conditions of the pretreatment techniques for maximum reducing sugars yield with minimum inhibitors production.

Chapter 6: Subcritical water hydrolysis of spent citronella biomass and its comparison with bagasse

This chapter describes the production of total reducing sugars from spent biomass of citronella and bagasse using subcritical water (SCW) hydrolysis. The process parameters affecting the SCW hydrolysis of lignocellulosic biomass such as temperature, reaction time, and biomass loading were evaluated and optimised using response surface methodology. Furthermore, the reaction kinetics of the process was studied based on the first-order reaction model and kinetic parameters such as activation energy, rate constant and pre-exponential factors were evaluated. The residual biomass obtained after hydrolysis was characterised with FTIR, XRD and SEM for its morphological and structural changes. The results obtained in this study will be useful to understand the significance of spent biomass of aromatic plant for the production of value added chemicals.

Chapter 7: Levulinic acid production from bagasse and spent citronella biomass

This chapter discusses the production of levulinic acid from spent citronella biomass and sugarcane bagasse by using homogeneous methanesulfonic acid (MSA) as catalysts. Sulfonic acids are non-oxidising, strong, biodegradable and environmentally friendly acid catalysts. They are less corrosive than mineral acids like sulphuric acid, HCl etc. which are widely used for levulinic acid production. Acid-catalysed hydrolysis of citronella biomass and sugarcane bagasse to levulinic acid were studied at different reaction conditions such as temperature: 200-240 °C; Reaction time: 30-60 min; and MSA concentrations: 0.5-1 M using response surface methodology with the aim to maximise levulinic acid yield.

Chapter 8: Conclusions and recommendations for future work

This chapter presents the various conclusions drawn from the research work. It also provides possible directions for future work.

Chapter 2

Materials and Methodology

This chapter describes about all the materials and process methodology including analytical techniques adapted for the complete study. The schematic flow chart of the work is designed in order to initiate the work in a systematic and organised way as summarised in **Fig. 2.1** and **Fig. 2.2**.

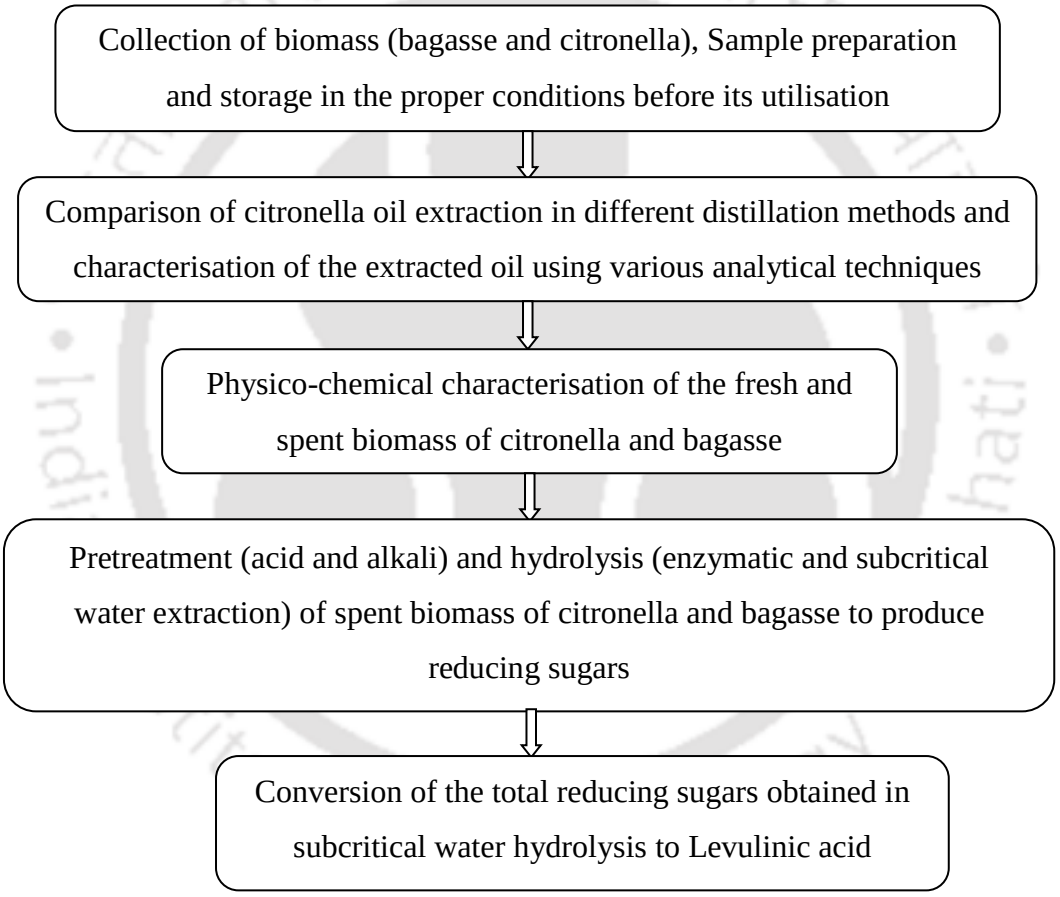


Fig. 2.1: Schematic design of research work.

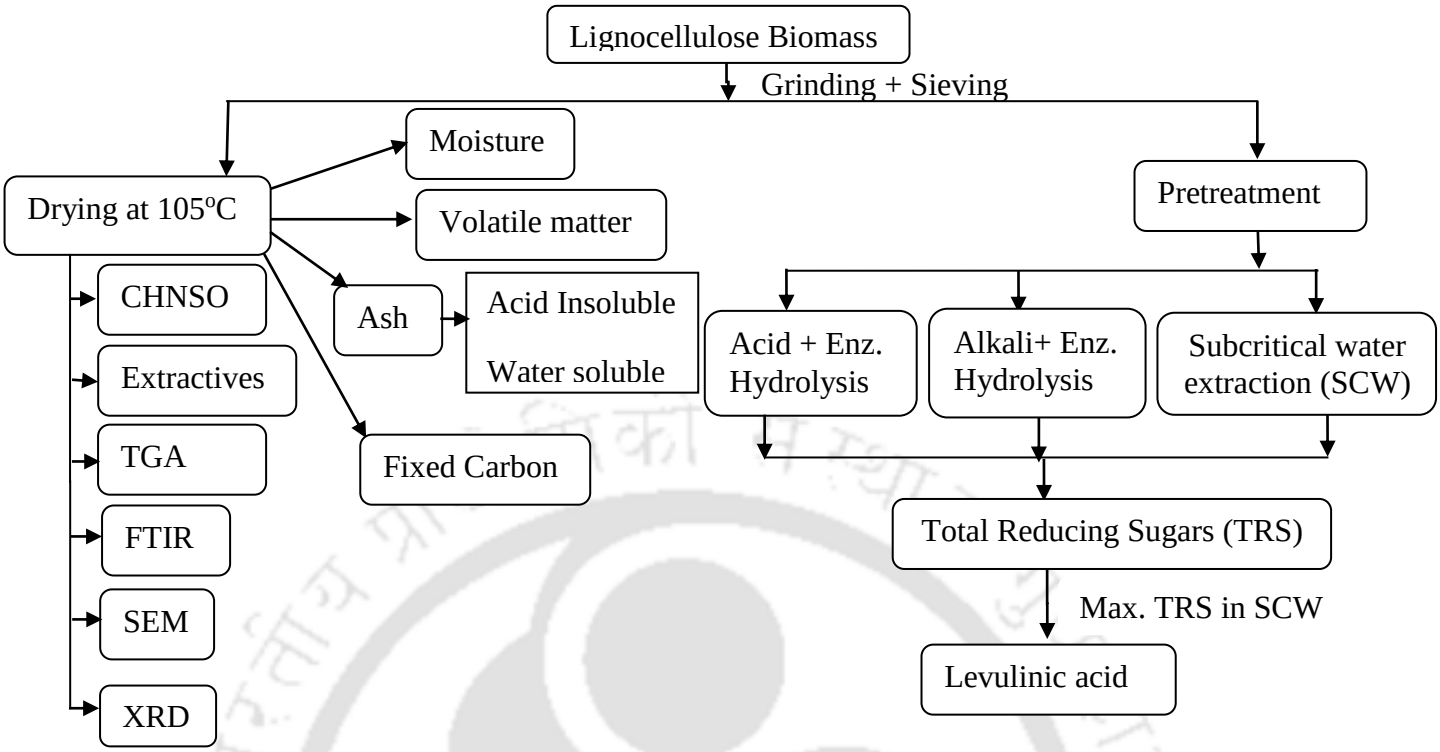


Fig. 2.2: Schematic representation for the characterisation of biomass.

2.1 Materials

Cymbopogon winterianus Jowitt (Java citronella) was collected from Karbi Anglong, Assam, Northeast India in the month of December (winter season). Leaf lamina or leaf blades were separated from the whole aerial parts and were used as leaves part. The remaining culm and leaf sheaths were considered as stem and leaf sheath part (**Fig. 2.3**). Whole aerial plants were initially shade dried in a semi-dark room for 10-15 days and then chopped into small pieces. The plant sample is then stored separately in zipped plastic bags at ambient temperature. Millipore water was obtained using Millipore water synthesis unit bearing Model No: Elix-3, Milli Q; Make: M/S Millipore, USA. 3451-Clevenger apparatus (1000 ml capacity) purchased from Borosil was used. Subcritical extractor, 1734-batch type (manufactured by Amar equipment Pvt. Ltd., India) equipped with stirrer and 500 ml reactor vessel (160 mm height and 75 mm i.d) made of stainless steel was used. Pressure gauge and a thermocouple is connected to the reactor to show the pressure and temperature readings. Sugarcane bagasse and nitrogen gas with 98% purity was procured from Guwahati, Assam. All the chemicals used for analysis were obtained from Merck India Pvt. Ltd, Himedia and Sigma Aldrich. Nylon filter paper (0.2 µm) was purchased from AXIVA. The standard sugars (maltohexaose, Maltopentaose, Raffinose, Cellobiose, Glucose, Xylose, Arabinose, Erythrose etc.), sugar

alcohols (Adonitol, erythritol, Mannitol, sorbitol etc.) as well as HMF and furfural were obtained from Himedia and Sigma Aldrich.

Fig. 2.3: Representation of different parts of Citronella plant.

For characterisation of citronella biomass, whole aerial plants were initially dried in a semi-dark room, chopped into small pieces, and then stored in zipped plastic bags at ambient temperature. After extraction of essential oil, the spent citronella biomass was dried, grinded and sieved to 1 mm sizes using 16 BSS mesh screen. Similar procedure was followed for the original citronella biomass and sugarcane bagasse for their physico-chemical characterisation. The stored spent citronella biomass and sugarcane bagasse were further used for acid, alkali pretreatment and subcritical water hydrolysis.

2.2 Methodology

2.2.1 Design of experiment (DOE) using response surface methodology

Conventional experimental approach requires lots of experiments to ascertain the optimum conditions of the process leading to high cost. It also requires more time and does not explains the interaction effect of the process variables. These limitations can be avoided by optimising the process parameters using statistical experimental design approach such as response surface methodology (RSM). RSM is a mathematical and statistical technique useful for modelling, improving and optimising the process in order to evaluate the relative significance of several process parameters even in the presence of complex reaction conditions (Montgomery, 2005; Peng et al., 2012; Galadima et al., 2012). It is an important tool to study the optimisation of process variables, and to analyse how the individual variable and their interactions affect the response variable. RSM saves time and effort by reducing the

number of experimental runs, and gives optimised and statistically significant results (Betiku et al., 2013). This methodology is widely used in chemical engineering, food industry, research and development and applied sciences to optimise process variables. The most common experimental designs used are Central Composite Design (CCD), Box-Behnken Design (BBD) and Doehlert designs. Among them, CCD and BBD are the commonly used but CCD is the most widely used design, as it is suitable for analysing the interaction effects between the variables and fitting a quadratic model thereby optimising the effective parameters with a minimum number of experiments. In this study, three factor-three level designs were used. The responses as a function of independent factors were described using the generated second order polynomial model. The model equation generated are given in **Eq. (2.1)**:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1+i}^{k} \beta_{ij} x_i x_j \qquad \textbf{(2.1)}$$

Where, Y is predicted response, β_o is intercept, β_i, β_{ii}, β_{ij} is coefficients for linear, quadratic and interaction effect, and x_i, x_j is the independent variables. The significance of the model and the effect of process variables towards the response were evaluated using coefficient of determination (R^2) and probability value (p-value).

2.2.2 Essential oil extraction from Java citronella

2.2.2.1 Hydro distillation technique

Variable amount (10-30 g) of different parts of plant material and millipore water (500 ml) was charged in a 1000 ml capacity round bottom flask of Clevenger apparatus for hydro distillation (**Fig. 2.4**).

Fig. 2.4: Hydro distillation process using Clevenger apparatus.

Heating was provided using a heating mantle (Ikon instruments, Delhi-95). Distillation time was accounted from the moment water starts boiling. The oil was collected, dried over anhydrous sodium sulphate and stored in an amber bottle at -4 °C until used. Extractions were performed at least three times, and the mean values were reported. The oil yield was calculated from the relation between the volume of oil collected and the initial weight of the raw material used as given in **Eq. (2.2)**.

$$\%\ \text{Yield}\left(\text{v.w}^{-1}\right) = \frac{\text{Volume in ml of extracted oil}}{\text{Weight in gram of the Citronella plant sample}} \times 100 \qquad \textbf{(2.2)}$$

2.2.2.2 Steam distillation technique

Steam distillation achieves the twin action of heat and moisture from the steam to break down the cell walls of the plant tissues and releases the essential oil. This method involves placing the flower, plant, or herb (which can be either fresh or dried) into a perforated still vessel and concentrate the pressurised steam in the area where the plants materials are placed (**Fig. 2.5**).

Fig. 2.5: Steam distillation process (Source:www.heartmagic.com/essential distiller).

The steam pressure is higher than the atmospheric pressure unlike water distillation. As the steam breaks down the cell walls of plant tissues, their essential oil released into a vapourised form and becomes saturated. Once it is saturated, the cell walls get burst, allowing the molecules to release the essence out. The steam containing essential oil is condensed, collected and separated into EO and the by-product, known as hydrosol or plant water essence. The oil yield was calculated according to **Eq. (2.2)** on the basis of weight of the oil collected. Initial oil extraction is faster by steam distillation than by hydro distillation as the

diffusion rate is higher for steam than for water. However, essential oil yield and the process duration are longer for water distillation than for steam distillation.

2.2.2.3 Ultrasonic assisted hydro and steam distillation

Ultrasonic processor is considered as a powerful lab homogeniser which is commonly used for sample preparation such as cell disruption, dispersion, degassing, dissolving and emulsification. Hence, ultra-sonication of Java citronella was performed to break the cell wall of the plant material using Ultrasonic device (UP100H – Compact Ultrasonic Laboratory Device (**Fig. 2.6**)).

Fig. 2.6: Ultrasonic Laboratory Device (UP100H).

The instrument is equipped with piezoelectric transducer at the bottom with 1 cm tip diameter, and has operating power of 100 W and frequency 30 kHz. In this study, 30 g of citronella plant sample and millipore water (500 ml) was charged into 1000 ml capacity round bottom flask and immersed in the water bath. The transducer was immersed into the mixture inside the flask and ultra-sonication was performed for 30 min. The round bottom flask with entire mixture after completed ultra-sonication was then subjected to hydro distillation and steam distillation as described above in sub-section 2.2.1 and 2.2.2 respectively.

2.2.3 Alkali and acid pretreatment of biomass

The biomass was treated with sodium hydroxide (NaOH) to remove lignin and increase the accessibility of cellulose in alkali pretreatment. 200 mg dry weight biomass samples were treated with 0.05- 0.5 M NaOH at 80-120 ºC (20 ºC interval) and 20-60 min (20 min interval). On the other hand, in the acid pretreatment process, the biomass was treated with dilute sulfuric acid (H_2SO_4) to hydrolyse hemicellulose and increase the accessibility of cellulose. The biomass samples were treated with 0.1, 0.3, 0.5, 0.7, and 0.9 M H_2SO_4 at a

solid to liquid ratio of 1:10 ($w.v^{-1}$) and temperature range of 80-120 ºC (20 ºC interval) and reaction time of 2 h (30 min interval). Reaction at 80 °C and 100 °C were performed in a water bath shaker (Lab Tech) whereas at 120 °C experiment was carried out in an autoclave at 15 lbs pressure. At most care was taken to ensure that the biomass was completely wetted by mixing for 5-10 min before the reaction. After reaction, the sample is allowed to cool and pipetted out specific volume (0.1 µl) of the liquid fractions to estimate the concentration of total reducing sugars (TRS) using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1951) using UV-Vis spectrophotometer (Model: lambda 45, Perkin Elmer) at 540 nm. The alkali and acid pretreated sample at which maximum TRS was obtained were neutralised using 1 M HCl and 1 M NaOH solution, respectively and further washed with distilled water to remove salts and other impurities until pH was neutral. The substrate was then air dried at room temperature and later subjected to cellulase enzyme production by *phanechaete chrysosporium* NCIM 1106 and enzymatic hydrolysis was performed by commercial cellulase solution Celluclast® 1.5L at different reaction conditions (40-60 °C, 24-72 h and 10-30 FPU). Specific volume (0.1 µl) of the hydrolysate was periodically pipetted out to analyse total reducing sugar (TRS) content. Experiments were carried out in triplicate and the average values obtained were reported.

2.2.4 Dinitrosalicylic acid (DNS) analysis

Dinitrosalicylic acid method has been widely used for the estimation of reducing sugar released during the pretreatment process of lignocellulosic biomass. In the study of chemical pretreatment (Dilute acid and alkali), DNS assay was used to determine the amount of reducing sugar as reported by Miller, (1959). In order to prepare 100 ml of DNS reagent, 1.6 g of NaOH was dissolved in 80 ml of distilled water, then 1 g of DNS was dissolved to the above solution. 30 g of sodium potassium tartrate was added and the final volume was made up to 100 ml. To estimate the amount of reducing sugar present in the sample, 0.1 ml of sample was taken in a test tube and distilled water was added to make up the volume of 1 ml, later 3 ml of DNS reagent was added and placed in the boiling water for 5 min. After that the sample was cooled to room temperature, 6 ml of distilled water was added to the test tubes and mixed well. Then the samples were analysed in UV-Vis spectrophotometer (Model: lambda 45, Perkin Elmer) at 540 nm. The concentrations of reducing sugar in the sample were determined from the calibration plot prepared using standard glucose sugar with at least 5 points of different concentrations.

2.2.5 Cellulase enzyme production

2.2.5.1 Seed culture preparation

The strain *phanechaete chrysosporium* NCIM 1106 was used for cellulase enzyme production. Spores were stored in Potato Dextrose ager (PDA) slants at 4 °C. For the seed culture preparation, spores of *P. chrysosporium* NCIM 1106 containing slants were streaked with sterile distilled water containing 0.1 % ($w.v^{-1}$) Tween 80. 1 (One) ml of this suspension was cultured into the mineral salt media (MSM) containing glucose as carbon source under the aerobic condition at 30 °C for 7 days. Protein content of this seed culture was determined by using the Lowry method (Lowry et al., 1951), and total protein content of the seed culture was adjusted to 150 $mg.ml^{-1}$, and 1 ml of this suspension was used as a inoculum to produce the cellulase enzyme by using pretreated biomass containing cellulose as the carbon source in 100 ml of MSM.

2.2.5.2 Enzyme production

Approximately one gram of pretreated citronella and sugarcane bagasse samples were placed in a 250 ml of erlenmeyer flasks and mixed with MSM consisting of ($g.L^{-1}$): $(NH_4)_2SO_4$ – 1.4; $CaCl_2.2H_2O$ – 0.4; $MgSO_4.7H_2O$ – 0.3; urea - 0.3; Tween 80 – 0.2; Peptone – 1; and elements such as $MnSO_4.7H_2O$ – 0.0016; $FeSO_4.7H_20$ – 0.005; $CoCl_2$ – 0.002 and $ZnSO_4.7H_2O$ – 0.0014. The media pH was adjusted to 5 with 1 M HCl. Flask were sterilised by autoclaving at 121 °C for 20 min at 15 lbs and then allowed to cool at room temperature in a laminar flow. 1 (One) ml of the seed culture (150 $mg.ml^{-1}$ total protein) was inoculated in a flasks containing 100 ml of liquid media and incubated for 7 days at 30 °C. Samples were collected at every 24 h interval and then filtered through 0.2 µm nylon filter membrane to estimate the cellulase enzyme activity by filter paper unit (FPU) assay.

2.2.5.3 Enzyme activity

Measurement of cellulase enzyme activity present in the fermentation broth was carried out according to the FPU assay (Ghose, 1987). This method is expressed in international units (IU). One IU of filter paper activity is the amount of enzyme which releases 1 µmole glucose (reducing sugar) per min during the reaction. The reducing sugar concentration was determined using DNS method (Miller, 1951).

2.2.6 Subcritical water (SCW) hydrolysis of biomass

Experiments were performed using a 500 ml capacity high pressure batch reactor (maximum working temperature and pressure is 500 °C and 350 kg.cm^{-2} respectively) as adapted by Mohan et al. (2015), at the temperature of 140-220 °C, 3-5 wt % biomass loading and reaction time of 5-40 min. An inert gas (Nitrogen) was used to maintain the pressure required such as 52, 89, 145, 225, 335 psi or slightly more at 140, 160, 180, 200 and 220 °C, respectively to keep water at liquid state and also to prevent unnecessary reaction in the process. Extraction time has been accounted from the moment steady desired reaction temperature is achieved. The temperature and pressure in the reactor were recorded by a temperature controller and pressure gauge, respectively. The reaction mixture was continuously mixed using stirrer at the set temperature. At the set conditions, hydrolysate samples were collected through sample outlet from 5-40 min at subsequent 5 min interval. At the completion of reaction, the whole system was cooled to room temperature, residue biomass was collected and air dried at 25 °C and characterised using XRD, FTIR and SEM. The liquid hydrolysate samples collected were filtered using 0.2 µm filter paper and analysed using High Performance Liquid Chromatography (HPLC). Experiments at the specified conditions were performed in duplicates and the average value obtained were reported. At the end of every experiment, the whole system was flashed using millipore water at approximately 220 psi to prevent choking of the system (sample port and gas inlet port).The total reducing sugar concentration (TRS) was estimated according to the following **Eq. (2.3)**:

$$TRS\ (\text{mg.g}^{-1}) = \frac{C_B \times V}{C_{A0}} \qquad \textbf{(2.3)}$$

Here, C_B is the concentration of TRS from the hydrolysis of biomass (mg.ml^{-1}), V is the volume of the obtained hydrolysate (ml), and C_{A0} is the initial mass of the biomass (g).

2.2.7 Levulinic acid production

The production of Levulinic acid (LA) from the spent citronella biomass and sugarcane bagasse by using homogeneous methanesulfonic acid (MSA) catalysts has been evaluated. The study was carried out at different reaction conditions such as temperature: 200-240 °C; reaction time: 30-60 min; and MSA concentrations: 0.5-1 M, using a 200 ml capacity high pressure batch reactor (maximum working temperature and pressure is 500 °C and 350 kg.cm^{-2} respectively). The biomass were initially treated with SCW at the condition in which maximum total reducing sugars was obtained (from the previous SCW hydrolysis study). The

subcritical treated hydrolysate sample at which maximum total reducing sugars was obtained were used for levulinic acid production. Experiments at the specified conditions were performed in duplicates and the average value obtained were reported. The yield of levulinic acid was calculated based on the following **Eq. (2.4)**:

$$\%\ \text{Yield}\left(\text{w.w}^{-1}\right) = \frac{\text{Concentrations of Levulinic acid (mg.g}^{-1})}{\text{Total sugars content of biomass sample (mg.g}^{-1})} \times 100 \qquad \textbf{(2.4)}$$

2.2.8 Analytical techniques

2.2.8.1 Physical properties of citronella oil

Refractive Index

The refractive index of the oil was estimated using a Refractometer having temperature indicator and intensity controller (Advanced Research Instruments Company, New Delhi) at 20 °C. 2-3 drops of citronella oil was placed on the prism with the help of a micropipette and the prism was firmly closed. The lens of the apparatus was adjusted to record the value from the display.

Optical rotation

Optical rotation of the oil was estimated by taking 10 ml of 1 % Citronella oil in hexane, in a 100 mm glass sample cell, and measured at 25 °C and 589 nm, using an automatic polarimeter (Rudolph Research Analytical, Auto pol I). The nature of rotation was determined by direction of rotation from the zero position. If the rotation is clockwise from zero position, it is levorotatory (-) type and if it is anti-clockwise, it is dextrorotatory (+).

Specific gravity

Specific gravity was determined using a specific gravity bottle (accuracy as per I.S. 5717, Borosil). A 10 ml specific gravity bottle was weighed (W_0) and filled with millipore water up to the brim and stopper was inserted. The spilled out water on the stopper and bottle were carefully wiped off and then reweighed the bottle (W_1). Following the same procedure by taking oil instead of water, W_2 was obtained. The specific gravity of the oil sample was calculated using the following equation **Eq. (2.5)**.

$$\text{Specific Gravity} = \frac{W_2 - W_0}{W_1 - W_0} \qquad \textbf{(2.5)}$$

Where, W_0 = Weight of empty specific gravity bottle, W_1= Weight of water + specific gravity bottle, W_2 = Weight of oil sample + specific gravity bottle.

Viscosity

Dynamic viscosity (μ) of the oil was determined using an Anton Paar Physica MCR 301 Rheometer at 25 °C and shear rate of 100 S^{-1}. The viscosity data estimated was the average value of 20 readings measured with the interval of 6 sec point duration and the average shear stress of 0.4053 Pa.

2.2.8.2 Gas chromatography mass spectrometry (GCMS) analysis

The qualitative and quantitative analysis of the extracted citronella oil was carried out using Perkin Elmer Clarus 680 GC/ 600 C MS system. Fused silica HP-5MS column cross-linked with 5% phenyl methylsiloxane of 60 m length and internal diameter of 0.25 mm was used. The initial oven temperature was maintained 50 °C for 2 min and then ramp at 5 °C.min^{-1} up to 260 °C. The holding time was 2 min and the solvent delay time was 8 min. Helium was used as a carrier gas at 1 ml.min^{-1}. 2 µl volume of the oil sample (2 % solution of citronella oil in hexane) was injected. Split ratio 50:1 and scan range of 50 to 600 Da was used. The identification of constituents was done on the basis of their retention time and mass spectra library search (NIST). The relative amount of individual components was estimated according to the GC peak area. The percentage area of compound was obtained by the electronic integration of the peak without taking the relative response factors into account.

2.2.8.3 Fourier Transform Infrared (FTIR) analysis

Infrared spectra of citronella oil and biomass samples were obtained from FTIR spectrometer "IR Affinity1" (Shimadzu Corporation, Japan), measured at the range between 4000-400 cm^{-1} with resolution of 2 cm^{-1} and 30 scans per spectrum. Potassium bromide (KBr) was used for the reference. For oil sample, KBr pellet was prepared and about 20 µl citronella oil was spread over the pellet and directly taken for analysis. Whereas, for biomass samples, KBr and powdered biomass samples were ground well for proper mixing and used for the analysis.

2.2.8.4 High performance liquid chromatography (HPLC) analysis

HPLC is an efficient technique for separating the components and to identify and quantify each component in a liquid mixture. SUPELCOGEL Ca HPLC column provides an excellent separation of monosaccharide and sugar alcohols. The hydrosol liquid obtained after HD as

well as the hydrolysate obtained after pretreatment and hydrolysis were filtered using 0.2 µm nylon membrane filter paper for composition analysis using HPLC (Perkin Elmer Series 200) equipped with RI detector, vacuum degasser and network chromatography interface (NCI) 900. SUPECOGEL Ca column with dimensions of 300 mm length and 7.8 mm inner diameter connected to Ca^{*} guard column purchased from Sigma-Aldrich was used. HPLC instrument was operated at an oven temperature of 80 °C at 0.5 $ml.min^{-1}$ or 0.8 $ml.min^{-1}$ flow rates for total run time of 40 min, and milli-Q water used as a mobile phase. The peaks obtained were identified from the retention time of standard sample and quantified using the calibration plots for pure compounds standardised with at least 5 points of different concentrations.

2.2.8.5 Proximate and ultimate analysis of the biomass

Moisture content of the biomass was evaluated using oven-dry method as reported in CEN/TC 335, (2004). 4 g of biomass sample, placed in crucible was kept in the oven at 105±2 °C for 24 h. Volatile matter was estimated keeping 1 g of the oven-dried sample in muffle furnace at 950 ± 5 °C for 7 min, while ash content was evaluated using 1 g of oven-dried sample in muffle furnace at 600 ± 5 °C for 4 h, as reported by Naik et al. (2010). The dry weight was measured after the successive cooling and drying process attaining constant weight on keeping the crucible in the desiccator. All the calculations were done on the basis of ratio between final dry weight and initial weight of the sample. Finally, the fixed carbon percentage was estimated by means of difference.

The major organic elements of biomass were estimated in Eurovector EA3000 CHNS-O elemental analyser. 1 g of biomass sample was placed in a tin boat assortment to evaluate the percentage composition of hydrogen, carbon, nitrogen, sulfur and the percentage of oxygen was determined by means of difference (Naik et al., 2010; Sasmal et al., 2012).

2.2.8.6 Calorific value determination

The calorific value of the biomass samples was determined using static bomb calorimeter; sealed parr 1108 according to the protocol reported by Naik et al. (2010). The biomass sample pellets approximately weighing 1 g each was put in contact with the platinum wire attached with cotton thread which was placed in the ignition port. Approximately 1 litre distilled water was added to the bomb and filled with 20 bar oxygen at 25 °C. In the isothermal jacket, calorimeter was placed with the air gap separation of 10 mm between all surfaces. The bomb calorimeter was submerged in a cane filled with distilled water, and the

calorimeter jacket was maintained at a constant temperature by circulating water at 25 °C. The discharged ignition energy from about 40 V platinum wires was evaluated using change in potential across a 1256 or 2900 μF capacitor.

2.2.8.7 Energy-dispersive X-ray (EDX) analysis

Energy-dispersive X-ray spectroscopic analysis of the biomass was carried out using Carl Zeiss Sigma VP FE-SEM with Oxford EDS sputtering system electron microscope to determine the percentage weight of the elements present in the biomass. The biomass sample was sprayed over the carbon tape which was glued in the sample stump and gold coating was done to prevent charging. The image of the sample was taken and specific area on the sample was selected to obtain the weight percentage of the elements present in the material. The results obtained are the average value of 5 spectrums.

2.2.8.8 Estimation of extractives content in the biomass

The amount of extractives in the biomass (citronella and bagasse) were estimated using NREL protocol (Sluiter et al., 2011; Naik et al., 2010; Sasmal et al., 2012). The biomass samples were extracted with n-hexane, ethanol and distilled water sequentially by Soxhlet extraction apparatus and each solvent extraction were carried out for 12 h and solvent after subsequent extraction was separated using rotary evaporator. Weight of the extractives was measured and percentage extraction was calculated from the ratio of final weight obtained to the initial weight. In order to extract the non-polar compounds such as lipids, hydrocarbons, terpeniods etc. the biomass was first extracted using hexane. The hexane raffinated biomass was extracted with alcohol to separate polar compounds such as polar waxes, chlorophyll, sterol and other minor constituents. The final raffinate biomass was extracted using millipore water to remove non-structural carbohydrates and inorganic materials. The solvent in each extract was removed in rotary evaporator under reduced pressure. The raffinate biomass after Soxhlet extractions was subjected to thermo gravimetric analysis (TGA) to estimate the lignin, cellulose and hemicellulose content in the biomass samples. Published literature on the thermal decomposition of different biomass revealed that decomposition of hemicelluloses occurred at temperatures ranging from 150 - 350 °C, cellulose decomposed at temperature range of 275–350 °C, and lignin gradually decomposed between the temperatures 250 °C and 500 °C (Sasmal et al., 2012). The present study followed the same approach to estimate lignin, hemicelluloses and cellulose content of the biomass samples.

2.2.8.9 TGA analysis for Hemicellulose, Cellulose and Lignin content

Thermo gravimetric (TG) analysis helps to study the degradation profile of the material and its kinetics with respect to temperature. Most of the literature published in the area of thermal degradation of biomass samples indicate that degradation of biomass follows 1st order reaction kinetics and hence the concentration of individual constituents in a biomass is directly proportional to the degradation rate (Ledakowicz and Stolerak, 2002, Saddawi et al., 2009). To determine the major chemical composition viz. hemicellulose, cellulose and lignin content of the biomass, TG analysis was performed using NETZSCH TG 209 F1 Libra® at the heating rate of 10 °C.min^{-1} from 30 °C to 700 °C under nitrogen atmosphere as reported by Naik et al. (2010) and Carrier et al. (2011).

2.2.8.10 X-ray diffraction (XRD) analysis

The crystallinity of biomass samples was estimated using wide angle X-ray diffraction measurement system (Bruker D8 Advanced X-ray diffraction measurement systems). The samples with particle size less than 120 μm were transferred into the glass sample holder and analysed under plateau conditions. The radiation was generated with an accelerated voltage of 40 KV and current of 40 mA. The scan scope was between 7° and 40° with a step size of 0.02°, determination time of 1 sec per 0.02° and constant rotation speed of 20 rpm. Crystallinity Index (CI) was calculated based on the intensity of amorphous region at ~18.5±0.05^{o} of 2θ (I_{amp}) and maximum intensity at ~ 22.1±0.3^{o} of 2θ for crystalline fraction (I_{002}) according to the **Eq. (2.6)**:

$$CI\ (\%) = \frac{I_{002} - I_{amp}}{I_{002}} \times 100 \qquad \textbf{(2.6)}$$

2.2.8.11 Scanning Electron Microscopic (SEM) analysis

Surface morphology of biomass samples, before and after hydrolysis was qualitatively studied using SEM (LEO, Model 1430vp, Germany). The dry samples were fixed with a conducting adhesive carbon tape on an aluminium stub followed by 2–3 times sputter coating of gold. Finally, the gold coated samples were observed in SEM, which was operating at 15 kV.

Chapter 3

Optimisation of essential oil extraction from Java citronella using hydro distillation and its comparison with steam distillation, ultrasonic assisted hydro distillation, and ultrasonic assisted steam distillation

This chapter describes the optimisation of essential oil extraction from Java citronella using hydro distillation (HD) technique and was optimised using response surface methodology. The effect of moisture content on citronella oil yield was also evaluated. The components of extracted oil were identified and quantified using GCMS. Further, quality of the citronella oil was determined from the physico-chemical characterisation of the extracted oil. HPLC analysis of the hydrosol samples was performed to determine its composition. The later part of this chapter describes the comparison of essential oil extraction process such as HD using Clevenger apparatus, Steam distillation (SD), Ultrasonic assisted HD (UA-HD) and Ultrasonic assisted SD (UA-SD) from fresh Java citronella. The comparison was done based on the citronella oil yield and composition. Whole aerial citronella plant was used for the citronella oil extraction process. Considering the condition of maximum oil yield i.e. 3 h extraction time, obtained in HD process in the previous study, the extraction using SD was also carried out for 3 h. On the other hand, Ultrasonication of 30 min followed by 3 h of HD and SD was performed for UA-HD and UA-SD, respectively. For the comparison of composition present in the extracted citronella oil using different methods, 5 major composition of the oil viz. citronellal, citronellol, geraniol, limonene and linalool were considered 100 % in the integration data of GCMS chromatogram. Further, the functional groups present in the extracted citronella oil were compared using FTIR.

3.1 Introduction

Essential oils are essences of aromatic plant species obtained by the extraction from whole plants or certain parts such as flowers, fruits, leaves, roots, barks and seeds (Mu'azu et al., 2012). Different methods of extraction like distillation (hydro and steam), solvent extraction and supercritical fluid extraction can be used to extract essential oil. However, the quality and

quantity of the oil yield depend on extraction technique used (Chanthai et al., 2012). Essential oil obtained by solvent extraction may contain traces of the solvent which may have deleterious effect. On the other hand, supercritical fluid extraction is although an efficient technique to produce high yield and good quality oil, but the initial cost of investment is very high. Hence, distillation methods which are relatively simple, cheap, environment friendly and produce good quality oil are generally preferred for essential oil extraction from grasses (Manaf et al., 2013). Even though essential oil content in the plant parts is very low (typically 1-8 % of total weight of the plant), but has high industrial importance and market value. Therefore, it is important to optimise the extraction process parameters in order to obtain a high yield and good quality oil (Luthria and Natarajan, 2009).

Citronella oil is an essential oil known for its natural insect repellent property and is of great interest for pharmaceutical and fragrance industry (Wany et al., 2013). It has various therapeutic uses as analgesic, anticonvulsant, anxiolytic, etc. and is a favourable agent towards anti-fungal, anti-bacterial, anti-parasitic and nematicidal activities (Almeida et al., 2001, 2004). Citronella essential oil is obtained from the aromatic citronella grass, belonging to the Kingdom- Plantae, Family- Poaceae, and Genus- *Cymbopogon Spreng* (APG III, 2009). From the genus *Cymbopogon* two species are commercially categorised: (1) Ceylon citronella (*Cymbopogon nardus* Rendle) and (2) Java citronella (*Cymbopogon winterianus* Jowitt) (Wijesekara, 1973). Java citronella oil containing about 85 % of commercially important compounds like citronellal, geraniol and citronellol are considered to be more superior to that of Ceylon citronella oil, which contains only 55-65 % of these three compounds (Wijesekara, 1973; Wany et al., 2014). Therefore, Java type is more widely cultivated in tropical and subtropical countries like India, Sri Lanka, Malaysia, Taiwan, Ecuador, Madagascar, Guatemala, Honduras, Brazil, Argentina, Mexico and West Indies (Wany et al., 2013). The Northeastern part of India is one of the most important biodiversity hotspot having diverse agro climatic zones, which provide ample conditions for the cultivation of Java citronella. The region experiencing well distributed rainfall all over the year is considered favourable for the cultivation of citronella plant. Sandy loamy soil with high moisture content, but without water logging is the appropriate soil condition for plant growth. The best planting period is the rainy season, and the most ideal season of harvesting is summer and the early winter. Citronella plants can be harvested 3-4 times annually with the interval of 2.5-3 months, starting with the first harvest six months after plantation. However, harvest during the late winter significantly

reduces the oil yield (Joy et al., 2001; Blank et al., 2007). In order to extend the shelf life of the plant material, post-harvest drying is important, which reduces the moisture content and prevents the microbial and enzymatic activity (Rocha et al., 2011). Although biosynthesis of essential oil is genetically regulated, but still environmental factors also have a high impact. The citronella plants grown in the Northeastern region of India are rich in citronellal content (Ahmed, 2005).

Although a lot of work has been reported on the citronella plant, but limited literature is available on the optimisation of essential oil extraction using hydro distillation technique; evaluation of oil content and its quality from different parts of the plant; and evaluation of the anti-bacterial activity of the oil. Hence, the present study has the objectives of optimisation of essential oil extraction from different parts of the Java citronella plant using hydro distillation technique, physico-chemical characterisation of extracted citronella oil and to evaluate the anti-bacterial activity of the extracted oil using selected bacterial strains. Further, HD process was compared with Steam distillation (SD), Ultrasonic assisted HD (UA-HD) and Ultrasonic assisted SD (UA-SD) in terms of essential oil yield and composition.

3.2 Results and Discussion

3.2.1 Effect of moisture content on oil yield

The moisture content of fresh and dried sample (whole aerial citronella plant) was found to be 76.01 ± 0.14 % and 9.43 ± 0.11 %, respectively. On dry weight basis, the fresh citronella plant showed higher oil yield (2.43 $v.w^{-1}$%) than dried sample (2.12 $v.w^{-1}$%) at 3 h distillation time (**Table 3.1**). These results revealed that oil yield is greatly affected by the moisture content which is influenced by dry weight of the plant sample.

Table 3.1: Effect of moisture content on oil yield.

State of plant (Whole aerial part)	Initial wt of plant (g)	Moisture content (wt%)	Final wt of plant (dry wt) (g)	Time (min)	Vol. of oil (ml)	%Yield ($v.w^{-1}$) Dry wt basis
Fresh	30	76.01 ± 0.14	7.20	180	0.175	2.43
Shade Dried	30	9.43 ± 0.11	27.16	180	0.575	2.12

These values are average of triplicate determination.

During drying of the plant sample, volatile compounds might percolate to the surface and evaporate along with water ensuing a decreased oil content. Cassel et al. (2006) obtained 0.78 % of citronella oil yield at the distillation time of 4 h using dry sample and 0.94 % yield using fresh sample. In the drying process, temperature is the most important parameter to preserve the essential oil in the gland. Drying methods significantly affects the oil content and composition. Jalal et al. (2009) evaluated the effect of drying on oil yield in Rosemary leaves and discovered that shade dried sample (1.8 %) had higher oil content than the sun-dried (1.63 %) and 45 °C oven dried sample (1.5 %). Rocha et al. (2000) studied air-drying of Java citronella at five different temperatures viz. 30, 40, 50, 60, 70 °C and found that the best air-drying condition for Java citronella plant was at 60 °C, where highest oil yield was obtained without affecting its quality. Kakaraparthi et al. (2014) observed that in the process of shade-drying and sun-drying, the oil content in Palmarosa increases up to 7-9 days after harvesting and decreases thereafter.

From the aforementioned discussion it was clear that the drying process affects the active ingredients of the essential oil. Therefore, in the present work, plant materials were shade dried in a semi dark room. Moreover, considering the traditional practice of distillation of citronella plant and non-availability of fresh material on regular basis, shade dried citronella plant parts were used in optimisation studies of HD process.

3.2.2 Preliminary experimental results

The preliminary experiments were carried out to evaluate the effect of different process parameters such as parts of citronella plant, solute to solvent ratio and distillation time on the oil yield. The oil yield was found to be increased with increasing distillation time but oil extraction almost gets completed within 2 h (**Fig. 3.1**). Further increase in extraction time up to 3 h increases the oil yield by only 1-5 % and beyond 3 h oil quality deteriorates (Ahmed, 2005). Comparative analysis of oil yield obtained from different parts of the plants revealed 40 % more essential oil in the leaves than the stems and 17 % more than whole aerial parts. On the other hand, whole aerial parts contain approximately 21 % more essential oil than the stem. The volume of water used in the HD process does not affect the oil yield in the range of parameters considered in the experiments (**Fig. 3.2**). However, sufficient quantity of water was maintained throughout the study to avoid insufficient evaporation of essential oil and burning of plant material. The yield of essential oil obtained from different parts of citronella plant such

as leaves, stems with leaf sheaths, and whole aerial parts was found to be 2.38 v.w^{-1}%, 1.58 v.w^{-1}%, and 2.0 v.w^{-1}%, respectively (**Fig. 3.3**).

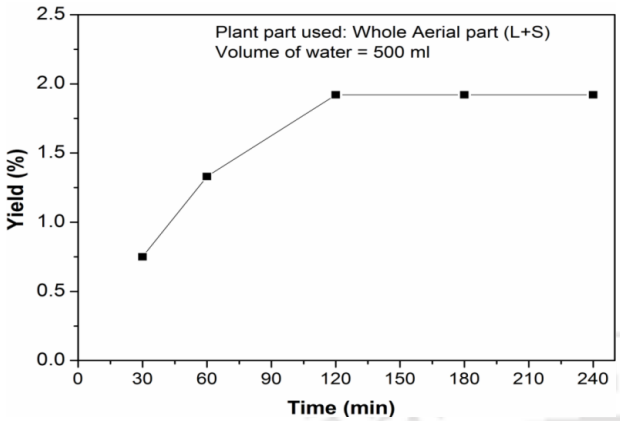


Fig. 3.1: Effect of distillation time on % oil yield.

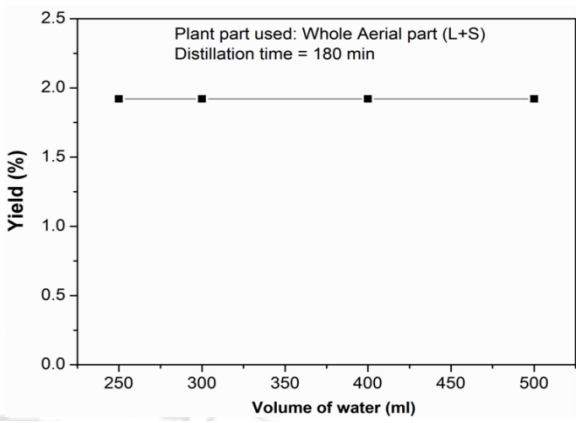


Fig. 3.2: Effect of volume of water on % oil yield.

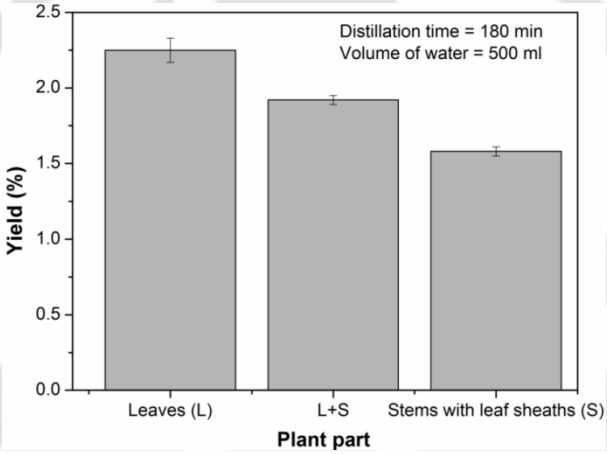


Fig.3.3: Effect of plant part used on % oil yield.

3.2.3 Box Behnken Design (BBD)

According to the preliminary experimental results, matrix was designed for the low and high level of each factor. The coded levels of the factors are given in **Table 3.2**. The design matrix and their corresponding experimental and predicted yields are shown in **Table 3.3**.

Table 3.2: Factors and coded levels for Box-Behnken design.

	Factor	Low	Medium	High
		-1	0	1
A	Distillation time (min)	60	120	180
B	Solute/solvent (g per 500 ml)	10	20	30
C	Plant part	Stems (S)	Whole aerial part (L+S)	Leaves (L)

Table 3.3: Box-Behnken design of the extraction process variables using coded factors and its corrresponding experimental and predicted responses.

Std. order	Run order	Distillation time (A)	Solute /solvent (B)	Plant part (C)	Oil Yield (v.w^{-1}%)	
					Expt. data	Pred. data
1	1	-1.00	-1.00	0.00	1.50	1.46
2	4	1.00	-1.00	0.00	2.00	1.94
3	9	-1.00	1.00	0.00	1.33	1.39
4	2	1.00	1.00	0.00	1.92	1.96
5	6	-1.00	0.00	-1.00	1.13	1.12
6	17	1.00	0.00	-1.00	1.50	1.51
7	8	-1.00	0.00	1.00	1.75	1.74
8	14	1.00	0.00	1.00	2.38	2.39
9	11	0.00	-1.00	-1.00	1.50	1.55
10	7	0.00	1.00	-1.00	1.58	1.53
11	13	0.00	-1.00	1.00	2.25	2.30
12	5	0.00	1.00	1.00	2.33	2.28
13	10	0.00	0.00	0.00	2.00	2.00
14	16	0.00	0.00	0.00	2.00	2.00
15	3	0.00	0.00	0.00	2.13	2.00
16	15	0.00	0.00	0.00	1.88	2.00
17	12	0.00	0.00	0.00	2.00	2.00

3.2.3.1 Effect of factors

The parameters affecting the oil yield can be examined from **Table 3.4** and **Table 3.5**. The large F-value and corresponding low P-value in **Table 3.5** depicts that the factors viz. distillation time, solute to solvent ratio and plants part used, had a significant effect on the response (oil yield) whereas factors with P-value > 0.05 showed an insignificant effect to the response. Similarly, the coefficient of factor with '95 % CI low' and '95 % CI high' both having positive values as represented in **Table 3.4** showed the factors had high effect on the response. It can be clearly observed that distillation time (A), plant part (C) had high individual effect on the response, and A^2 with P-value 0.0004 had significant effect on the response indicating the oil yield increases with an increase in the distillation time. The other factors and their interactions showed an insignificant effect on the oil yield.

The highest and lowest oil yield from every part was obtained at 3 h and 1 h distillation time respectively. Experimentally obtained highest and lowest yield for the leaves sample were 2.38 v.w^{-1}% and 1.75 v.w^{-1}%, respectively. For whole aerial parts, the experimentally obtained highest yield was 2 v.w^{-1}% and lowest was 1.33 v.w^{-1}%. Likewise, for stems with leaf sheaths sample, the highest yield was 1.58 v.w^{-1}% whereas lowest yield was 1.13 v.w^{-1}%. Blank et al. (2007) reported that dry leaves harvested at different day time during the winter season showed oil yield as 2.33 to 2.67 %, whereas leaves harvested during spring season yielded up to 3.55 % oil for HD process of 3 h. The plant material used in this study was also collected during the winter season and gave almost similar oil yield percentage (i.e. 2.38 v.w^{-1}%). Cassel and Vargas (2006) reported maximum oil yield of about 0.78 % from dry sample for 4 h steam distillation. Wany et al. (2014) reported yield of 1 %, by applying HD to fresh sample and 0.7 % by implementing steam distillation to the dry sample for 8-10 h.

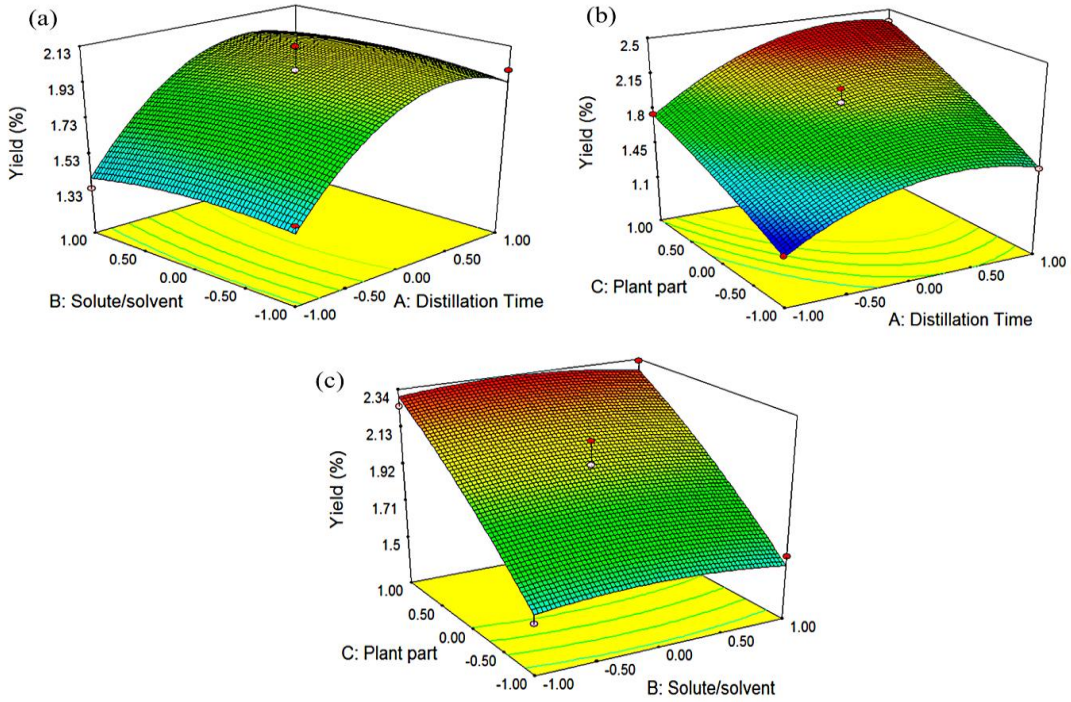


Fig. 3.4: 3D response plot to show the interaction effects of process variables on % oil yield: (a) Distillation time and solute-solvent ratio; (b) Distillation time and Plant part; (c) Plant part and solute to solvent ratio.

Fig. 3.4 represents 3D response surface plots for interaction effects of independent factors on the percentage oil yield. The effect of mutual interactions of individual factors on oil yield can be observed from the curvature nature of surface plots. There is minuscule interactions between

plant part and distillation time (AC), as well as between distillation time and solute to solvent ratio (AB), but no mutual interaction between the plant part and solute to solvent ratio (BC), which can be observed from the response plots as well as from the data presented in **Table 3.4 and Table 3.5**.

Table 3.4:Significance of the response quadratic model.

Factor	Coefficient	DF	Std. error	95 % CI low	95 % CI high	VIF
Intercept	2.00	1	0.04	1.91	2.09	-
A: distillation time	0.26	1	0.03	0.19	0.33	1.00
B: solute / solvent ratio	-0.01	1	0.03	-0.08	0.06	1.00
C: plant part	0.38	1	0.03	0.30	0.45	1.00
AB	0.02	1	0.04	-0.08	0.13	1.00
AC	0.07	1	0.04	-0.04	0.17	1.00
BC	0.000	1	0.04	-0.10	0.10	1.00

Table 3.5:Analysis of variances (ANOVA) for response surface quadratic model.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model	2.03	9	0.23	29.58	<0.0001
A: Distillation time	0.55	1	0.55	77.70	<0.0001
B: Solute/Solvent	1.01E-003	1	1.01E-003	0.13	0.73
C: Plant Part	1.13	1	1.13	147.73	<0.0001
AB	2.03E-003	1	2.03E-003	0.27	0.62
AC	0.017	1	0.017	2.22	0.18
BC	0.000	1	0.000	0.000	1.0000
A^2	0.31	1	0.31	40.23	0.0004
B^2	8.43E-003	1	8.43E-003	1.11	0.33
C^2	7.52E-003	1	7.52E-003	0.99	0.35
Residual	0.05	7	7.62E-003	-	-
Lack of fit	0.02	3	7.34E-003	0.94	0.50
Pure error	0.031	4	7.820E-003	-	-
Cor Total	2.08	16	-	-	-

3.2.3.2 Model equation

The model equation developed for the extraction of essential oil from the citronella plant using HD technique in terms of both experimental and coded values are given in **Eq. (3.1)** and **Eq. (3.2),** respectively.

$$Y=2.00+0.26A–0.01B+0.38C+0.02AB+0.07AC+0.00BC–0.27A^2–0.04B^2–0.04C^2 \quad \textbf{(3.1)}$$

$$Y=2.00+0.26A–0.01B+0.38C+0.02AB+0.07AC+0.00BC–0.27A^2–0.05B^2–0.04C^2 \quad \textbf{(3.2)}$$

3.2.3.3 Validation of the model

The analysis of variance showed that the experimental data was best fitted to the second order polynomial model. The significance of the model was checked by coefficient of determination (R^2). For a good fit of a model, R^2 value should be minimum of 0.80 (Joglekar and May, 1987; Guan and Yao, 2008). The statistical parameters of the model such as R^2 = 0.9744, standard deviation = 0.087, F-value of 29.58 and P-value <0.0001 showed a highly significant model. The true coefficient (2.00) of the model intercept with positive values of both 95 % CI low (1.91) and 95 % CI high (2.09) also depicted the model as highly significant. The P-value of 0.5006 for lack of fit indicated that, lack of fit which was not significant relative to the pure error or in other words the model was significant. Hence, the model can be used to navigate the design space. The comparison of the actual and predicted oil yield as represented in **Fig. 3.5** also exhibited a good fitness of experimental data to the model.

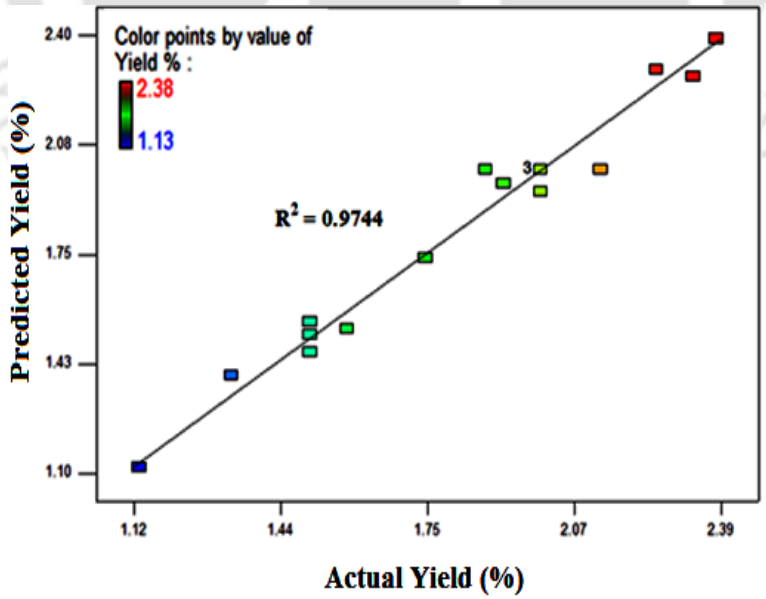


Fig. 3.5: Comparison of the actual and predicted percentage oil yield.

3.2.4 FTIR analysis

The qualitative analysis of different organic compounds can be ascertained from the characteristics vibration bands appeared in the infrared spectral region at a particular frequency influenced by specific functional groups. The percentage transmittance corresponding to the wave number is deduced in the attenuated total reflectance IR spectra (spectra of citronella oil acquired from leaves part at highest oil yield, standard citronellal, citronellol, geraniol and Limonene) as shown in **Fig. 3.6**.

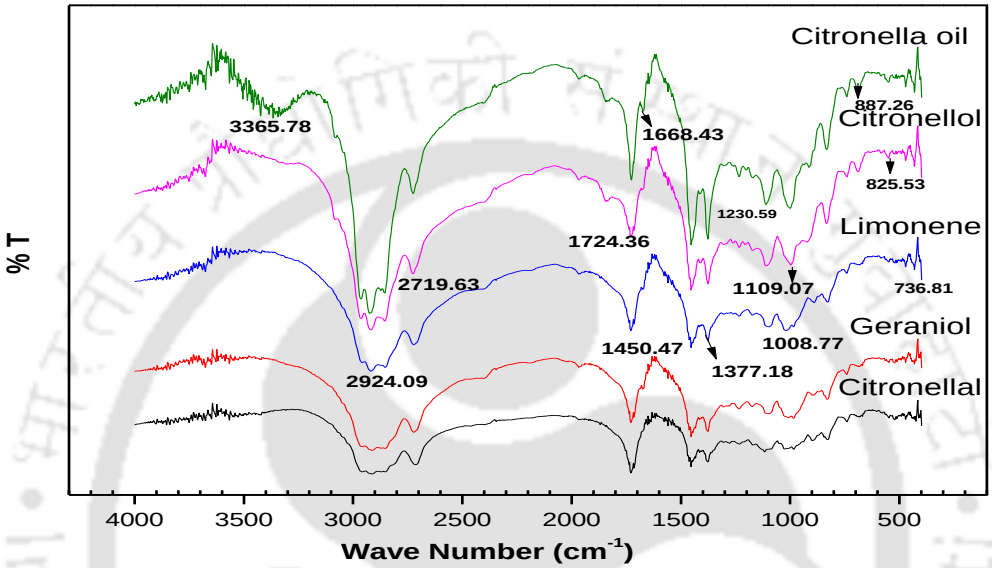


Fig. 3.6: FTIR spectra of hydro distilled citronella oil, standard citronellal, citronellol, geraniol and Limonene.

There was an intense broad peak in the range of 3600-3200 cm^{-1} particularly at 3365.78 cm^{-1} corresponding to the polymeric hydroxyl (OH) group. Another intense and bifurcated peak in the range of 2935-2915 cm^{-1} which corresponds to the C-H methyl and methylene asymmetric stretch, mostly aliphatic alkyl groups were observed. The medium peak at 2719.63 cm^{-1} validated a terminal aldehydic C-H stretch of carbonyl compound. Another distinct and sharp peak in the range of 1750-1705 cm^{-1} signified aldo, keto, estero and or acido (C=O) stretch. A strong and relatively narrow absorption peak at 1668.43 cm^{-1} contributed to olefinic unsaturated C=C group. The sharp and strong peaks were observed for methylene C-H (1485-1445 cm^{-1}), methyl C-H symmetric bend (1380-1371 cm^{-1}) and aryl-O-H stretch (1270-1230 cm^{-1}). Moreover, C-O bend (1140-1050 cm^{-1}), simple –OH stretch (1200-1000 cm^{-1}) and CH=CH trans-unsaturated (910-860 cm^{-1}) functional groups with medium peaks were also observed. A medium peak depicting di or tri-substituted alkenes (C-H) stretch was detected at

825.53 cm^{-1}. Minor vibrations in the range of 750-660 cm^{-1} attributed to the presence of aromatic, vinyl C-H group. These results were in absolute accord with the previous work carried out by Wany et al. (2014). The spectras shows that the hydrodistilled oil contains all the standard compounds being analysed.

3.2.5 GCMS analysis

The compositional analysis of hydro distilled citronella oil was studied based on the peaks obtained in the GC chromatogram. The oil extracted from different parts (leaves, stems and whole aerial parts) obtained at highest yield were analysed using GCMS (**Fig. 3.7**). A total of 50 compounds shown at different retention time were generated according to the electronic integration of the chromatogram plots. Out of these 50, a total of 13 distinct peaks can be observed in the chromatogram of leaves sample constituting 99.21 % of the total composition which were identified as citronellal, citronellol, geraniol, 3-hydroxy-5-isopropyl-2-methylbenzo-1-4-quinone, citronellyl propanoate, limonene, β-elemene, patchoulane, linalool, germacrene D, δ-cadinene, germacrene A and germacrene D-4-ol respectively. Other 37 trace compounds constituting 0.79 % were not identified. Whereas, whole aerial parts and stems parts had these 13 compounds with 95.67 % and 81.20 % of the total composition of oil respectively. This implied that the composition of other trace compounds of oil was dispersed at a higher percentage in the whole aerial parts and more distributed in the stems part. The first major peak obtained was citronellal at 26.71 min suggesting citronellal as the most volatile compound in the citronella oil and the last major visible peak was germacrene D-4-ol (39.34 min). Silva et al. (2011) and Manaf et al. (2013), in their study also reported citronellal as the first major peak. The presence of two peaks for geraniol at 29.73 min and 29.82 min in the oil obtained from leaf parts might be due to the presence of its isomer nerol as discussed by Madivoli et al. (2012). Higher area percentage of the peak signified higher percentage of the corresponding compound in the oil. The chromatogram plot obtained from the leaves part revealed a high concentration of commercially important major three compounds i.e. citronellal (55.23 %), geraniol (26.29 %) and citronellol (13.41 %) accounting for 94.94 % of total constituents. Whereas, whole aerial parts had 89.15 % of these three compounds and the stems part constituted only 69.84 %. This compositional distribution analysis indicated that the oil obtained from leaves part is of good quality compared to whole aerial parts and stems parts. Cassel and Vargas (2006) during their study on essential oil extraction from *Cymbopogon winterianus* using steam distillation reported the composition as citronellal (35.90 %),

citronellol (5.2 %) and geraniol (20.90 %) quantifying 62 % of the total oil composition. Beneti et al. (2011) obtained 71.32 % constituting citronellal (40.23 %), citronellol (13.39 %) and geraniol (17.70%) in their study on fractionation of citronella (*Cymbopogon winterianus*) essential oil. Similarly, other researchers have also reported these three major constituents of citronella oil in the range of 60-75 % (Li et al., 2013; Pinheiro et al., 2013; Songkro et al., 2012).

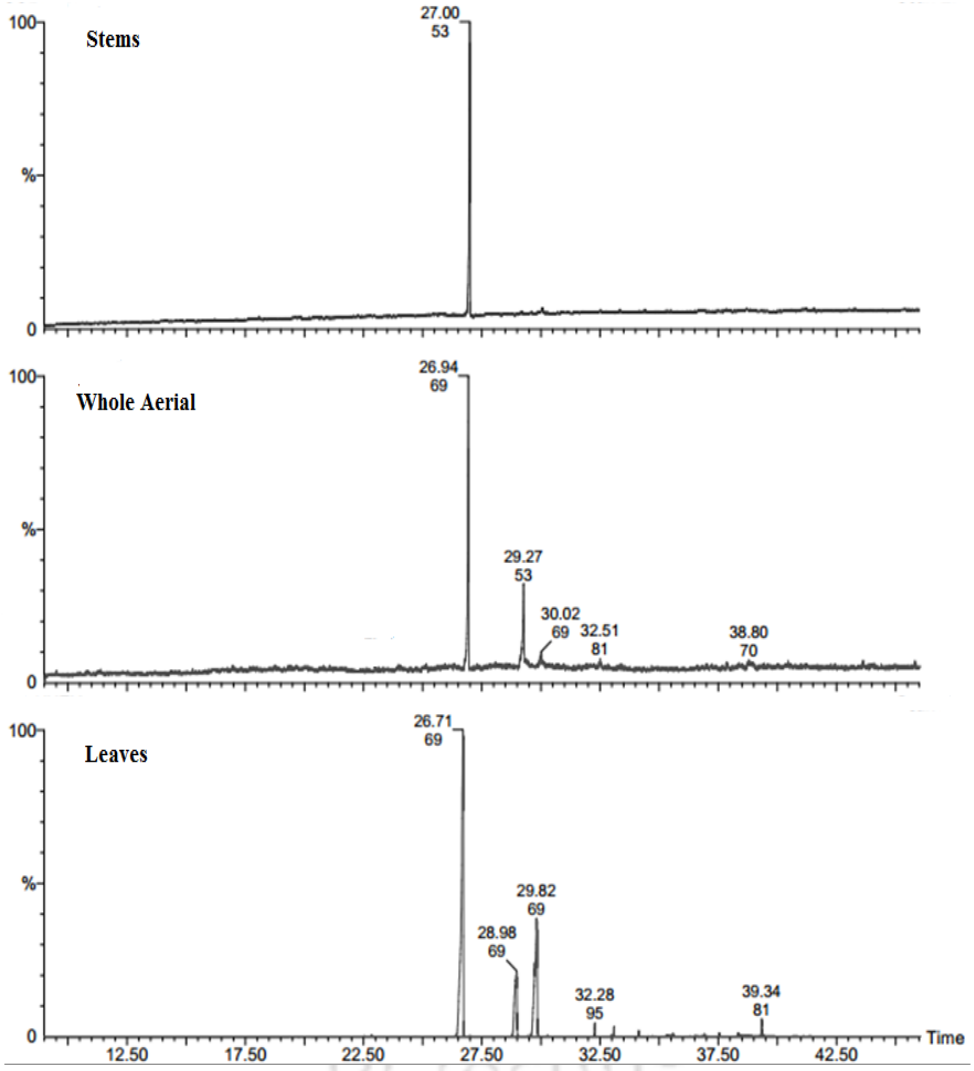


Fig. 3.7: GCMS chromatogram of hydro distilled citronella oil.

In this study, the composition of these three commercially important compounds (i.e. citronellal, geraniol and citronellol) in the leaves and whole aerial parts extracted oil were found to be 94.94 % and 89.15 %, respectively, which indicates that the extracted oil was of superior quality. The overall compositional distribution of extracted Java citronella oil identified using GCMS is given in **Table 3.6**.

Table 3.6: Chemical composition of citronella oil identified using GCMS.

Retention time (min)	Compound name	Abundance %		
		Leaves	Whole Aerial	Stems
26.71	Citronellal	55.24	48.39	54.64
28.91	Citronellol	13.41	24.86	5.03
29.82	Geraniol	26.29	15.89	10.16
30.28	3-Hydroxy-5-Isopropyl-2-Methylbenzo-1-4-Quinone	0.05	0.11	0.56
32.28	Citronellyl propanoate	0.64	3.81	1.05
33.09	Limonene	0.51	0.10	1.15
34.14	β-Elemene	0.37	0.11	1.11
35.35	Patchoulane	0.08	0.08	1.03
35.59	Linalool	0.21	0.09	1.09
36.92	Germacrene D	0.15	0.11	0.97
37.55	δ-Cadinene	0.22	0.10	0.95
38.35	Germacrene A	0.82	0.09	2.58
39.34	Germacrene D-4-ol	1.22	1.93	0.88
Percentage of total compounds identified		**99.21**	**95.67**	**81.2**
Other trace compounds		**0.79**	**4.33**	**18.8**

3.2.6 SEM Analysis

SEM analysis of the sample showed an apparent change in the structure of citronella plant after HD. **Fig. 3.8 (a)** represents the micrograph of untreated plant material. The cells of the plant are intact and a smooth and linear surface can be observed. When the plant material is subjected to HD process, the cellular content of plant tissues gets distorted. The extensive thermal stress and severe mechanical strain induced due to rapid disintegration and intense vaporisation of water causes fast dehydration leading the plant cells including the oil glands to collapse or crumble promptly. Ferhat et al. (2006) also observed similar kind of deformation in the SEM analysis of hydro distilled residue of orange peel. The significant deformation on the external surface of the citronella plant material after HD can also be seen in **Fig. 3.8 (b)**.

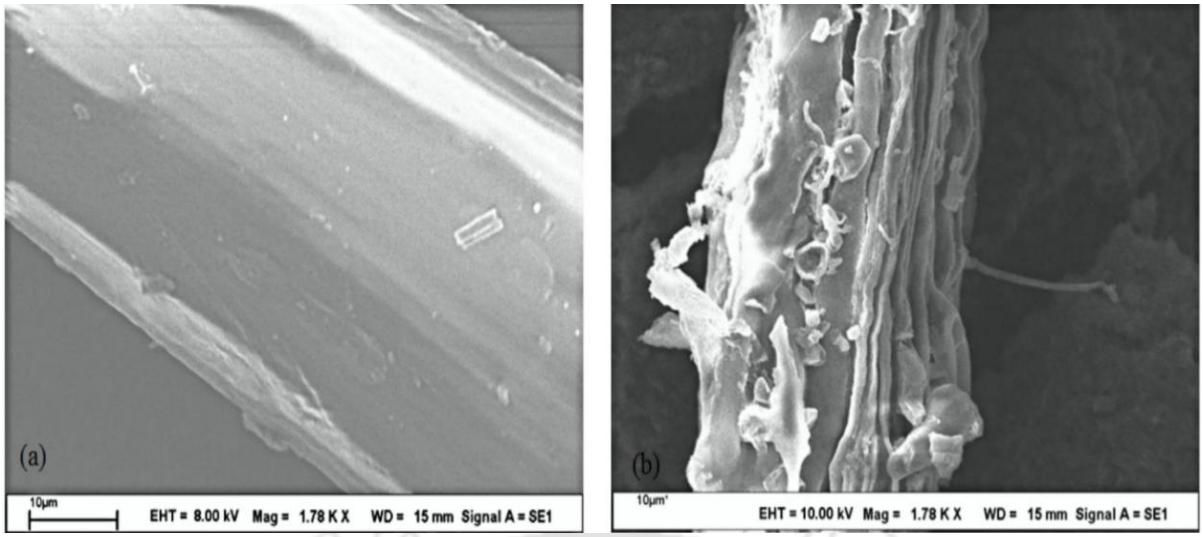


Fig. 3.8: Electron micrograph of citronella plant; (a) before HD and (b) after HD.

3.2.7 HPLC analysis

The hydrosol liquids obtained after distillation of aromatic plants are generally not analysed. The significance of such liquid is therefore not efficiently determined. Thus, in the present study, HPLC analysis of the hydrosol obtained at highest oil yield were performed to determine their composition. The hydrosol obtained after HD process was found to contain minuscule amount of pentose sugar and sugar alcohols such as xylose, adonitol, erythritol and mannitol (**Fig. 3.9**). The composition of sugars was found to be differed with the plant materials. The leaves part contains 0.07 $mg.ml^{-1}$ of erythritol and 0.03 $mg.ml^{-1}$ of mannitol. Stems of citronella plant contain higher quantity of erythritol (4.49 $mg.ml^{-1}$) relative to leaves part (0.07 $mg.ml^{-1}$) and whole aerial parts. The hydrosol of whole aerial parts was distributed with 0.09 $mg.ml^{-1}$ xylose, 0.63 $mg.ml^{-1}$adonitol, 0.03 $mg.ml^{-1}$ erythritol, 0.02 $mg.ml^{-1}$ mannitol. These sugar alcohols are of great importance for human health and medication. Erythritol is a useful component for food industry and oral health. Mannitol has diuretic effect which is useful for kidney. This composition analysis of hydrosol evidenced its high importance and projected extensive scope for further utilisation.

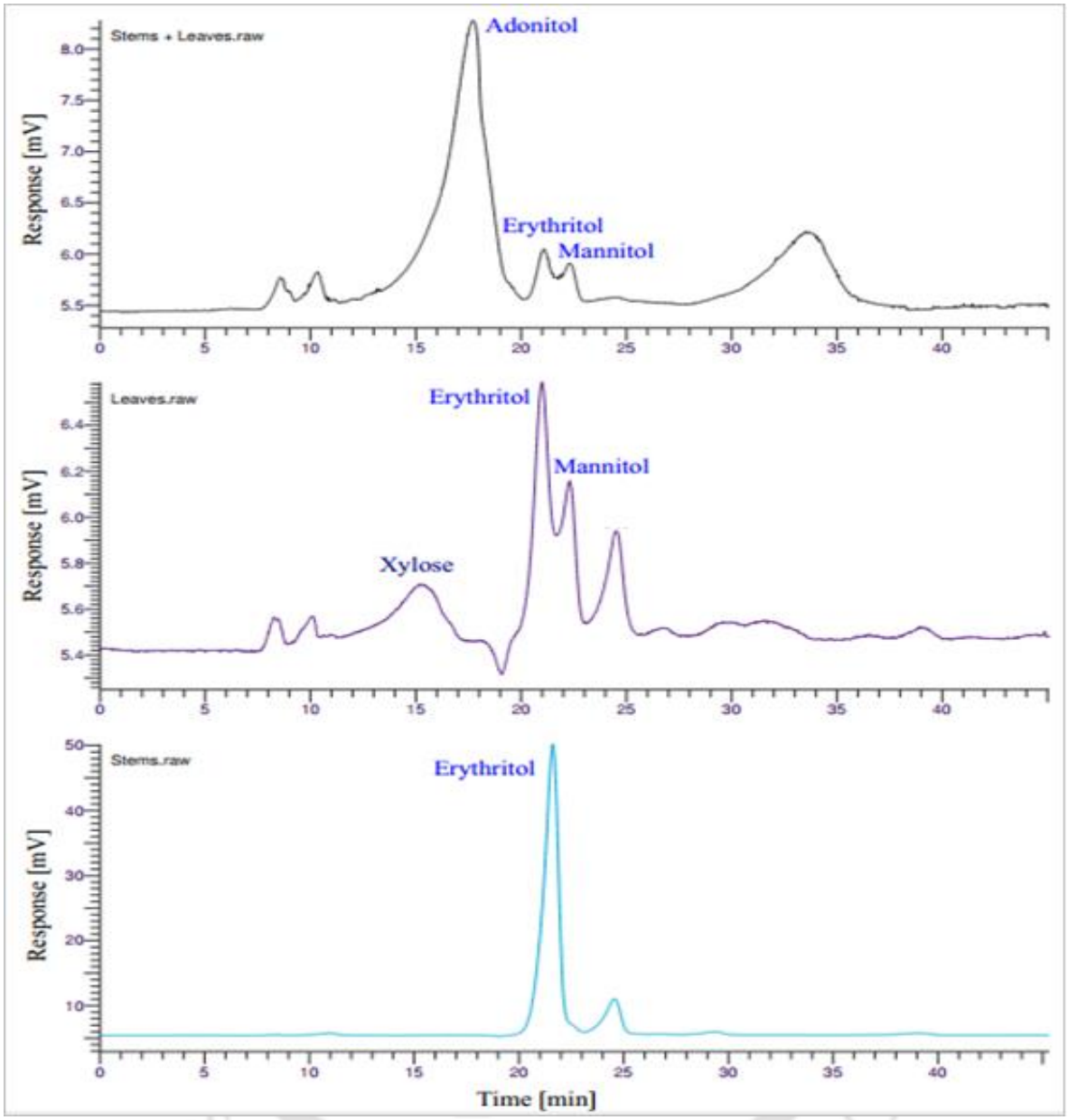


Fig. 3.9: Comparison of HPLC chromatogram of hydrosol obtained after HD from different parts of citronella plant.

3.2.8 Physical properties of citronella oil

The physical properties of the extracted citronella oil were evaluated for the oil obtained from leaves sample at highest oil yield. The colourless to pale yellow citronella oil had a characteristic citronellal aroma. The oil obtained from a particular citronella cultivar used to have its unique constituents strongly influencing the overall weight of the oil and degrees at which they tend to refract light when a beam of light pass through it. These characteristics can be determined by evaluating the refractive index, optical rotation and specific gravity, which

are also helpful to evaluate adulterated citronella oil. The optical rotation value (-4.0 °@ 25 °C) confirmed that the extracted citronella oil was optically active and had a levorotary behaviour. The dynamic viscosity of the oil was found to be 4.053 Centipoise (cP) at 25 °C. The specific gravity and refractive index of the extracted citronella oil were 0.9005 and 1.473, respectively and were comparable with the literature data (New direction aromatics Inc., 2014; Natural sourcing, 2010) as given in **Table 3.7**.

Table 3.7: Physical properties of citronella oil.

Property	Experimental value	Literature data	
		New direction aromatics Inc., 2014	Natural sourcing, 2010
Appearance	Pale yellow to colorless Oily liquid	Clear light yellow to brownish liquid.	Clear yellow to pale brown
Odor	Lemon characteristic	Strong lemon-like odor.	Characteristic of lemon, herbaceous
Specific gravity	0.9005 @ 25 °C	0.880-0.922 @ 20 °C	0878-0.895
Refractive index	1.473@ 20 °C	1.466-1.474 @ 20 °C	1.466-1.475 @ 25 °C
Optical rotation	-4.0 º @ 25 °C	-	-5 ° to 0 °
Dynamic Viscosity	4.053 cP @ 25 °C	-	-

3.2.9 Anti-bacterial activity of citronella oil

Citronella oil was found effective against all the six tested bacterial strains which could be observed from the developed zone of inhibition as presented in **Table 3.8**.

Teixeira et al. (2013) in their study on the chemical composition, anti-bacterial activity and anti-oxidant properties of 17 commercial essential oils also observed growth inhibition of different pathogenic bacteria by citronella oil. The susceptibility of oil towards *Bacillus subtilis* and *Staphylococcus epidermidis* was higher compared to other selected strains, as the zone of inhibition was more than 10 mm even at the lowest concentration (10 %v.v^{-1}) of the oil considered. The increased in anti-bacterial activity was observed with an increase in the oil

concentrations in all the strains irrespective of being gram positive or gram negative. Similar pattern was observed by Naik et al. (2010) in their study on the anti-bacterial activity of lemongrass oil. On the other hand, Victoria et al. (2011) reported that aldehyde (e.g. citronellal) and alcohol (e.g. citronellol) have same effects on both gram-positive and gram-negative bacteria.

Table 3.8: Zone of Inhibition of the citronella oil.

Bacterial Strains	**Zone of Inhibition at different oil concentrations (mm)**				
	10 %v.v^{-1}	20 %v.v^{-1}	30 %v.v^{-1}	40 %v.v^{-1}	50 %v.v^{-1}
Gram +ve					
Bacillus subtilis	10 ± 1.41	11	11.5 ± 2.12	19 ± 1.41	21
Staphylococcus epidermidis	10.5 ± 0.71	12.5 ± 0.70	13	18 ± 2.82	23 ± 1.41
Staphylococcus aureus	0	0	5	5	6
Gram -ve					
Pseudomonas aeruginosa	4 ± 1.41	9	10.5 ± 0.70	13.5 ± 2.12	15
Klebsiella pneumonia	0	6	11	17	23
Escherichia coli	0	4	7.5 ± 0.70	10.5 ± 0.70	12

Maximum zone of inhibition of around 23 mm was observed for *Staphylococcus epidermidis* and *Klebsiella pneumonia* at highest concentration of citronella oil (50 %v.v^{-1}). The anti-bacterial activity of citronella oil is attributed to the major components in the oil viz. citronellal, citronellol and geraniol as reported by Lertsatitthanakorn et al. (2008). Hence, citronella oil with selection of specific concentration could act as an anti-bacterial supplement for the treatment of various bacterial infections.

3.2.10 Comparison of essential oil extraction methods

With the objective of determining and developing an efficient process for citronella oil extraction, different extraction methods such as HD, SD, UA-HD and UA-SD have been compared and evaluated for their effectiveness in the extraction of oil from citronella biomass. The yield of citronella oil obtained in different processes is presented in **Table 3.9**. In the HD process essential oil yield obtained was 2.31 $v.w^{-1}$% which was less than in the SD process (2.63 $v.w^{-1}$%). On the other hand, ultrasonic pre-treatment of 30 min to the material before hydro distillation increases the oil yield to 2.56 $v.w^{-1}$% and 2.85 $v.w^{-1}$% in UA-HD and UA-SD process, respectively. In the ultrasonication process, the plant material is subjected to ultrasonic waves which might results in the maximum disruption of the plant cell wall providing easy extraction of oil during distillation process. Blank et al. (2007) reported 2.33 % to 2.67 % essential oil yield obtained from the citronella plant sample harvested at different day time during the winter season, whereas those harvested during spring season yielded up to 3.55 % oil. Wany et al. (2014) reported yield of 1 % by applying HD to fresh sample and 0.7 % by implementing steam distillation to the dry sample for 8-10 h. Cassel and Vargas (2006) obtained 0.78 % of Citronella oil yield for the distillation time of 4 h using dry sample and 0.94 % yield using the fresh sample. The above discussions revealed that the essential oil yield obtained in the present study are comparable to the literature. An initial pretreatment of the biomass with ultrasonication can improve the oil yield by approximately 8-10 % in the distillation process. Also steam distillation is more preferred process compared to hydro distillation.

Table 3.9: Comparison of citronella oil yield in different extraction method.

Extraction method	% Yield ($v.w^{-1}$)
Hydro distillation	2.31 ± 0.04
Steam distillation	2.63 ± 0.07
Ultrasonic assisted HD	2.56 ± 0.03
Ultrasonic assisted Steam distillation	2.85 ± 0.07

*% Yield calculation was done considering moisture content (76.01 ± 0.14 %)

3.2.10.1 Composition Analysis using GCMS

The compositional analysis of citronella oil obtained under different extraction process were studied based on the peaks of 5 (five) major components obtained in the GC chromatogram (**Fig. 3.10**). Higher area percentage of the peak signified higher percentage of the corresponding compound in the oil. The chromatogram plot obtained from oil in UA-SD process revealed a high concentration of citronellal (49.50 %). Whereas highest quantity of citronellol (25.44 %) and geraniol (34.49 %) was obtained in UA-HD. The oil contained in UA-SD contains geraniol (30.59 %), citronellol (13.50 %), limonene (5.58 %) and linalool (0.84 %) which has almost similar composition compared to that obtained using SD. Cassel and Vargas (2006) during their study on essential oil extraction from *Cymbopogon winterianus* by steam distillation reported the oil composition as citronellal (35.90 %), citronellol (5.2 %) and geraniol (20.90 %). Beneti et al. (2011) obtained citronellal (40.23 %), citronellol (13.39 %) and geraniol (17.70 %) in their study on fractionation of citronella (*Cymbopogon winterianus*) essential oil.

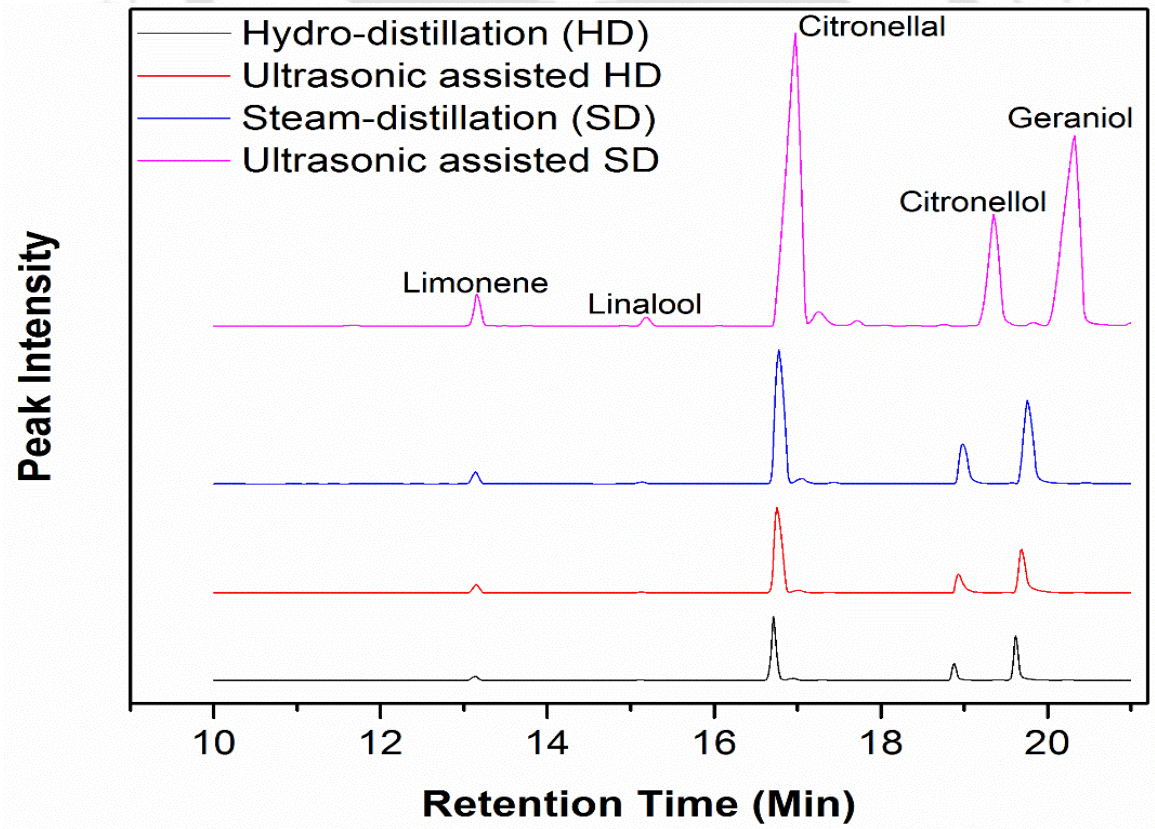


Fig. 3.10: GCMS chromatogram of citronella oil obtained in different extraction methods.

The differences in the composition of oil may be driven by various factors such as variation in the method of extraction, distillation time, genetical makeup, geographical locations and environmental conditions (Sarma et al., 2001; Sarma, 2002; Cannon et al., 2013). In the present work, the overall compositional distribution of extracted Java citronella oil identified using GCMS are given in **Table 3.10**.

Table 3.10: Composition of Citronella oil obtained using different extraction methods.

Compound	Abundance %			
	HD	**SD**	**Ultrasonic assisted HD**	**Ultrasonic assisted SD**
Citronellal	48.02	48.68	35.12	49.50
Citronellol	14.37	14.01	25.44	13.50
Geraniol	33.55	31.48	34.49	30.59
Limonene	3.52	4.97	4.25	5.58
Linalool	0.54	0.86	0.69	0.84

3.2.10.2 FTIR Analysis

No significant differences were observed in the FTIR spectra of citronella oil extracted using different extraction method (**Fig. 3.11**). The above results were in accordance with the work as discussed in section 3.2.4 (**Fig. 3.6**), as well as with the previous work carried out by Wany et al. (2014).

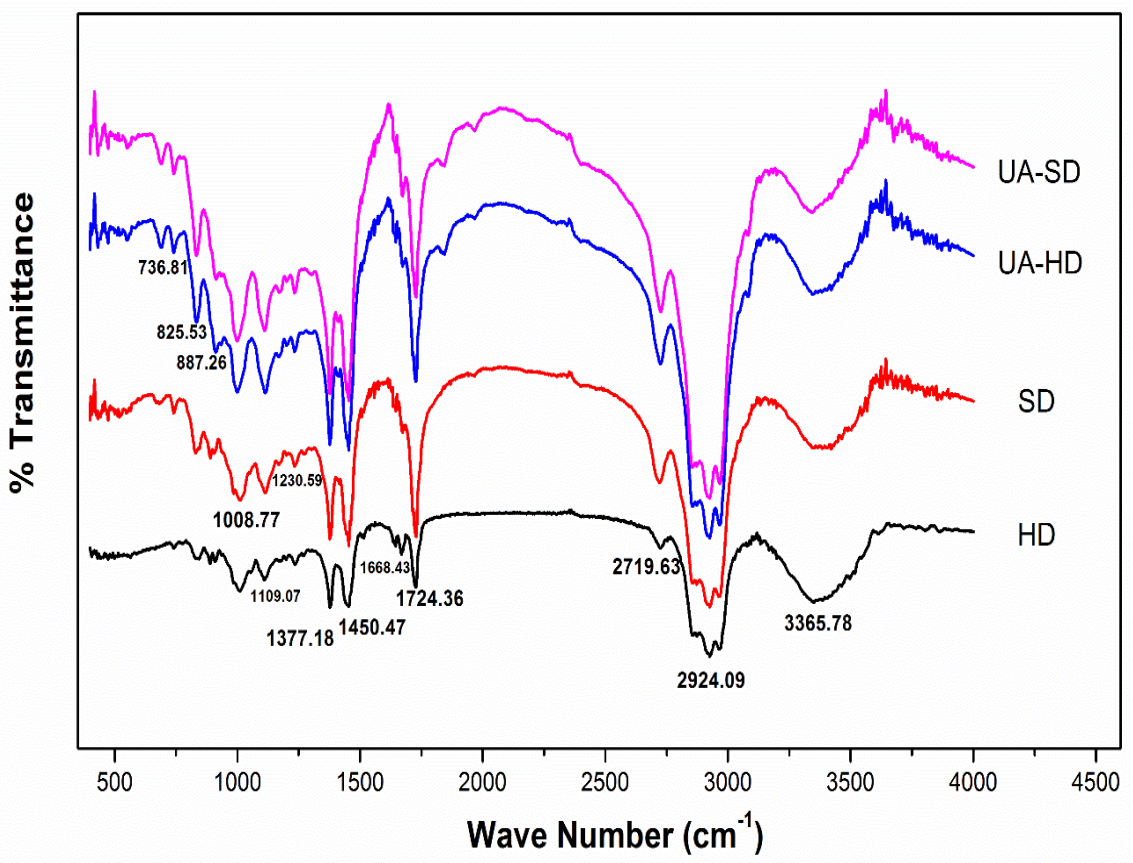


Fig. 3.11: FTIR spectra of citronella oil obtained in different process.

3.3 Summary

Results of the present study revealed that the essential oil from the citronella plant can be easily extracted using HD technique. High regression coefficients of the response (R^2 = 0.9744) with Model P-value << 0.05 showed that the developed model fitted well to the experimental data. Hence, Box-Behnken design of response surface methodologies can be applied to study the citronella oil extraction process using HD. Study reveals that the citronella oil yield is influenced by the state (fresh or dried) and parts of plant material used. The leaves of a citronella plant contain more and comparatively better quality essential oil than the stems or whole aerial parts. Citronella oil, therefore should be extracted from leaf part in order to obtain high quantity and quality. Although UA-SD process gave highest citronella oil yield, but the relative constituent's percentage of the oil extracted using different processes were comparatively the same. Hence, UA-HD could be considered as a simpler, rapid and more efficient process with high oil yield and good recovery of oil constituents compared to UA-SD and conventional SD process. Higher percentage citronellal was obtained in UA-SD whereas more geraniol and citronellol was obtained in the citronella oil extracted using UA-HD. The

high percentage (about 95 %) of commercially important compounds such as citronellal, geraniol and citronellol obtained in the extracted citronella oil from leaf part and around 89 % from whole aerial parts indicates a superior quality citronella oil suggesting that the geographical locations and environmental conditions of Karbi Anglong, Assam, Northeast India is exceptionally favourable for the growth of citronella plant. The hydrosol obtained during extraction can be used to produce sugar alcohols such as erythritol, mannitol etc. which can be further utilised in the nutraceutical and pharmaceutical industries.

In order to evaluate the feasibility of citronella biomass for bioenergy and biochemical production, the essential oil extracted biomass (spent biomass) was thoroughly characterised to determine its physico-chemical properties. Later the spent biomass was chemically pretreated (using dilute acid and alkali) followed by enzymatic hydrolysis to determine the yield of total reducing sugars from the biomass. Further, subcritical water hydrolysis (greener technique) process was employed for the production of total reducing sugars in order to compare its performance with the conventional treatment processes. The good TRS yield obtained in the SCW hydrolysis was further used in the production of levulinic acid. The next subsequent chapters discuss about in depth study of above mentioned processes and their results.

Chapter 4

Physico-chemical characterisation of citronella and bagasse biomass

The main emphasis of this chapter is to evaluate the potential of aromatic Java citronella biomass as a sustainable source for the production of bioenergy and biochemicals. The biomass used in the present work has been characterised extensively before and after (spent biomass) essential oil extraction. Besides proximate and ultimate analysis, physico-chemical characterisation of the biomass samples have been performed using bomb calorimeter, energy dispersive X-ray spectroscopy (EDX), thermo gravimetric analysis (TGA), x-ray diffraction (XRD), fourier transform infra-red spectroscopy (FTIR), and CHNS-O. The subsequent sections of this chapter elaborate upon the investigations carried out to realise the biomass composition and its potential for bioenergy and biochemicals production. Citronella biomass was compared with bagasse; as it is the mostly studied biomass for biochemical or bioenergy production from lignocellulosic biomass.

4.1 Introduction

The consumption of energy is increasing at a faster rate due to urbanisation and development all over the world. Lignocellulosic biomass remains the potential alternative energy resource to meet the rising energy demand. There has been excessive research on lignocellulosic biomass to use as an alternative to fossil fuels due to its abundance and less environmental threat (Abbasi et al., 2010; Fatma et al., 2018). But, the practicality and feasibility of conversion of agricultural biomass to bioenergy generation mainly depends on the characteristics of available biomass. The conversion of biomass can be carried out through various processes, such as biochemical, chemical, thermo-chemical etc. The physico-chemical properties of biomass have a remarkable impact on each of the conversion processing steps. Thus, the selection of process for design and development of biomass conversion scheme specifically relies on various properties of biomass such as heating value, moisture content, and ash content (Sheng et al., 2005). The dissimilarities among the available lignocellulosic biomass and the conversion processes offer both opportunities for potential synergies as well as new challenges. For example, woody biomass resources, as

well as residues from the pulp and paper industry, including other lignin-rich waste products, are not well apt for fermentation but are responsive for thermo-chemical conversion (Irmak, 2017, Ahorsu et al., 2018). In the thermal conversion of biomass, proximate analysis is essential, as it has high influence on the combustion characteristics. Some of the critical parameters which need to be considered for gasification and combustion include the content of volatile components, moisture, ash, and heating value. While biochemical conversion is typically more dependent on the carbohydrate content of biomass and less susceptible to ash content (Irmak, 2017). Thus, knowing the wide range of biomass and conversion process evaluation of the chemical composition of biomass becomes a primary requisite. Apart from that it also gives clear understanding of how the natural variability and pre-processing operations affect the physico-chemical properties. Moreover, how these altered properties of the material helps improve conversion to energy and fuels is the key to develop a sustainable production of fuels, chemicals, and other bio-based products (Ahorsu et al., 2018). The determination of different characteristics of biomass requires various analytical techniques such as thermogravimetric analysis (TGA), X-ray diffraction (XRD), FTIR, CHNS, bomb calorimeter, energy dispersive x-ray (EDX) spectroscopy etc. TGA helps in determining weight change as a function of temperature, and time. On the other hand, differential thermo-gravimetric analysis (DTG) provides information on the rate of weight change as a function of temperature and time (Razafindralambo et al., 2019; Nirav, 2017). It also helps in estimating the type of reaction, whether endothermic or exothermic. The data of weight loss with respect to temperature or time can be used to quantify volatile matter, moisture content, char, ash, and structural biomass composition. FTIR is a useful technique, particularly for isolating and characterising organic contamination. It can be used to identify organic compounds and, to a certain extent, some inorganic chemicals (Chen et al., 2015). The crystallinity of the biomass can be determined using XRD. On the other hand, EDX can be used to determine the elemental compositions of biomass. Besides high calorific value, ideal biomass for bioenergy production should also have a high cellulose and hemicellulose content and comparatively very less lignin, ash, and sulfur content. Hence in this chapter, Java citronella biomass (fresh and spent), which is widely available in the region of Northeast India, are evaluated to check its potential as a possible alternative feedstock for sustainable production of fuels, chemicals, and other bio-based products.

4.2 Results and discussion

4.2.1 Proximate and ultimate analysis

Proximate and ultimate analysis of the biomass is an efficient conventional way to evaluate its quality as a raw material for bioenergy and biochemical production. Higher moisture content leads to the decomposition of biomass during storage, which results in loss of energy and accordingly reduces its conversion efficiency. The volatile matter, which is another important parameter to consider, is present mostly in the form of gas and hydrocarbons, which can be devolatilised readily than solid fuel. The biomass with higher volatile matter and relatively less fixed carbon content are suitable candidates for gasification and pyrolysis process. The ash content in the biomass is an inherent constituent of plant material comprising mineral matters such as a salt of silica, potassium, zinc, calcium, copper, magnesium, and manganese, having enzyme inhibition effect which reduces the hydrolysis of cellulose into fermentable sugars. The ultimate and proximate investigations of the biomass is shown in **Table 4.1**. Citronella biomass contains higher volatile fractions due to essential oil content and other volatile fractions such as aldehydes, alcohols, and esters compared to bagasse. Essential oil-less biomass (spent citronella biomass) contains lesser volatile fraction due to obvious reasons. Elementary analysis (i.e. CHNS-O) of the above-mentioned biomass shows higher content of carbon, hydrogen, and oxygen with low nitrogen content and almost negligible sulfur content. Lower the ratios between H and O as well as between C and O, higher its energy content. This correlation is related to the degree of aromaticity and carbonation. The materials with a relatively low C and O ratio have more energy density and higher heating values (HHV) because there is more chemical energy in C-C bonds than C-O bonds.

Table 4.1: Proximate and Ultimate analysis of biomass.

Biomass Type	Proximate Analysis (%)				Ultimate Analysis (%)					Calorific Value ($MJ.Kg^{-1}$)
	Moisture (%)	Volatile Matter (%)	Ash (%)	Fixed Carbon (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulfur (%)	Oxygen (%)	
Original Citronella	9.47 ± 0.14	85.30 ± 0.21	0.39 ± 0.02	4.84 ± 0.11	43.74 ± 0.04	5.94 ± 0.07	2.20 ± 0.11	0.3 ± 0.02	47.82 ± 0.14	17.01 ± 0.26
Spent Citronella	10.06 ± 0.08	82.77 ± 0.33	0.57 ± 0.11	6.20 ± 0.17	42.55 ± 0.10	5.83 ± 0.03	3.44 ± 0.13	0.1 ± 0.01	48.08 ± 0.12	16.98 ± 0.18
Bagasse	6.2 ± 0.6	78.44 ± 0.2	1.7 ± 0.04	13.62 ± 0.3	42.1 ± 0.7	5.20 ± 0.04	0.43 ± 0.2	0.06 ± 0.1	52.5 ± 0.6	17.20 ± 0.13

Low sulfur content signifies lesser SO_x emission during gasification unveiling a potential feedstock for gasification. Original citronella biomass has more carbon, hydrogen, and sulfur but lower content of nitrogen and oxygen compared with spent biomass and bagasse. Bagasse has lower nitrogen content. Calorific value is inversely proportional to the moisture content of biomass (Ezeike, 1984). The calorific value of around 17 $MJ.Kg^{-1}$ for both original and spent citronella biomass was comparable with bagasse (17.20 $MJ.Kg^{-1}$) and other biomass used for energy production such as barley straw (15.7 $MJ.Kg^{-1}$), flax straw (17.0 $MJ.Kg^{-1}$), timothy straw (16.7 $MJ.Kg^{-1}$) reported by Naik et al. (2010). Abdullah et al. (2010) reported the calorific value of rice husk as 14.79 $MJ.Kg^{-1}$ and paddy straw 13.74 $MJ.Kg^{-1,}$ whereas Sasmal et al. (2012) reported 17.83 $MJ.Kg^{-1}$ for areca nut husk. The low ash and sulfur content with good calorific value proposes the utility of citronella biomass as a potential substrate for bioenergy and biochemical generation.

4.2.2 Elemental analysis using energy-dispersive X-ray spectroscopy (EDX)

Besides CHNS-O, other elements present in citronella biomass were determined using EDX as represented in **Table 4.2**. The original biomass contains a higher amount of K, Mg, Ca, Cu, Si, Cl, P, F, and Fe but a lesser amount of Al and tungsten. Those elements are mostly the uptake of plants from soil and atmosphere. The presence of those elements in biomass results in inhibition effect during enzymatic hydrolysis. The lesser content of these elements in the spent biomass than the original biomass suggests that spent biomass would be more accessible for the enzyme to hydrolyse compared to original biomass.

Table 4.2: EDX analysis of biomass.

Citronella Biomass Type	Elements (wt%)											
	K	Mg	Ca	Cu	Si	Cl	P	F	Fe	Co	Al	W
Original Biomass	1.15 ± 0.05	0.43 ± 0.27	0.63 ± 0.28	0.55 ± 0.05	3.86 ± 0.16	1.0 ± 0.01	0.56 ± 0.37	0.65 ± 0.05	0.3 ± 0.1	0.1 ± 0.02	0.5 ± 0.1	0.6 ± 0.26
Spent Biomass	0.15 ± 0.05	0.26 ± 0.14	0.33 ± 0.17	0.23 ± 0.11	1.05 ± 0.15	0.2 ± 0.1	0.1 ± 0.02	0.1 ± 0.03	0.2 ± 0.01	0.1 ± 0.04	0.9 ± 0.8	1.05 ± 0.65

4.2.3 Estimation of extractives

The compositional analysis of carbohydrates and lignin in biomass is profoundly affected by its extractives. The composition analysis of the biomass are given in **Table 4.3.**

Table 4.3: Extractives (g) from 100 g biomass sample.

Biomass Type	Hexane Extract	Ethanol Extract	Water Extract	Hemicellulose	Cellulose	Lignin
Original Citronella Biomass	3.62 ± 0.13	2.95 ± 0.21	7.46 ± 0.17	32.66 ± 0.23	31.70 ± 0.11	18.97 ± 0.14
Spent Citronella Biomass	3.06 ± 0.11	1.79 ± 0.15	6.68 ± 0.26	32.22 ± 0.28	28.15 ± 0.12	18.78 ± 0.19
Bagasse	0.9 ± 0.3	6.5 ± 0.3	7.7 ± 0.4	22.78 ± 0.2	40.15 ± 0.3	20.81 ± 0.20

Extractives in biomass consist of non-structural aromatic compounds, which include volatile oils, chlorophyll, fatty acids and their esters, waxes, resins, tannins, terpenes etc. and can be used for the production of green chemicals (Sasmal et al., 2012). These materials are either polar or nonpolar and can be extracted based on their polarity by using a range of solvents. Thus, biomass was first extracted using hexane to remove non-polar compounds such as lipids, hydrocarbons and terpenes, followed by ethanol to extract polar compounds such as chlorophyll, waxes and sterols and finally by water (Millipore water) to remove non-structural sugars and other inorganic compounds. Comparative analysis of the results revealed that the water extractives content was more in both the biomass compared with ethanol and hexane, unveiling the presence of more polar compounds in the biomass samples.

Estimation of hemicellulose, cellulose, and lignin, which are the major chemical components of lignocellulosic biomass, is essential to understand the conversion efficiency of biomass. From the study, it is revealed that citronella has lower cellulose content (31.70 % in original biomass and 28.15 % in spent biomass) than hemicellulose (32.66 % in original biomass and 32.22 % in spent biomass). The lignin content is approximately the same (~19 %) in both the biomass. Rolz et al. (1986) reported 30 % hemicellulose, 28.5 % cellulose, and 11.1 % lignin in Java citronella bagasse. On the other hand, bagasse found to have higher cellulose (40.15 %) compared to hemicellulose (22.78 %) and lignin (20.81 %).

4.2.4 Thermogravimetric (TGA) analysis

The lignocellulosic biomass can be characterised by its degradation intensity. Thus, the thermal decomposition of biomass was evaluated using a constant isothermal ramp program using TGA. The prominent slope of TG curve can be observed at 200-500 °C, which represents the weight loss due to the degradation of hemicellulose, cellulose, and some fraction of lignin, as depicted in **Fig. 4.1**. The initial mass loss between 40-100 °C was due to the vaporisation of moisture. Further, de-volatilisation of volatile compounds are generally observed between 130-200 °C. The degradation of hemicellulose takes place from 200-350 °C, followed by cellulose decomposition from 300-375 °C. On the other hand, lignin steadily degrades from 200-500 °C due to its thermally more stable nature compared to hemicellulose and cellulose (Naik et al., 2010, Carrier et al., 2011). In order to gain more insight about the degradation of biomass, TG analysis of standard xylan and cellulose was performed. The onset temperature of xylan and cellulose was observed to be 200 °C and 250 °C, respectively. Degradation range of xylan was between 200-320 °C. On the other hand, cellulose degrades between 250-375 °C. Xylan showed two distinct peaks at 242 °C and 284 °C, whereas the

peak temperature of cellulose was observed to be 340 °C. The third peak of xylan and the second peak of cellulose might be due to the presence of minuscule lignin or impurities. Both the biomass followed almost similar de-volatilisation trend with three distinct peaks showing the degradation of hemicellulose, cellulose, and lignin (**Fig. 4.1**). The original biomass has a peak temperature of 297 °C, 330 °C, and 428 °C. In contrast, the spent biomass showed peak temperature at 309 °C, 333 °C, and 433 °C, which corresponds to the maximum degradation temperature of hemicellulose, cellulose, and lignin, respectively. Further, the relative percentage of cellulose (~28 %), hemicellulose (~32 %), and lignin (~18 %) content of the biomass was estimated by comparing it with the degradation range of standards such as xylan and cellulose. Both the biomass used in the present study showed approximately the same composition (i.e. cellulose, hemicellulose, and lignin) except slightly lower cellulose observed in the spent biomass, which attributes to the dissolution of accessible soluble holocellulose content during hydro distillation at the boiling temperature of the water.

In order to get more insight about the thermal behaviour of biomass, researchers emphasise on studying the degradation temperature at 50 % weight loss (T_{50} (°C)) and reactivity of biomass or rate of weight loss at T_{50} (i.e. R_{50} (%.min^{-1})). The higher value of R_{50} signifies that spent biomass was relatively active at T_{50} (Abdullah et al., 2010). The R_{50} values for the standards and citronella biomass are depicted in **Fig. 4.2**.

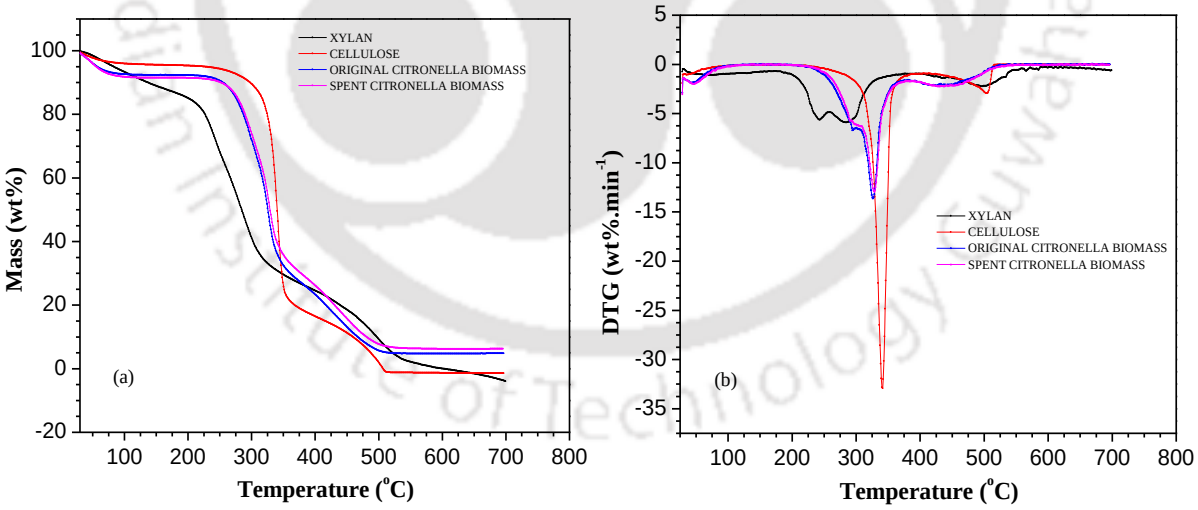


Fig. 4.1: Thermal analysis of biomass and standard samples; (a) TGA and (b) DTG.

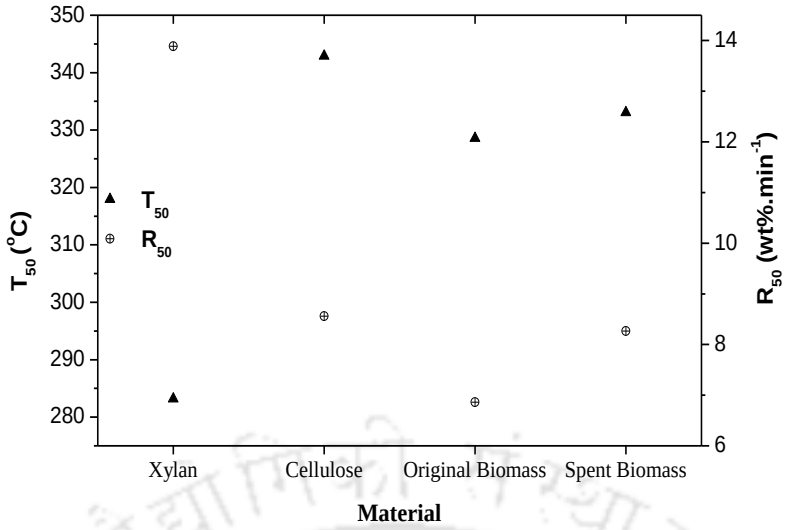


Fig. 4.2: T_{50} and R_{50} profile of biomass and standard samples.

From the comparative analysis of T_{50} and corresponding R_{50} values, It is observed that xylan has the lowest T_{50} (283.17 °C) and the highest R_{50} value (13.89 wt%.min^{-1}). On the other hand, standard microcrystalline cellulose showed T_{50} and R_{50} value of 342.86 °C and 8.56 wt%.min^{-1}, respectively. The higher R_{50} value of spent citronella biomass (8.27 wt%.min^{-1} at T_{50}: 333.04 °C) compared with original biomass (6.86 wt%.min^{-1} at T_{50}: 328.57 °C) signifies that spent biomass is more reactive at T_{50}, which implies that the spent biomass can act as a potential feedstock for pyrolysis and gasification process. Sasmal et al. (2012) have reported T_{50} and R_{50} value in the range of 320-356 °C and 7.2-7.4 wt%.min^{-1}, respectively, for areca nut husk, bonbogori, and moz biomass. Abdullah et al. (2010) reported T_{50} and R_{50} value in the range of 363-410 °C and 2.9-3.19 wt%.min^{-1} for rice husk, paddy straw, oil palm shell, and oil palm frond.

4.2.5 XRD analysis

The XRD analysis of biomass samples is presented in **Fig. 4.3**. The crystallinity of the surface is mainly related to the wax content in the biomass. Whereas the overall crystallinity of biomass depends on the cellulose, wax, and complex nature of bonding between holocellulose and lignin (Naik et al., 2010). The higher peak intensity signifies the higher crystallinity of the sample. The native biomass, before undergoing any pretreatment process showed a crystallinity index of 32.97 %. Whereas hydro distilled (spent) biomass showed increased crystallinity of 34.91 % indicating removal of water extractives in the hydro distillation process.

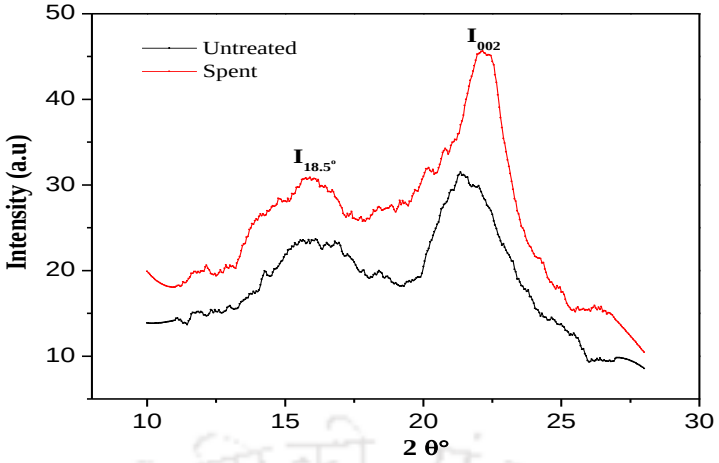


Fig. 4.3: XRD analysis of citronella biomass.

4.2.6 FTIR analysis

The IR spectra showing the percentage transmittance corresponding to wave number is shown in **Fig. 4.4**. From the figure, it can be seen that there is no significant difference in the spectra of untreated and spent citronella biomass. There is an intensely strong and sharp peak in the range of 3800-3200 $cm^{-1,}$ particularly at 3649.32 in both samples, which mostly represents polymeric free hydroxyl (O-H) stretch. Another intense and diverged peak in the range of 2935-2911 cm^{-1} corresponds to the aliphatic alkyl group C-H methyl and methylene asymmetric stretch. Another distinct and sharp peak observed in the range of 1750-1705 cm^{-1} in both the samples signifies un-conjugated xylan as well as aldo, keto, estero, and or acido (C=O) stretch. Methylene C-H stretch (1485-1445 cm^{-1}) and methyl C-H symmetric band indicate the presence of cellulose and hemicellulose (1380-1371 cm^{-1}) in the samples. At around 1220 cm^{-1}, the stretch corresponds to asymmetric glucose ring, and 1145 cm^{-1} corresponds to C-O-C asymmetric vibrations within cellulose.

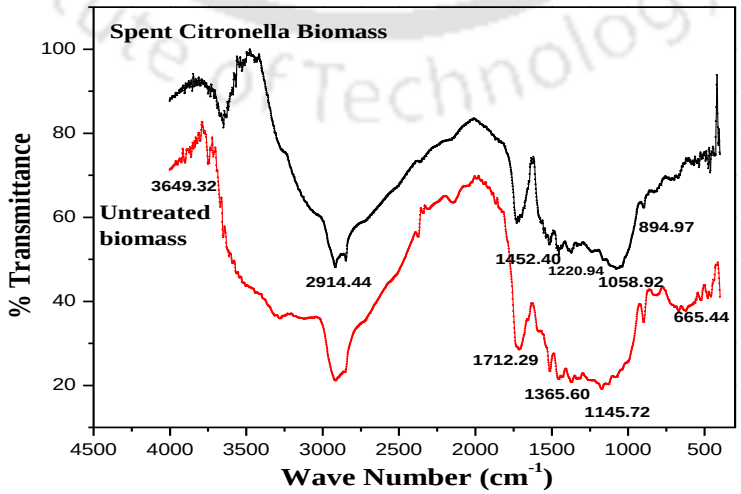


Fig. 4.4: FTIR spectra's of citronella biomass.

Moreover, sharp peaks at 910-860 cm^{-1} of CH=CH trans-unsaturated functional group corresponds to beta-glucosidic bonds of amorphous cellulose. Boeriu et al. (2004) reported most of the above stretches for lignin, which indicates that lignin is dispersed in the residue. The peak at ~1450 and ~894 cm^{-1} represents the crystalline and amorphous fractions of cellulose, respectively (Sasmal et al., 2012).

4.3. Summary

This work has evaluated and projected the commercial significance of citronella biomass. Besides the essential oil, due to its high holocellulose content, citronella biomass can act as a potential feedstock for the production of bioenergy and value added products. Likewise, high calorific value, high volatile matter with a high content of carbon, hydrogen, and oxygen along with low ash and fixed carbon with negligible sulfur content, revealed its suitability as an ideal feedstock for energy production. The spent citronella biomass found to contain fewer elements compared to original biomass suggesting its use for hydrolysis to extract total reducing sugars. Further, the application of citronella biomass for the production of bioenergy and value added chemicals need to be thoroughly assessed in order to gain a clear idea of its potential as a possible alternative feedstock. Thus, in this thesis, the spent citronella biomass was pretreated and hydrolysed to produce reducing sugars. Further, the obtained reducing sugars was utilised for the production of levulinic acid which is a useful biochemical. The detailed discussions about the above-mentioned processes are elaborated in the subsequent chapters.

Chapter 5

Pretreatment of spent citronella biomass using alkali and dilute acid pretreatment techniques and its comparison with bagasse

This chapter describes the investigation of citronella biomass as a possible alternative feedstock for the production of bioenergy and value added products. The potential of citronella biomass as a possible alternative feedstock has been evaluated based on its total reducing sugar (TRS) yield using chemical pretreatment techniques. Further, the yield of TRS obtained using citronella was compared with sugarcane bagasse which is a standard biomass generally studied for the production of bioethanol and other value added chemicals. This chapter also comprises of comparative analysis between alkali and dilute acid pretreatment followed by enzymatic hydrolysis using cellulase enzyme. The results and discussion of this chapter has been divided into two sub-sections; sub-section 5.2.1 for alkali pretreatment followed by sub-section 5.2.2 for dilute acid pretreatment. Later the pretreated solid biomass which remained after pretreatment and enzymatic hydrolysis have been characterised using X-ray diffraction and scanning electron microscope to evaluate its morphological and structural changes.

5.1 Introduction

The upsurge of biofuel production paves the way to search for lignocellulosic biomass materials. Unlike fossils and nuclear materials, availability of lignocellulose materials is not limited around the world. Almost all countries are abundant with the lignocellulosic resources depending on their geographical locations. The compositions of biomass within the same species may altered with varying seasons and climatic conditions. The relative quantities of principle building block elements of lignocellulosic material, i.e. cellulose, hemicellulose and lignin content are often required in studies involving biofuel production. Lignocellulosic biomass can be converted to biofuels or biochemicals by using efficient pretreatment technique to hydrolyse its holocellulosic content. On the other hand, pretreatment methods remains the most important steps in order to reduce the recalcitrance of substrate to achieve higher hydrolysis rate. Pretreatment of cellulosic materials can affect its physical properties, such as its degree of polymerisation, crystallinity and surface area. The primary objective of the pretreatment process is to disrupt the lignin shield and break the crystalline structure of

cellulose while increasing the cellulose porosity. Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass to fermentable sugars conversion (Jacobsen et al., 1999). Effective pretreatment is characterised by several principles: avoiding size reduction, preserving hemicellulose fractions, limiting the formation of inhibitors due to degradation of products, minimising energy input, and cost-effectiveness. Furthermore, appropriate pretreatment is a key step for the efficient utilisation of cellulosic biomass, due to its fractious nature. The conversion efficiency of biomass to fermentable sugars depends on the choice of biomass and pretreatment technique employed. Sugarcane bagasse is a potential feedstock due to its high growth rate and high content of holocellulose. Several literatures reported 32-43 % cellulose, 19-34 % hemicellulose and 25-32 % insoluble lignin for sugarcane bagasse (Krongtaew et al., 2012; Martin and Thomsen, 2007; Martin et al., 2007). On the other hand, Rolz et al. (1986) reported 30 % hemicellulose, 28.5 % cellulose and 11.1 % lignin in Java citronella biomass. Thus, sugarcane bagasse and Java citronella biomass have been chosen as a feedstock in the present work and pretreated using alkali and dilute acid pretreatment technique followed by enzymatic hydrolysis.

Alkali pretreatment is an effective technique for the pretreatment of biomass, but further enzymatic hydrolysis is required for the better yield of reducing sugars. It is often reported that pretreatment step is key for ensuing enzymatic hydrolysis in order to maximise the productivity of monosaccharides. Hence, the pretreatment process remains a vital step to provide suitable condition for enzymatic hydrolysis. Enzymatic hydrolysis operating at mild conditions produce higher yield with no by-products. Alkali pretreatment has proven a useful method among different existing pretreatment technologies because alkali pretreated feedstocks yield good amount of fermentable sugar after enzymatic hydrolysis with less amount of inhibitors formation (Xu and Cheng, 2011). On the other hand, it is a non-hazardous chemical agent, less expensive and requires less energy (Chang et al., 1998). The process involves use of bases like calcium, potassium, and sodium hydroxide. The NaOH solution is mostly preferred in the pretreatment process due to its lower molecular weight and better sugars yield compared to KOH and $Ca(OH)_2$. Alkali pretreatment with NaOH causes a swelling of the biomass, which increases the separation of linkages between carbohydrates and lignin, furthermore disruption of lignin structure. The major effect of alkali pretreatment is delignification as well as partial cellulose decrystallisation and dissolution of hemicellulose. Delignification reactions involve the formation of several different acids during the degradation of carbohydrates, which introduce hydrophilic groups into the lignin structure (Klinke et al., 2002). In the process of

alkali pretreatment, lignin fractions get removed without loss of structural carbohydrate such as glucose, xylose, arabinose etc. It is also reported that alkali pretreatment remove acetyl groups from hemicellulose, which improves hydrolysis by lowering steric hindrance of enzymes (Falls et al., 2011). The sole disadvantage of this process is longer reaction time, but researchers have overcome this drawback by elevating the reaction temperature to restrict the reaction period (Chang et al., 2001).

On the other hand, dilute acid pretreatment are practiced either at low acid concentration and high temperature (dilute-acid pretreatment) or at high acid concentration and low temperature (concentrated-acid pretreatment) (Kosaric et al., 2001). At high acid concentration the hydrogen bonding of the cellulose chains gets disrupted reducing the crystallinity of the cellulose to relatively amorphous state and releasing very less glucose (Orozco et al., 2007). However, use of high acid concentration in the process makes it extremely corrosive and dangerous (Sivers and Zacchi, 1995). In fact, dilute acid hydrolysis is an established method and provides less probability of inhibitors formation such as 5-HMF or furfural, but requires more time and higher temperature to achieve higher reducing sugar release rate (Luo et al., 2002). Several other sugar decomposition products such as organic acids (p-coumaric acid, ferulic acid, gallic acid, acetic acid etc.), phenolic compounds (vanillin, coniferyl aldehyde etc.) and non-phenolic aromatic compounds (benzoic acid, cinnamic acid, benzyl alcohol etc.) are also formed during the pretreatment process. The sugar inhibitors like metal ions of iron, nickel, copper etc. are usually formed during acid pretreatment at high concentration due to possible corrosion of the reactor or equipment. Aromatic compounds have negative effects on saccharification process using enzymes. Dilute acid pretreatment mostly depolymerises the hemicellulose, which enhances the cellulose digestibility during the enzymatic hydrolysis (Mcmillan, 1994; Lee et al., 1999; Nguyen, 2000). Different acids like sulfuric acid, phosphoric acid, nitric acid, hydrochloric acid etc. have been evaluated, but sulfuric acid has been commonly used for the pretreatment of biomass (Mosier et al., 2005; Torget et al., 1992). A typical dilute acid hydrolysis process follows the acid concentration range of 1-6 %, temperature from 100-200 °C and reaction time up to 300 min.

Since very few reports are available on alkali and dilute acid pretreatment of spent aromatic plants biomass. Therefore, this work is an endeavour to evaluate the effect of subsequent alkali or acid pretreatment followed by enzymatic hydrolysis on TRS production from spent biomass of Java citronella and its comparison with standard lignocellulosic feedstock viz. sugarcane bagasse, which is mostly used biomass for bioenergy and biochemical

production. Spent citronella biomass has been chosen due to its abundance as a raw material in the northeast part of India. The physical changes in the biomass sample have been identified using X-ray diffraction and scanning electron microscope. Results of the study revealed that the spent aromatic plants biomass could act as a potential lignocellulosic material for the bioenergy and value added chemicals production.

5.2 Results and Discussions

5.2.1 Alkali Pretreatment

The main purpose of alkali (NaOH) treatment is to remove or disrupt the lignin structure, increase cellulose content and sample porosity, which subsequently influences enzymatic hydrolysis of biomass. The effect of NaOH concentration (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M), reaction time, (20, 40 and 60 min) and temperature (80, 100 and 120 °C) on TRS yield of sugarcane bagasse and spent citronella biomass were evaluated. Results of the study revealed that substrate yield gradually decreases with the increase of NaOH concentration and temperature for both the biomass. This is mainly attributed due to delignification and partial dissolution of hemicellulose content of the biomass. Maximum weight loss was observed at 0.5 M NaOH and 120 °C, which was 18.10 % and 19.06 % for bagasse and citronella biomass, respectively. While, the maximum substrate yield achieved for bagasse and citronella biomass was 96.99 % and 96.86 % at 0.05 M NaOH and 80 °C, respectively (**Fig. 5.1**). Similar trend of maximum weight was also observed by Shankarappa and Geeta (2012) for the biomass pretreated with NaOH. Increasing the reaction temperature and concentration of NaOH cause swelling to the biomass and subsequently results to weight loss of the substrate, which might be attributed due to the effect of Na+ on the carboxylic fractions of the biomass (Lawther and Runcang, 1996).

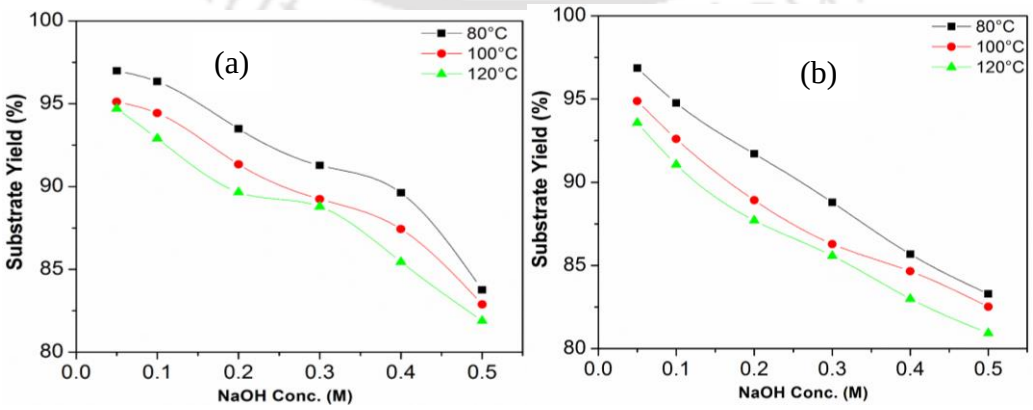


Fig. 5.1: Substrate yield after alkali pretreatment of (a) bagasse; and (b) citronella.

The effect of reaction temperature and NaOH concentration at different reaction time on TRS yield are depicted in **Fig. 5.2** and **Fig. 5.3,** respectively. For bagasse, there was abrupt decrease in TRS with an increased NaOH concentration from 0.05-0.1 M, and thereafter the change was insignificant. Whereas, for citronella biomass, initially TRS yield increased with an increase in the NaOH concentration upto 0.3 M and decreased thereafter. The maximum TRS yield for bagasse (45.22 $mg.g^{-1}$) was obtained at 0.05 M NaOH, 40 min and 80 °C, whereas for citronella (73.88 $mg.g^{-1}$) it was achieved at 0.3 M NaOH, 60 min and 80 °C. The variation in TRS in case of both the biomass attributed to the difference in cell structure and their compositions. Chang et al. (1998) studied alkali pretreatment of bagasse and wheat straw using calcium hydroxide and observed that, less pretreatment time (1-3 h) require higher temperature (85-135 °C), whereas longer pretreatment time (e.g., 24 h) require lower temperature (50-65 °C) to achieve high sugar yield. Cheng et al. (2010**)** compared alkali pretreatment of rice straw using sodium hydroxide (NaOH) and calcium hydroxide at 55 °C and 95 °C, respectively and observed delignification of 8.6-23.1 % for NaOH and 13.1-27 % for calcium hydroxide.

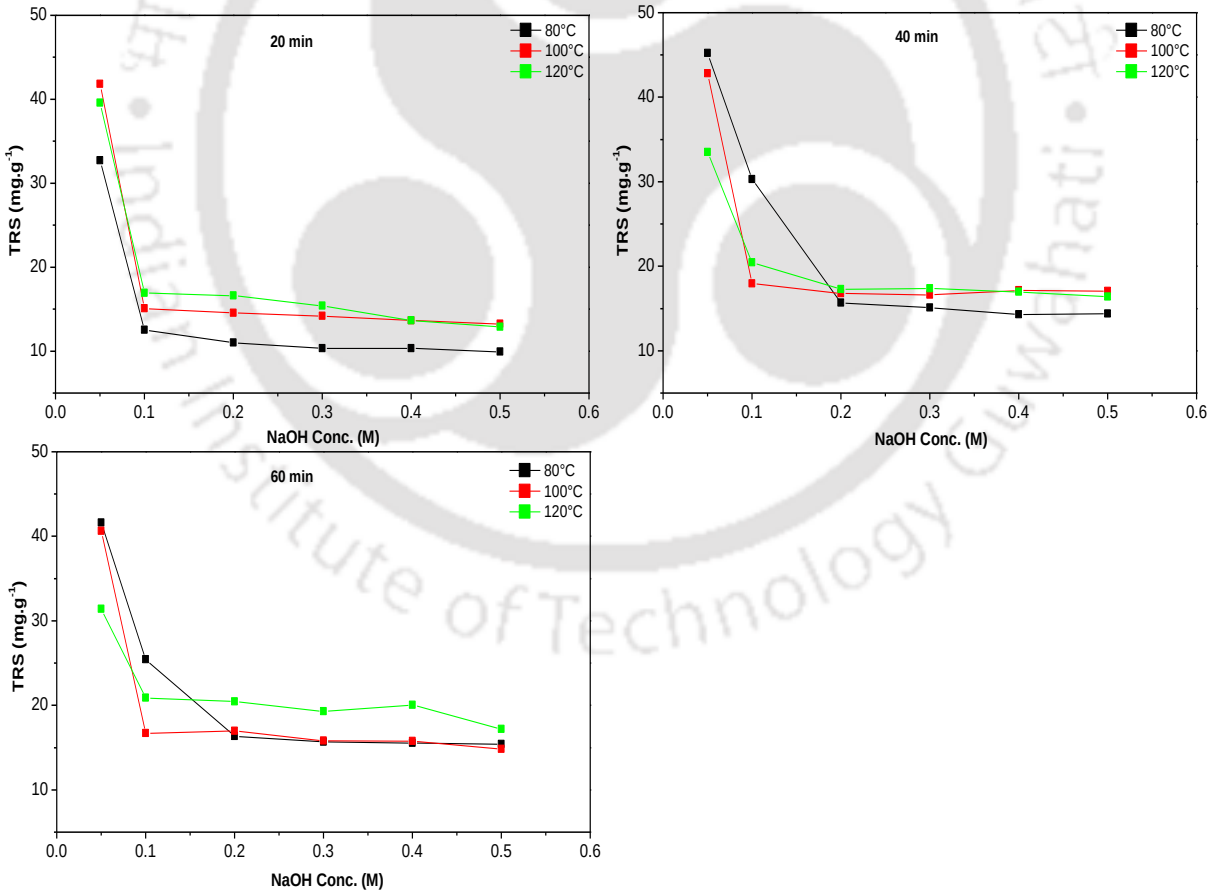


Fig. 5.2: Effect of temperature and NaOH concentration at different reaction time on the release of TRS from bagasse.

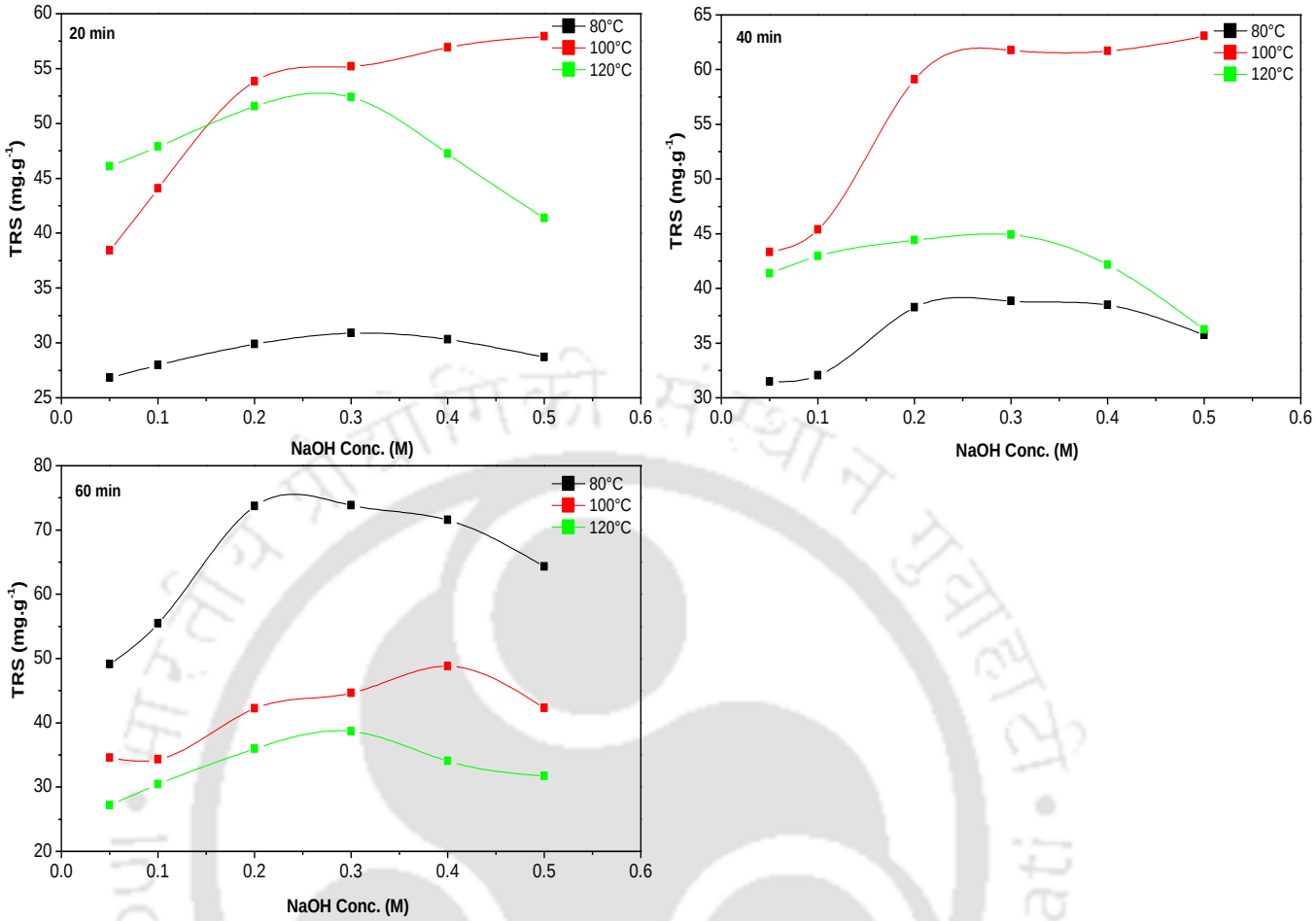


Fig. 5.3: Effect of temperature and NaOH concentration at different reaction time on release of TRS from citronella.

Similarly, the TRS yield obtained in this study was compared with those reported to be optimal for other biomass in the literature (**Table 5.1**). Considering total holocellulose content of the substrate (629.3 and 603.7 $mg.g^{-1}$ in sugarcane bagasse and spent citronella biomass, respectively) the maximum TRS yield in the present work for sugarcane bagasse and spent citronella biomass was found to be 7.19 % and 12.24 %, respectively. The optimum conditions to attain maximum sugar yield were almost identical for both the biomass types, whereas the reducing sugar yield was comparatively higher in the citronella biomass, which may be due to higher hemicellulose content in citronella compared to sugarcane bagasse.

Table 5.1: Comparison of the present study in production of total TRS with reported literature.

Biomass source	Reaction conditions	Maximum TRS yield	Reference
Sugarcane bagasse	0.05-0.5 M NaOH, 80-120 °C, 20-60 min	45.22 $mg.g^{-1}$	This study
Spent citronella	0.05-0.5 M NaOH, 80-120 °C, 20-60 min	73.88 $mg.g^{-1}$	This study
Oil palm fronds	1 % NaOH, 121°C, 60 min,	92.72 $mg.g^{-1}$	Hong et al., 2008
Miscanthus	0.2-0.4 M NaOH, 180 °C, 5-30 min	3 µmol. g^{-1}	Zhu et al., 2015
Bermuda grass	0.5-3 % NaOH, 100-210 °C, 15-90 min	86.00 %	Wang et al., 2008
Bagasse	3 % NaOH, 120 °C, 30 min	58.00 %	Singh and Trivedi, 2013
Oil palm fronds	0.5-10 % NaOH, 50-100 °C, 15-90 min	2.93 $mg.g^{-1}$	Sukri et al., 2013
Rice straw	2-5 % NaOH, 30-120 °C	55.48 $g.L^{-1}$	Remli et al., 2014
Rice straw	2-5 % KOH, 30-120 °C	21.10 $g.L^{-1}$	Remli et al., 2014
Rice straw	2-5 % KOH, 30-120 °C	59.90 $g.L^{-1}$	Remli et al., 2014
Rice straw	2-5 % $Ca(OH)_2$, 30-120 °C	41.46 $g.L^{-1}$	Remli et al., 2014

5.2.1.1 Cellulase enzyme production

The alkali pretreatment prevents condensation of lignin leading to its high solubility and removal. In this process sugars degradation is minimal; however the structure altered during the process makes cellulose accessible to hydrolytic enzymes and thus enzymatic hydrolysis greatly improves after the alkali treatment. Hence, for cellulase production and enzymatic hydrolysis, alkali pretreated biomass which yield highest reducing sugar was used. The cellulase activity of the bagasse treated with 0.05 M NaOH at 80 °C increased from 2.21 FPU at 24 h to 16.17 FPU at 96 h and decreased thereafter. Whereas, cellulase activity increased from 1.81 FPU at 24 h to 14.48 FPU at 120 h for citronella biomass treated with 0.3 M NaOH at 80 °C and decreased to 13.86 FPU at 168 h (**Fig. 5.4**). The higher cellulase activity for

bagasse compared to citronella biomass attributed to the fact that sugarcane bagasse has higher cellulose conversion efficiency compared to citronella; citronella might contain more lignin compared to sugarcane bagasse after the pretreatment; moreover aliphatic and aromatic constituents of lignin can also hinder the cellulase activity (Reddy et al., 2014).

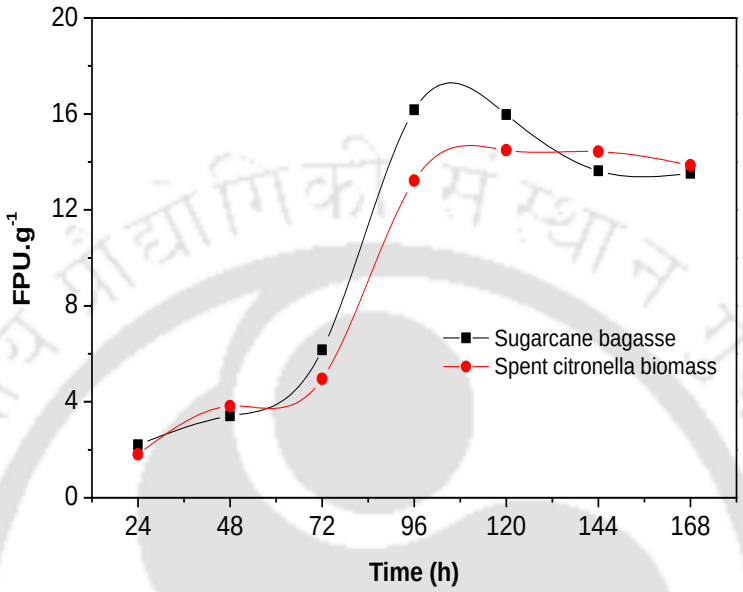


Fig. 5.4: Effect of time on the production of cellulase enzyme for pretreated bagasse and citronella.

5.2.1.2 Enzymatic hydrolysis

The untreated biomass are not an appropriate feedstock for enzymatic hydrolysis due to minuscule release of reducing sugars. Therefore, pretreatment of biomass is necessary before hydrolysis to provide easy access for enzymes to convert cellulose to chains of glucose and its isomers. Mostly glucose is released during enzymatic hydrolysis (Idrees et al., 2014). The effect of temperature (40-60 °C), time (24-72 h) and enzyme loading (10-30 FPU) on the release of TRS were evaluated for both the biomass and tabulated in **Table 5.2.** From the table, it can be seen that the yield of TRS increased with an increase in the reaction time from 24 to 48 h and decreased thereafter. The maximum sugars released was recorded at 48 h and 50 °C (396.70 ± 0.5 $mg.g^{-1}$ and 345.49 ± 0.3 $mg.g^{-1}$ from bagasse and citronella respectively). The decreased of TRS production with increased in reaction temperature and time could be due to instability of the enzyme at higher temperature and elevated reaction time. On the other hand, enzyme concentration is inversely proportional to FPU, therefore the production of TRS

decreased with an increase in the enzyme loading from 10 FPU to 30 FPU. Optimum TRS yield was attained at 50 °C, 48 h and 10 FPU.g^{-1} enzyme loading for both the biomass.

Table 5.2: Effect of temperature, time and enzyme concentration on TRS production during enzymatic hydrolysis of citronella and bagasse.

Temp (°C)	FPU (IU)	TRS (mg.g^{-1} substrate)					
		24 h		48 h		72 h	
		Bagasse	Citronella	Bagasse	Citronella	Bagasse	Citronella
40	10	286.83±0.18	255.79±0.29	353.26±0.33	297.76±0.21	327.39±0.37	241.69±0.26
	20	243.19±0.31	201.23±0.17	283.79±0.21	235.24±0.49	247.90±0.24	206.82±0.47
	30	218.81±0.43	188.49±0.51	244.43±0.47	206.37±0.43	216.67±0.51	173.27±0.28
50	10	320.98±0.22	268.60±0.38	**396.70±0.36**	**345.49±0.62**	349.07±0.33	324.80±0.64
	20	278.73±0.13	229.81±0.41	338.18±0.42	328.25±0.34	293.71±0.38	306.74±0.52
	30	242.26±0.16	200.87±0.13	304.19±0.58	271.35±0.47	283.53±0.29	215.47±0.33
60	10	216.21±0.37	208.10±0.41	237.13±0.44	214.21±0.38	216.34±0.27	187.83±0.22
	20	168.16±0.23	173.37±0.32	183.53±0.39	187.69±0.41	148.87±0.49	131.68±0.61
	30	137.19±0.19	125.33±0.27	147.47±0.15	141.22±0.25	132.30±0.34	116.59±0.42

Han et al. (2012) reported maximum reducing sugars yield of 343.95 mg.g^{-1} for NaOH pretreated wheat straw at 50 °C, 30 h and enzyme loading of 25 FPU.g^{-1}. On the other hand, Nomanbhay et al. (2013) observed reducing sugar yield of 71 mg.g^{-1} during their study on enzymatic hydrolysis of 3 % NaOH treated empty fruit bunch fiber at 50 °C and 120 min. The release of more amount of TRS in case of sugarcane bagasse is mainly because of the higher conversion of cellulose to glucose. Another possible cause leading to this effect is high initial content of cellulose in sugarcane bagasse compared with citronella. Rajan et al. (2014) also reported highest glucose recovery (89 %) for enzymatic hydrolysis of wheat straw at 48 h. Fileto-Perez et al. (2013) reported 50 °C as the optimum temperature for the hydrolysis of cellulose using enzymes. The overall schematic representation of the process has been shown in **Fig. 5.5** to depict the summary of this work.

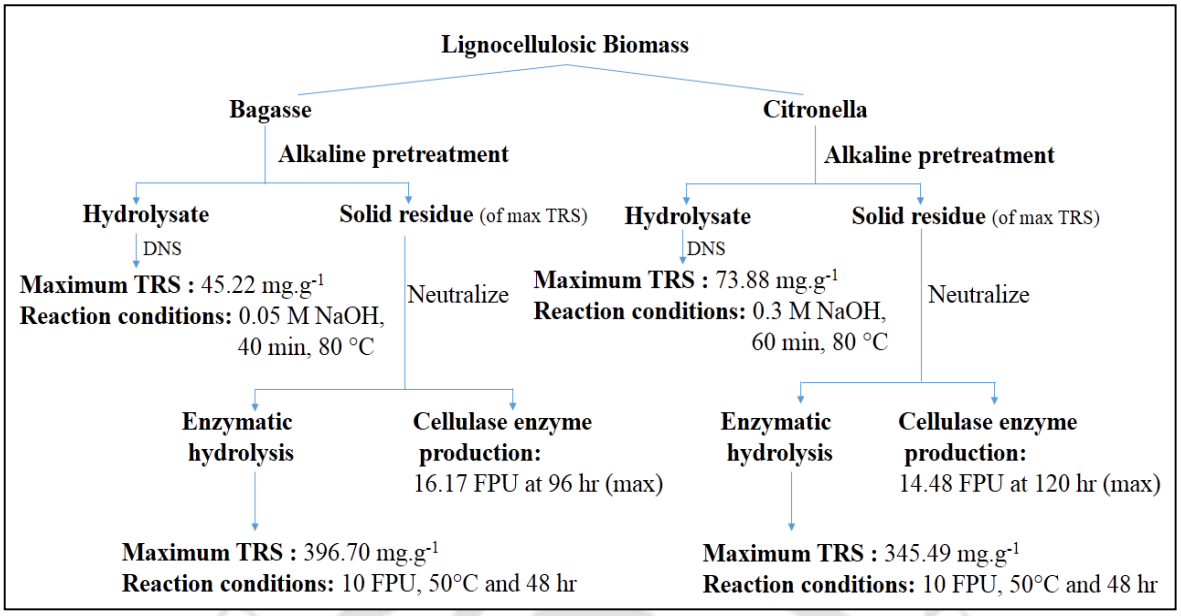


Fig. 5.5: Schematic representation of overall methodology and major results.

5.2.1.3 Characterisation of alkali pretreated residual samples

The x-ray diffraction analysis was performed to investigate the structural changes that occurred with respect to the untreated and treated biomass. The crystalline structure of biomass is mainly because of the strong hydrogen bonding of cellulose chains and Van der Waals forces of glucose molecules in the hemicellulose and cellulose (Naik et al., 2010; Sasmal et al., 2012). The crystallinity index (CI) of untreated citronella and bagasse was found to be 32.97 % and 33.15 %, respectively whereas for alkali treated samples CI value increased from 48.62 % to 58.15 % for bagasse and 49.77 to 57.35 % for citronella. This increase in CI of biomass sample after pretreatment suggests the removal of lignin and partial dissolution of hemicellulose to pentose sugars, thereby exposing the crystallinity of cellulose with increased NaOH concentration from 0.05-0.5 M. The maximum CI of bagasse (58.15 %) and spent citronella biomass (57.35 %) was attained at 0.4 M NaOH concentration and 120 °C.

Further, increase in the NaOH concentration to 0.5 M NaOH decreases the CI value for both the biomass which indicates either change in the crystallinity structure of cellulose to amorphous (Cellulose I to Cellulose II) (**Fig. 5.6 (a)**). The CI of both the biomass decreases abruptly in enzymatic hydrolysis process due to depolymerisation of holocellulose which simultaneously gets converted into amorphous state. The least CI value of enzymatic hydrolysed residual biomass obtained at 50 °C, 10 FPU and 72 h revealed maximum cellulose degradation, further increase in the hydrolysis temperature to 60 °C showed minimal change

in CI value compared to 50 °C. Thus, 50 °C was considered as the optimum temperature for the enzymatic hydrolysis of spent citronella biomass and bagasse at which minimum CI value achieved was 35.72 % and 36.53 %, respectively (**Fig. 5.6 (b)**).

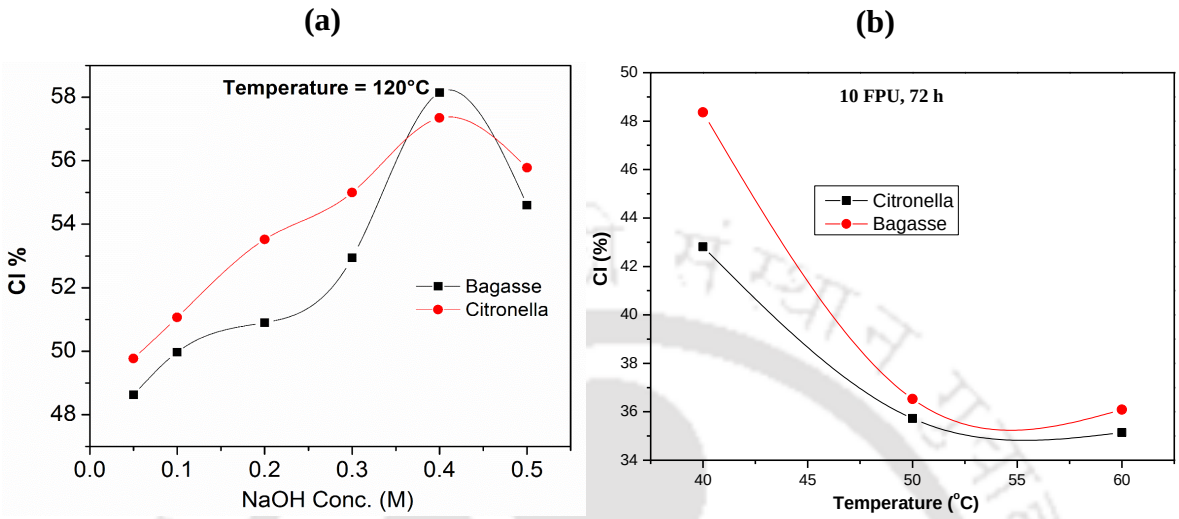


Fig. 5.6: Crystallinity index plot; (a) after alkali pretreatment at different NaOH concentration; (b) after enzymatic hydrolysis at different temperature.

The microscopic images of samples before the pretreatment are shown in **Fig. 5.7 (a)**. The untreated bagasse has intact, tight with smooth cell tissues, whereas the cells of spent citronella biomass sample are distorted due to extensive thermal stress on the oil glands during the oil extraction process causing rapid expansion and disruption of cells while releasing the oil content. The structural changes with micro-pores in the alkali treated sample of both the biomass as shown in **Fig. 5.7 (b)** indicates the partial dissolution of hemicellulose and significant lignin removal providing sufficient accessible surface area for enzyme during enzymatic hydrolysis. Analysis of enzymatic hydrolysed biomass samples showed more micro-pores and cracks on the surface compared to alkali pretreated sample for both the biomass as depicted in **Fig. 5.7 (c)** which revealed substantial depolymerisation of cellulose content in the substrate (Giese et al., 2013).

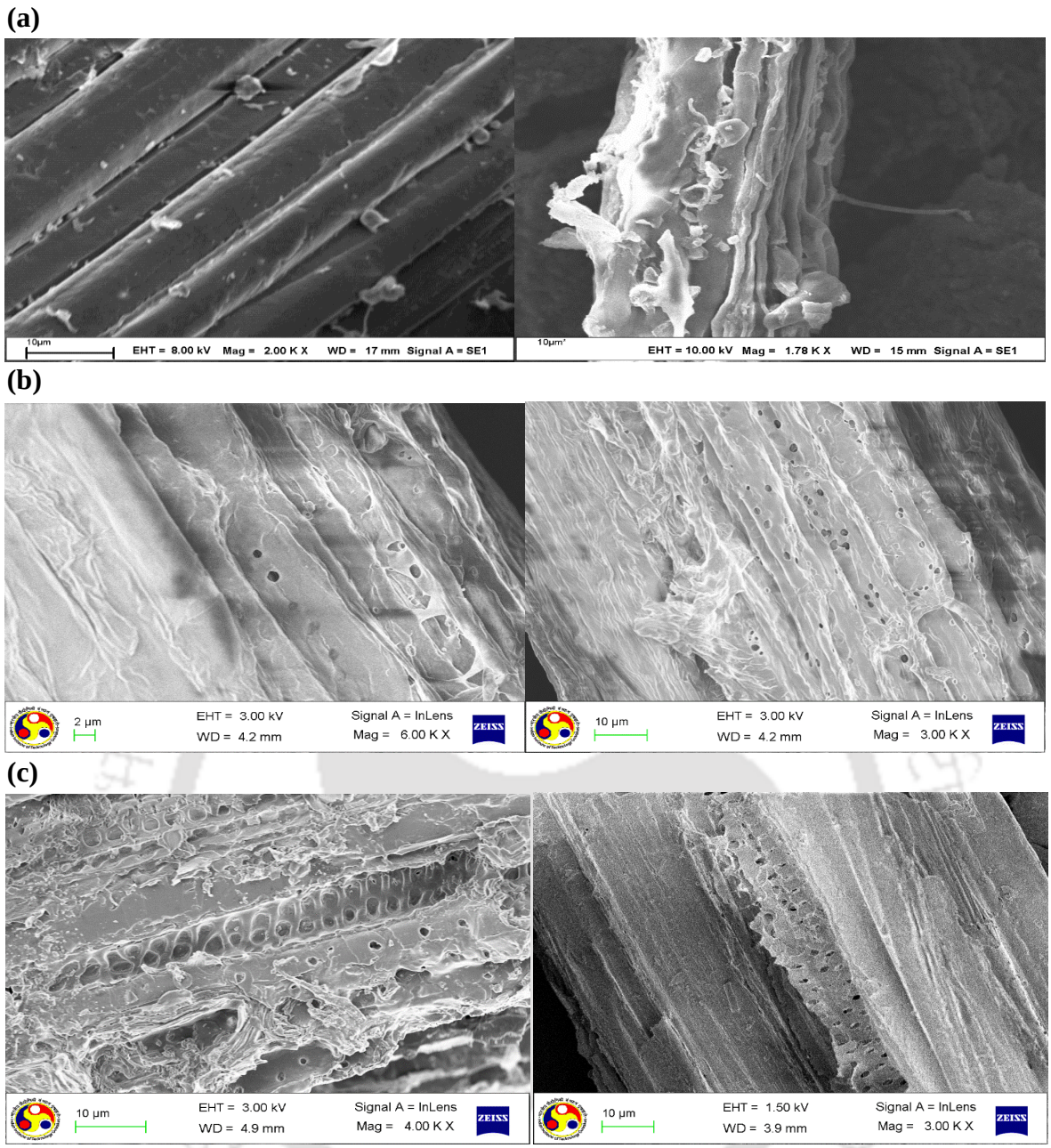


Fig. 5.7: Microscopic image of bagasse and citronella biomass respectively; (a) before NaOH treatment; (b) after NaOH pretreatment; (c) after enzymatic hydrolysis.

5.2.2 Dilute acid (DA) hydrolysis

The effect of acid (H_2SO_4) concentration (0.1, 0.3, 0.5, 0.7 and 0.9 M), reaction time (30, 60, 90 and 120 min) and temperature (80, 100 and 120 °C) on TRS yield of both the biomass types were evaluated individually. DNS method was used to quantify sugar concentration in the process. Significant amount of reducing sugars were obtained in the process. Both the biomass exhibits similar trend of TRS production within the studied range. The effect of reaction time and H_2SO_4 concentration at different temperature on the release of TRS from sugarcane

bagasse and spent citronella biomass is depicted in **Fig. 5.8** and **Fig. 5.9,** respectively. From the figures, it can be seen that TRS yield increases with acid concentrations (0.1-0.9 M) and reaction time (30-120 min) at 80 and 100 °C, but at 120 °C TRS yield increases with increasing acid concentration (0.1-0.9 M) between 30-90 min and thereafter decreases at 120 min, which may be due to the formation of degradation products from hydrolysate containing pentose and hexose sugars. Xylose, a pentose sugar and glucose, a hexose sugar are responsible for the formation of fermentative inhibitors known as furfural and 5-hydroxymethyl furfural (5-HMF), respectively. The concentration of furfural and 5-HMF may increases with increasing acid concentration, temperature and time (Ezeji et al., 2007; Gonzales et al., 1986; Taherzadeh and Karimi, 2007). The minimum TRS yield (97.40 $mg.g^{-1}$ and 11.98 $mg.g^{-1}$) was obtained at 80 °C, 0.1 M and 30 min from sugarcane and spent citronella biomass respectively. Whereas, the maximum (296.64 $mg.g^{-1}$ and 274.93 $mg.g^{-1}$) TRS yield was obtained at 80 °C, 0.9 M and 120 min from sugarcane and spent citronella biomass respectively. These results indicate that the deformation rate of polymeric carbohydrates of sugarcane bagasse was higher than spent citronella biomass at 80 °C. On the other hand, the dissolution of hemicellulose from spent citronella biomass released more sugars than sugarcane bagasse with increase in the process temperature from 100 to 120 °C during the acid hydrolysis. The optimum conditions of both the biomass for maximum sugar yield are almost the same, whereas the reducing sugar yield was comparatively higher in citronella biomass, which may be due to higher hemicellulose content in the citronella compared to sugarcane bagasse. The maximum TRS yield for sugarcane bagasse (452.27 $mg.g^{-1}$) and spent citronella biomass (487.50 $mg.g^{-1}$) was obtained at 120 °C, 30 min, and 0.1 M H_2SO_4.Considering the total holocellulose content of the substrate, conversion efficiency of polymeric to monomeric sugars was 71.87 % for sugarcane bagasse and 80.75 % for spent citronella biomass. These results suggest that, hydrolysis of soft biomass yields more sugars at higher temperature and reaction time with low acid concentration (Ogawa et al., 2008).

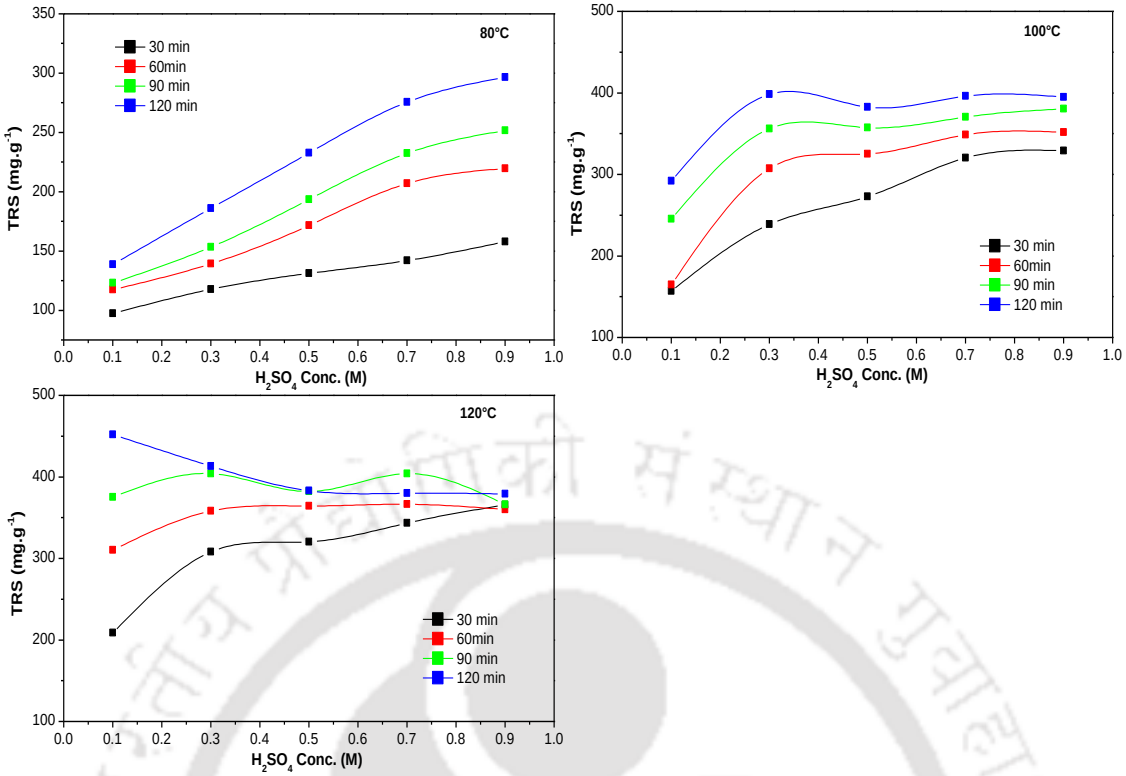


Fig. 5.8: Effect of reaction time and H_2SO_4 concentration at different temperature on the production of TRS from sugarcane bagasse.

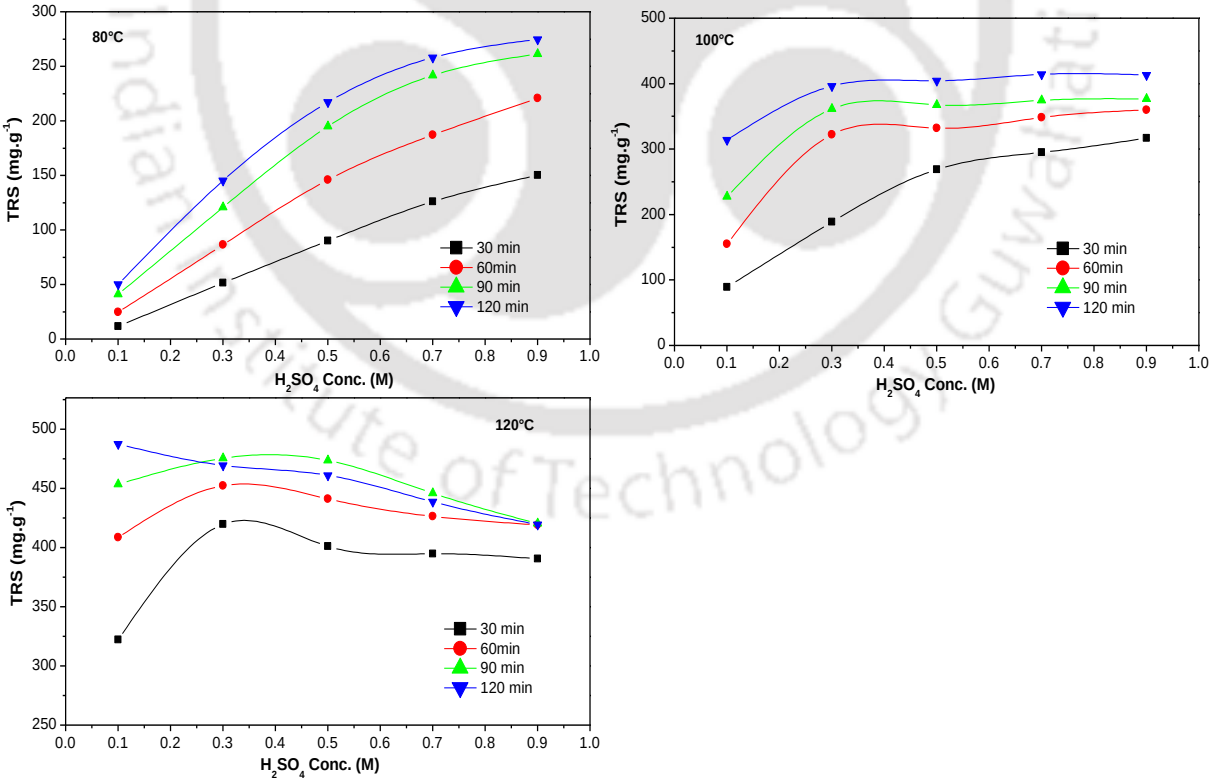


Fig. 5.9: Effect of reaction time and H_2SO_4 concentration at different temperature on the production of TRS from spent citronella biomass.

The results obtained in this study have been compared with the other reports on various alternative feedstocks and presented in **Table 5.3**.

Table 5.3: Comparison of the results obtained in this study with published literature.

Biomass source	Reaction conditions	TRS yield	Reference
Sugarcane bagasse	0.1-1 M H_2SO_4, 80-120 °C, 30-120 min	487.50 $mg.g^{-1}$	This study
Spent citronella	0.1-1 M H_2SO_4, 80-120 °C, 30-120 min	452.27 $mg.g^{-1}$	This study
Rice hulls	0.25-1 % H_2SO_4, 140-180 °C, 15-60 min,	189 $mg.g^{-1}$	Saha et al., 2005
Oil palm frond	1 % H_2SO_4, 121 °C, 15 min	43.61 $mg.g^{-1}$	Hong et al., 2008
Water hyacinth	0.5-3 % H_2SO_4, 100-120 °C, 0-120 min	540 $mg.g^{-1}$	Fileto-Perez et al., 2013
Water hyacinth	2 % H_2SO_4, 121 °C, 60 min	327 $mg.g^{-1}$	Satyanagalakshmi et al., 2011
Water hyacinth	3 % H_2SO_4, 110 °C, 90 min	510 $mg.g^{-1}$	Nigam, 2002
Wood waste	0.5-0.6 % H_2SO_4, 150-180 °C, 2.3-3 h	35-49 %	Harris and Beglinger, 1946
Olive tree biomass	0.2-1.4 % H_2SO_4, 170-210 °C	48.6 %	Cara et al., 2008
Corn fibres	0.5-1.5 % H_2SO_4, 120 °C and 140 °C, 5-20 % biomass	56.8 %	Noureddini and Byun, 2010
Eulaliopsis binata	0.5 % H_2SO_4, 160 °C, 30 min, solid to liquid ratio 1:5	21.02 %	Tang et al., 2013
Sugarcane tops	3 % H_2SO_4,60 min, 25 % $w.w^{-1}$ biomass	68.5 %	Sindhu et al., 2011
Barley straw	1.0 % H_2SO_4, 110 °C, 210 min	70.83 %	Aguilar-Rivera and Canizalez-Leal, 2004
Water hyacinth	0-3 % H_2SO_4, 108 °C and 120 °C, 0.11 MPa, 1-3 h	0.57- 33.47 $g.L^{-1}$	Idrees et al., 2014

Studies reported in literature in this area indicate that pretreatment of biomass required more reaction time or higher acid concentration and higher temperature. Increasing reaction time, temperature and acid concentration leads to the formation of fermentative inhibitors like HMF,

furfural, formic acid, acetic acid (Rajan et al., 2014, Kootstra et al., 2009), which inhibits the growth of microbes during the fermentation of carbohydrates into biofuels and production of other value added products. This study has thus established that proper optimisation of pretreatment parameter reduces the adverse effect of fermentative inhibitors on fermentation process and production of value added products. With the optimised condition used in this study, pretreatment of both the biomass has resulted in higher TRS yield which are at par with yield for most the biomass reported in table 5.3. The effect of reaction temperature and H_2SO_4 concentration at different reaction time on TRS production from sugarcane bagasse and spent citronella biomass is shown in **Fig. 5.10** and **Fig. 5.11,** respectively.

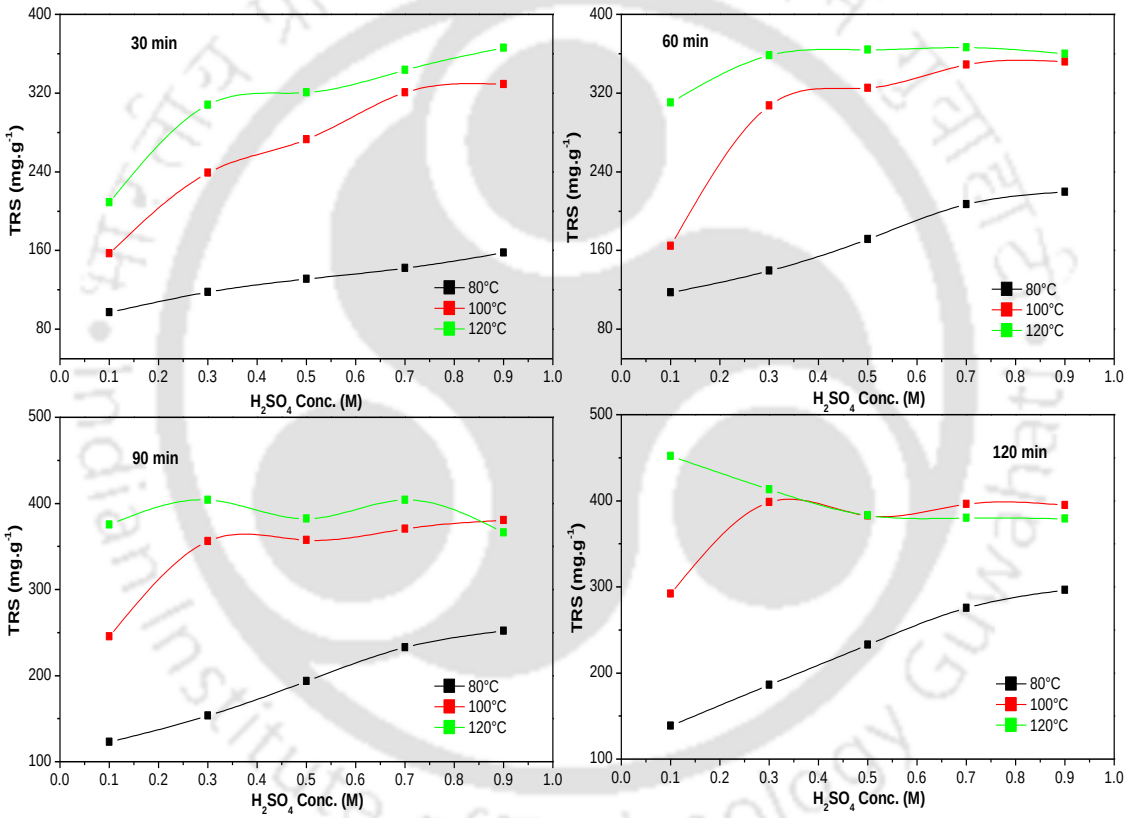


Fig. 5.10: Effect of temperature and H_2SO_4 concentration at different reaction time on the production of TRS from sugarcane bagasse.

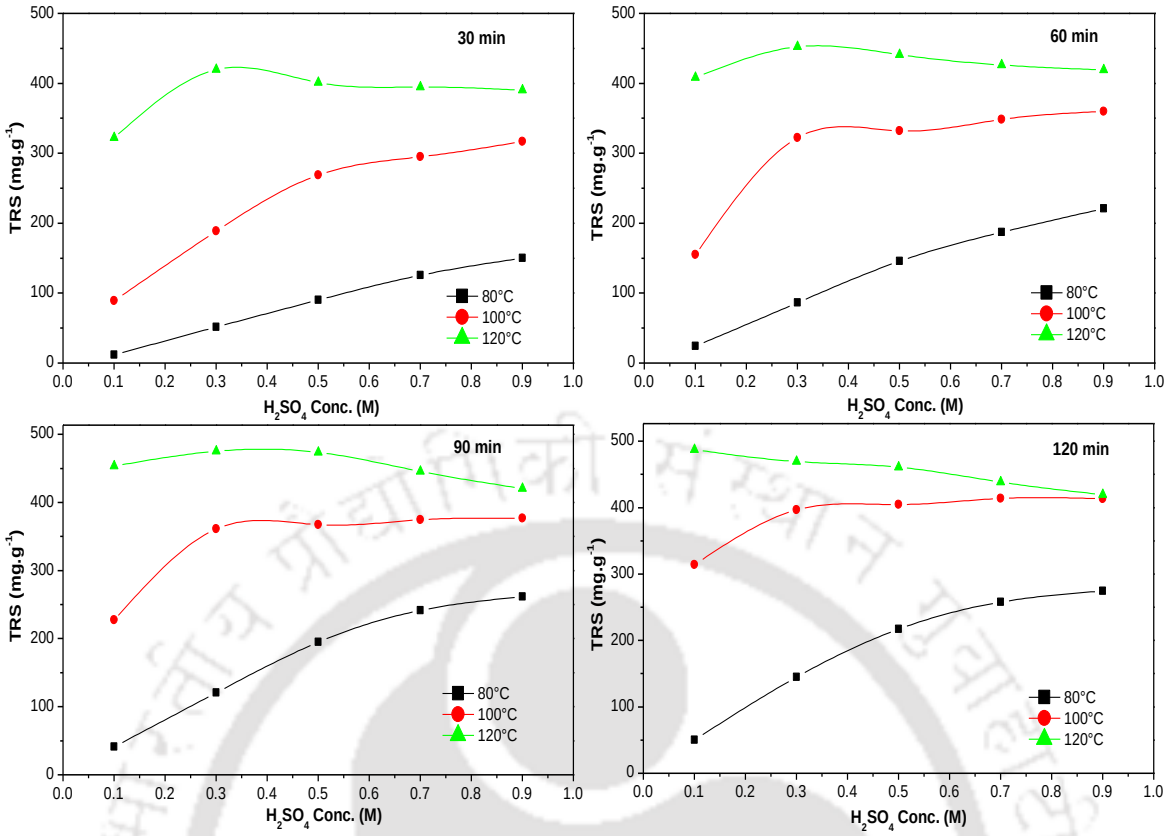


Fig. 5.11: Effect of temperature and H_2SO_4 concentration at different reaction time on the production of TRS from spent citronella biomass.

5.2.2.1 Cellulase production

The pretreated materials at optimised condition (0.9 M H_2SO_4, 120 min and 120 °C) were neutralised using 1 M NaOH solution and further washed with distilled water for the removal of other impurities. The substrate was then air-dried at room temperature and later one gram of material (on dry weight basis at 105 °C for 4 h) was used as a substrate for the production of cellulase enzyme by *phanechaete chysosporium* NCIM 1106. While using the pretreated sugarcane bagasse as a substrate, the enzyme activity (measured by FPU assay) was increased from 3.70 FPU to 18.81 FPU with an increase in the reaction time up to certain period, thereafter decrease to 17.60 FPU at 168 h. Whereas, in case of citronella biomass enzyme activity increased from 3.19 FPU to 20.39 FPU with an increase in the time from 24 to 144 h then decreased to 19.03 FPU at 168 h (**Fig. 5.12**).

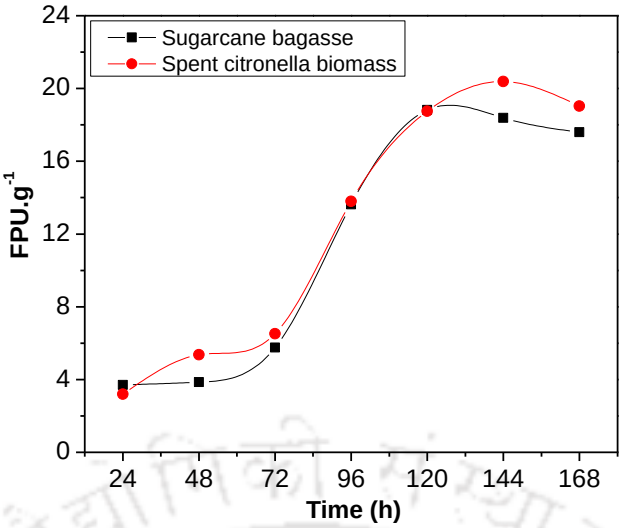


Fig. 5.12: Effect of time on the production of cellulase enzyme from bagasse and spent citronella biomass.

The Cellulase activity obtained in this study were higher than those obtained by Xiaomin et al. (2011) with *Trichoderma sp.* B-8 producing 2.67 FPU using mixture of corncob and wheat bran as substrate. Potumarthi et al. (2012) found the highest cellulase activity of *phanechaete chysosporium* NCIM 1106 (38.46 FPU) from corncobs treated at 0.05 M H_2SO_4 for 144 h. Biotechnological conversion of cellulosic biomass is potential and is a novel approach to develop various type of enzyme. Most of the hemicellulose content will be washed out during the dilute acid pretreatment. The pretreated biomass may contain mostly cellulose and lignin. The removal of lignin is not an easy task during the acid treatment and may require harsh conditions like high temperature and pressure, which may be harmful to the environment. Lignin can be degraded by microorganisms which are eco-friendly. In the microbial approach, lignin degradation can be possible by microbial secreted enzymes like lignin peroxidases (Shangxian et al., 2015). Production of consortium of extra cellular enzymes by cheap source of pretreated lignocellulosic biomass may reduce the production cost on a larger scale.

5.2.2.2 Enzymatic hydrolysis of dilute acid pretreated biomass

Pretreatment of the biomass is necessary before enzymatic hydrolysis to provide easy accessibility to convert the cellulose to glucose and hemicellulose to xylose and arabinose by various types of enzymes. During the dilute acid pretreatment major portion of hemicellulose can be hydrolysed to form its constituents. The residual biomass may contain major portion of cellulose and minor amount of lignin. As discussed above, the biomass pretreated with

optimum condition at which maximum TRS yield was obtained were subjected to subsequent enzymatic hydrolysis using commercially available cellulase (Celluclast 1.5 L) for cellulose conversion. The use of pretreated substrate after water wash can reduce the concentration of inhibitory products (Palmqvist and Hahn-Hagerdal, 2000; Rajan et al, 2014). In the present work, the biomass pretreated with optimum condition at which optimum TRS yield was obtained were neutralised, water washed and air dried before enzymatic hydrolysis. The effect of temperature (40-60 °C), reaction time (24-72 h) and enzyme loading (10-30 FPU.g^{-1} of substrate) on TRS yield were evaluated for both the biomass. The TRS production increases with time up to 48 h and decreased thereafter. Similarly, TRS release rate also decreases with an increase in the temperature from 50 to 60 °C. The Optimum TRS yield was obtained at temperature of 50 °C for 48 h and 10 FPU.g^{-1}. Maximum TRS from spent citronella biomass was 226.99 mg.g^{-1} and from bagasse was 282.85 mg.g^{-1}, as depicted in **Table 5.4**.

Table 5.4: Effect of temperature, time and enzyme concentration on production of TRS during enzymatic hydrolysis of citronella and bagasse.

Temp (°C)	FPU (IU)	TRS (mg.g^{-1} substrate)					
		24 h		48 h		72 h	
		Citronella	Bagasse	Citronella	Bagasse	Citronella	Bagasse
40	10	134.88±0.18	181.48±0.11	178.76±0.31	212.36±0.24	174.98±0.15	204.34±0.35
	20	126.77±0.23	136.32±0.41	139.83±0.66	152.76±0.31	132.95±0.26	136.93±0.41
	30	116.35±0.38	108.25±0.66	126.34±0.39	137.54±0.43	112.85±0.29	123.87±0.57
50	10	204.56±0.27	246.96±0.48	**226.99±0.18**	**282.85±0.23**	218.81±0.33	253.89±0.61
	20	185.75±0.36	238.42±0.17	203.33±0.29	266.21±0.35	194.12±0.41	228.63±0.32
	30	163.89±0.42	232.63±0.28	174.82±0.41	248.41±0.27	169.44±0.58	216.64±0.53
60	10	105.93±0.68	126.77±0.31	123.10±0.36	137.11±0.14	102.17±0.19	117.54±0.49
	20	84.23±0.26	92.91±0.45	102.19±0.22	109.35±0.18	83.72±0.11	96.02±0.36
	30	53.26±0.37	88.28±0.44	91.61±0.17	97.23±0.22	79.94±0.43	83.48±0.38

Enzyme concentration is inversely proportional to FPU, therefore the production of TRS decreased with an increase in the enzyme loading from 10 FPU to 30 FPU. On the other hand increase in the reaction temperature (60 °C) decreased the TRS yield which could be due to denaturation of cellulase enzyme at higher temperature. In the present study, enzymatic hydrolysis of pretreated sugarcane bagasse found to yield more TRS than pretreated citronella.

This might be due to higher cellulose conversion efficiency from sugarcane bagasse compared to citronella. Also, citronella biomass might contain more lignin compared to sugarcane bagasse after the pretreatment; moreover aliphatic and aromatic constituents of lignin can also hinder the cellulase activity (Reddy et al., 2014). Rajan et al. (2014) also obtained the highest glucose recovery (89 %) for enzymatic hydrolysis of wheat straw at 48 h. Fileto-Perez et al. (2013) in their study reported 50 °C as the optimum temperature for hydrolysis of cellulose using enzymes. On the other hand, Saha et al. (2005) obtained 215 $mg.g^{-1}$ of fermentable sugars on the enzymatic hydrolysis of dilute H_2SO_4 pretreated rice hulls by using the combinations of cellulase and β-glucosidase. The overall schematic representation of the process is depicted in **Fig. 5.13**.

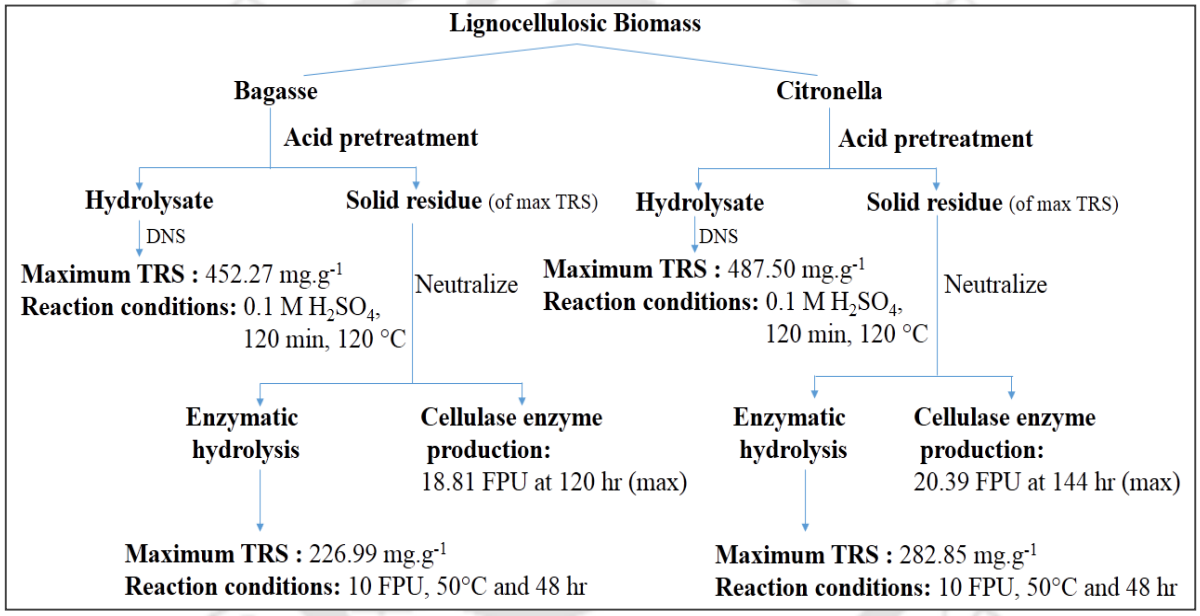


Fig. 5.13: Schematic representation of overall methodology and major results.

5.2.2.3 Characterisation of residual pretreated biomass

XRD analysis of lignocellulosic biomass, before and after pretreatment was performed to compare the structural changes that occurred during the pretreatment of biomass samples. The crystalline structure of biomass is mainly due to the strong hydrogen bonding of cellulose chains and Van der Waals forces of glucose molecules in the cellulose (Naik et al., 2010; Sasmal et al., 2012). In the process of acid pretreatment, the crystallinity index (CI) of the residual biomass samples increased from 48.04 % to 56.18 % in case of spent citronella biomass and 61.90 % to 64.41 % in case of bagasse, with an increase in the acid concentration

from 0.1- 0.7 M H_2SO_4 at 100 °C. The higher CI value implied removal of more hemicelluloses and leaving the crystalline cellulose fraction intact in the pretreated solid residues. Hence, crystallinity of cellulose was found to increase with increased acid concentration. Maximum CI of spent citronella biomass (56.18 %) and bagasse (64.41 %) was obtained at 0.7 M and 100 °C. Further, increase in the temperature to 120 °C decreases CI from 56.18 % to 55.14 % for citronella and 61.60 % to 60.84 % for bagasse at 0.9 M and 120 °C, which indicates either change in the crystallinity structure of cellulose allomorphs (Cellulose I to Cellulose II) or the disruption of crystalline cellulose structure at higher temperature (**Fig. 5.14 (a)**).

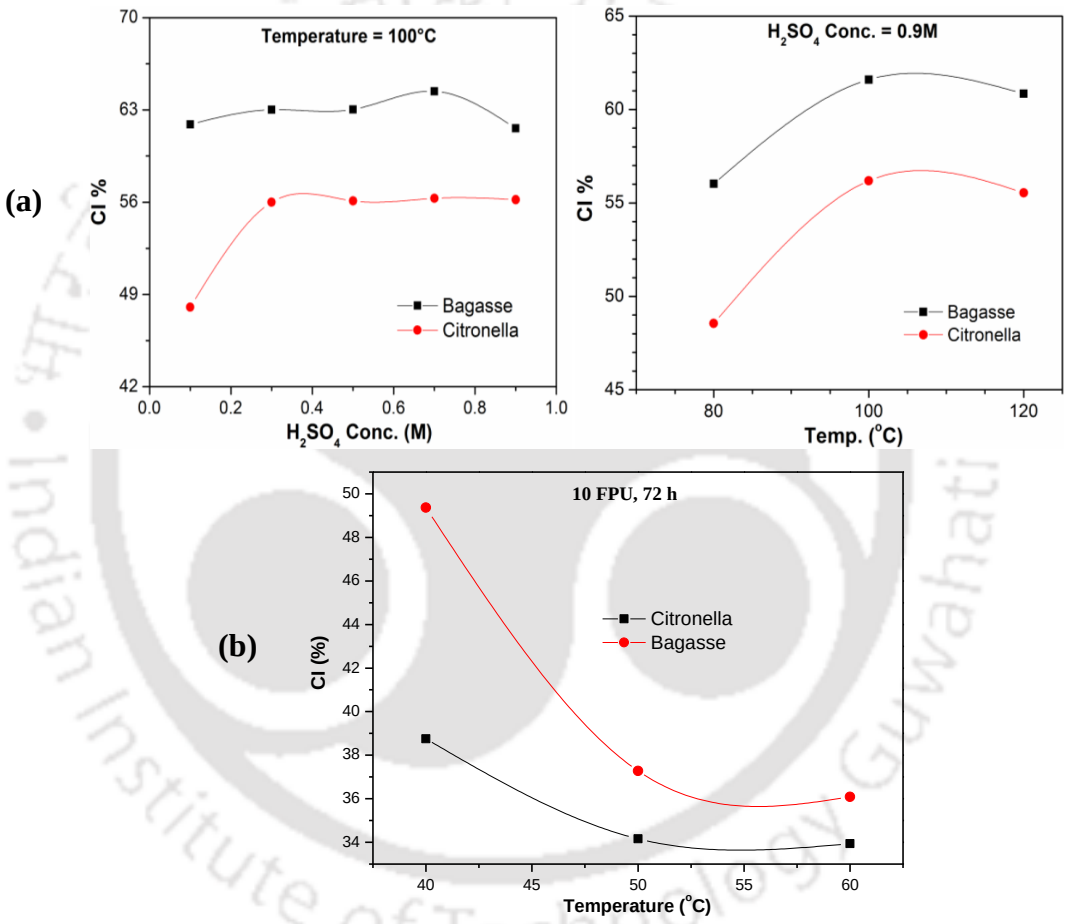


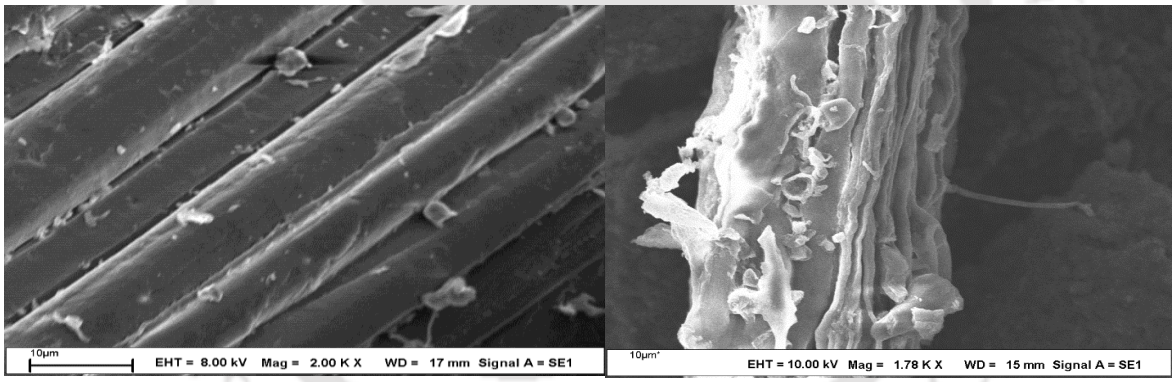
Fig. 5.14: XRD results of acid pretreated biomass at: (a) different H_2SO_4 concentration at 100 °C and different temperature at 0.9M H_2SO_4; (b) after enzymatic hydrolysis at different temperature.

The degradation of hemicellulose and amorphous cellulose occurs at low temperature followed by degradation of crystalline cellulose at higher temperature (Quintana et al., 2015). The CI of both the biomass decreased abruptly in enzymatic hydrolysis process which might be due to

the degradation of crystalline cellulose to glucose. The least CI of enzymatic hydrolysed residual biomass was observed at 50 °C, 10 FPU and 72 h which revealed maximum degradation of cellulose; further increasing temperature to 60 °C showed only minimal change in CI compared with 50 °C. The minimum CI for spent citronella biomass and bagasse at 50 °C was found to be 34.16 % and 37.28 %, respectively (**Fig. 5.14 (b)**).

SEM analysis of the biomass samples revealed significant morphological changes after acid pretreatment. The cells of spent citronella biomass sample were distorted due to extensive thermal stress on the oil glands causing rapid expansion and disruption in the essential oil extraction process, whereas the untreated bagasse showed intact cell tissues as represented in **Fig. 5.15(a)**. The structural changes in the acid treated samples of both the biomass with exposed pores as depicted in **Fig. 5.15(b)** revealed depolymerisation of holocellulose and removal of significant amount of lignin (Giese et al., 2013). SEM analysis of enzymatically hydrolysed biomass samples showed no significant changes in the structure compared with acid pretreated sample for both the biomass.

(a)



(b)

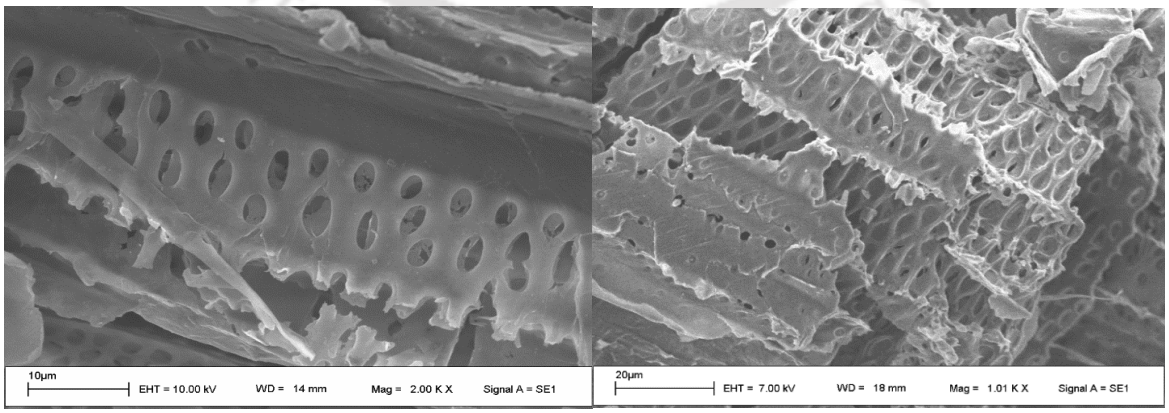


Fig. 5.15: SEM results of bagasse and spent citronella biomass respectively: (a) before acid treatment (b) after acid treatment.

5.2.3 Comparative analysis of the above study

The comparison of above chemical pretreatment methods is difficult due to the fact that every individual method has its advantages and disadvantages. Also, DNS method is accompanied with certain disadvantages such as: It is not suitable for quantification of individual sugar concentration; presence of phenolic impurities in the preparation process of DNS assay might increase the concentration of sugar due to which exact sugar concentration might not be quantified; Inhibitory products of sugar such as HMF or furfural which are produced during pretreatment process might react with 3, 5-dinitrosalicylic acid ensuing more colour intensities leading to increased sugar concentration (Teixeira et al., 2012; Wood et al., 2012; Saqib et al., 2011; Kamireddy et al., 2013; Turek et al., 2013). Due to the above mentioned reasons, the quantification of sugars using DNS might not provide the exact or appropriate value of sugar concentration. Among the pretreatment processes used in this study, chemical pretreatment process results in a rapid and good yield of sugar after enzymatic hydrolysis, but chemical pretreatments requires recovery of corrosive chemicals and hydrolysate neutralisation prior to fermentation, whereas physical treatments requires high energy. Therefore a greener approach for utilising the lignocellulosic biomass in the production of different value added bio-products is an emerging field for researchers. Hence, in the next chapter, the potential of subcritical water hydrolysis of lignocellulosic biomass as an emerging green technology to recover reducing sugar from the biomass has been explored.

5.3 Summary

In present study, we have explored the pretreatment of biomass with alkali and acid pretreatment techniques. The results of this study have revealed that pretreatment of biomass with NaOH results in the swelling of the sample, which leads to the breakdown of linkages between lignin and carbohydrates, furthermore disrupt the lignin structure. Alkali pretreatment was found to be an efficient technique to delignify bagasse and spent citronella biomass; and preserved the polysaccharide fraction. Considering total holocellulose content of the substrate, maximum TRS yield of 7.19 % and 12.24 % was obtained for sugarcane bagasse and spent citronella biomass, respectively. Increasing the reaction time and temperature up to certain interval enhances TRS production during hydrolysis of the pretreated substrate with cellulase enzyme, but further increase in the time and temperature decreases the TRS yield. Maximum TRS yield of 63.04% for sugarcane bagasse and 57.23% for spent citronella biomass was achieved during enzymatic hydrolysis of samples at 10 FPU, 48 h reaction time and 50 °C.

While, in case of dilute acid pretreatment, higher acid concentration (0.9 M H_2SO_4) and higher operating temperature (120 °C) was considered as an optimum condition for acid pretreatment process. At this optimum condition maximum TRS yield obtained was 71.87 % and 80.75 % for sugarcane bagasse and spent citronella biomass, respectively. Subsequent enzymatic hydrolysis of pretreated samples revealed decrease in CI of spent citronella biomass and bagasse to 34.16 % and 37.28 %, respectively indicating significant conversion of crystalline cellulose to glucose. Maximum TRS yield of 44.95% for sugarcane bagasse and 37.60% for spent citronella biomass was achieved during enzymatic hydrolysis of samples at 10 FPU, 48 h reaction time and 50 °C. Considering the high sugar yield, dilute acid pretreatment process using sulfuric acid could be considered as a good choice for the hydrolysis of spent citronella biomass and sugarcane bagasse. Furthermore, the enzyme produced from the substrates could be purified and utilised for enzymatic hydrolysis of the biomass.

Chapter 6

Subcritical water hydrolysis of spent citronella biomass and its comparison with bagasse

This chapter describes the hydrolysis of spent citronella biomass and bagasse using subcritical water (SCW) treatment in a batch reactor. SCW hydrolysis technique is an efficient and one step process to convert biomass to reducing sugars. The parameters affecting SCW hydrolysis such as temperature (140-220 °C), reaction time (5-40 min), and biomass loading (3-5 wt%) on total reducing sugars have been evaluated and optimised using response surface methodology. Further, first-order reaction kinetic model is used to fit the experimental data to obtain kinetic parameters such as activation energy, rate constant and pre-exponential factor. The physical and chemical changes in the treated (SCW treated) and untreated samples were evaluated using XRD, FTIR and SEM. Apart from that, this chapter also include the comparative analysis on SCW hydrolysis of spent citronella biomass and bagasse. Finally, the reducing sugars obtained using SCW technique has been compared with conventional alkali and dilute acid pretreatment technique already discussed in the previous chapter (Chapter 5).

6.1 Introduction

A suitable and environmentally friendly pretreatment method is required to effectively decrystallise the cellulose and lowers the lignin content of lignocellulosic biomass (Agbor et al., 2011). The effective conversion of biomass to fermentable sugars depends on the choice of biomass and pretreatment method employed. Ideal biomass for the production of bioenergy should possess high holocellulose (cellulose + hemicellulose) content and calorific value (Carrier at al., 2011). Lignocellulosic material is very resistant to enzymatic breakdown; hence pretreatment is required in order to enhance the susceptibility of biomass to enzymes. Pretreatment technologies have been extensively studied to process lignocellulosic biomass for bioenergy production. Among all the pretreatment processes, the chemical pretreatment process results in a rapid and good yield of fermentable sugars after enzymatic hydrolysis. However, chemical pretreatment suffers from additional cost of solvent recovery, toxicity, and hydrolysate neutralisation prior to fermentation. An ideal pretreatment

process generally should fulfil the following criteria: (1) breaks-down the covalently cross-linked matrix of hemicellulose and lignin that embeds the cellulose fibres; (2) perturb the strong intra and intermolecular hydrogen linkages in crystalline cellulose, and; (3) increase the porosity and surface area of cellulose for the significant enzymatic hydrolysis. Among the pretreatment methods, SCW treatment has gained more attention as an environmentally friendly technique with an extensive variety of attractive properties. SCW treatment is an auto hydrolysis process, requires less reaction time, cheap, non-flammable, non-toxic, and has many other advantages, mainly in the field of "green chemistry". SCW is defined as hot water at a temperature ranging from 100 °C to 374 °C maintained in a liquid state under high pressure. Under SCW condition, the physical properties of water, such as dielectric constant, density, and viscosity changes with increase in the temperature. SCW has wide range of dielectric constant, density, viscosity and high ion products and also act as an effective catalyst for hydrolysis (Adachi, 2015; Khajenoori et al., 2009; Prado et al., 2014). Water under subcritical condition targets the cellulose and hemicellulose fractions of the biomass to solubilise and liberates the hexose and pentose sugars by disrupting the complex structure of biomass (Rostagno et al., 2014; Zhu et al., 2013; Zhao et al., 2009). However, the hydrolysis rate and release of sugars depend on the biomass cell structure and composition. In the SCW treatment, polysaccharides degrade to release reducing sugars (monosaccharides and disaccharides) and further, the reducing sugars are decomposed to form inhibitor products depending on the operational temperature and time (Cardenas-Toro et al., 2014; Mohan et al. 2015). Depending on the operational temperature and reaction time of SCW hydrolysis of biomass, polysaccharides degrades to oligosaccharides (Degree of polymerisation (DP) <10) or monosaccharides and monosaccharides further decompose to other degradation products (Cardenas-Toro et al., 2014). The conventional experimental approach requires numerous experiments to establish the optimum condition of the process and is time consuming. These bounds can be avoided by optimising the process variables using statistical technique namely response surface methodology (RSM) (Montgomery, 2005; Singh and Sharma, 2012). The commonly used experimental design techniques are Central Composite Design (CCD), Box-Behnken Design (BBD) and Doehlert Design. Among them, CCD is the most widely used design technique, as it is suitable for analysing the interaction effects between the variables and fit in a quadratic model thereby effectively optimising the process with minimum number of experiments. The RSM technique has many advantages: it is inexpensive, less time consuming and can investigate several numbers of process variables at a time with minimum

number of experiments. It further correlates the interaction between the independent variables and the response of interest. There is an extensive literature on hydrolysis of lignocellulosic biomass using SCW treatment (Pourali et al., 2010; Lin et al., 2015). Researchers explored saccharides extraction from different feedstocks such as cellulose, oil palm fronds hemicellulose, coconut meal, sugarcane bagasse, cotton stalk, tobacco stalk, wheat stalk, sunflower stalk etc. (Mohan et al., 2015; Akpinar et al., 2009; Sasaki et al., 2000; Norsyabilah et al., 2013; Khuwijitjaru et al., 2014). Moreover, in literature, SCW also operated at high temperatures ranging from 250-430 °C (Zhao et al., 2012; Pourali et al., 2010; Lin et al., 2015). However, literature in the area of optimisation and estimation of kinetics parameters using SCW hydrolysis technique is rather limited. These kinetic data and parameter optimisation models play an effective role in process development.

Hence, the present study aims to hydrolyse the spent citronella biomass and sugarcane bagasse under SCW temperature (140–220 °C) and pressure using a batch-type reactor. Spent citronella biomass has been chosen due to its abundance in the Northeast part of India. On the other hand, sugarcane bagasse is a by-product generated from the sugar industry with high holocellulose content and is abundantly available on earth. Furthermore, the process variables, such as temperature, biomass loading and reaction time are optimised by RSM technique. An attempt has also been made to study the kinetics of biomass hydrolysis and evaluate its kinetic parameters. Moreover, to understand the structural changes after SCW treatment, biomass samples are characterised by using X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy and Scanning electron microscopy (SEM).

6.2 Results and Discussion

6.2.1 Effect of reaction temperature and time on sugar yield

Temperature remains the most influencing factor in SCW hydrolysis. Reaction time is dependent on temperature and matrix of the targeted compound. The pH of hydrolysate decreases with reaction time (Meillisa et al., 2015; Chao et al., 2013). On the other hand, increase in reaction temperature provides higher mass transfer and reduces surface tension of water thereby increases the solubility of the targeted material and provides higher diffusivity through the sample matrix (Khajenoori et al., 2009; Zhao et al., 2009; Akpinar et al., 2009). However, the sugar yield depends on the competitive reactions between biomass hydrolysis and monosaccharides degradation. The increase in TRS production with an increase in the temperature from 140-160 °C for citronella; and 140-180 °C for bagasse revealed that

hydrolysis rate increases with increased temperature, beyond which there was decrease in the TRS yield, which suggests that degradation rate of saccharides to inhibitors such as 5-HMF and furfural increases at higher temperature (**Fig. 6.1**). Thus, in the present work we have not considered temperature beyond 220 °C for further experimentation.

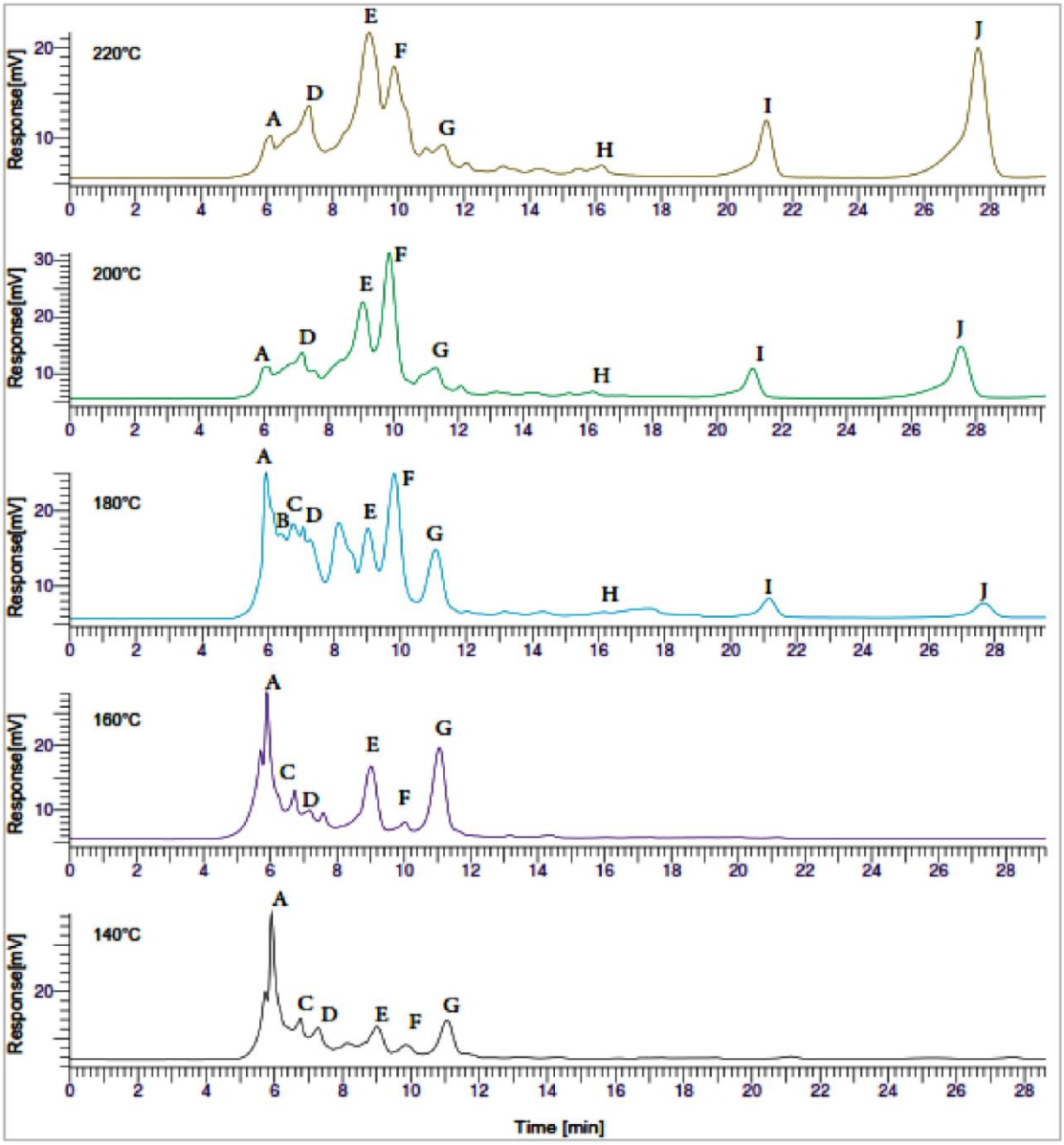


Fig. 6.1: HPLC chromatogram of SCW treated hydrolysate samples at 30 min reaction time of different temperatures; A: Maltohexaose, B: Maltopentaose, C: Raffinose, D: Cellobiose, E: Glucose, F: Xylose, G: Arabinose, H: Erythrose, I: 5-HMF, J: Furfural.

In case of citronella, maximum sugar obtained was 281.66 $mg.g^{-1}$ at 160 °C and 30 min, which includes 52.24 $mg.g^{-1}$of maltohexaose, 2.73 $mg.g^{-1}$ of maltopentaose, 1.13 $mg.g^{-1}$ of

raffinose, 32.27 mg.g^{-1} of cellobiose, 74.33 mg.g^{-1} of glucose, 98.30 mg.g^{-1} of xylose, and 20.67 mg.g^{-1} of arabinose. Whereas, at the reaction time of 30 min, the release of total sugars at 140 °C, 180 °C, 200 °C and 220 °C were 178.53 mg.g^{-1}, 265.69 mg.g^{-1}, 212.94 mg.g^{-1} and 161.17 mg.g^{-1}, respectively (**Fig. 6.2** and **Fig. 6.3**). The production of furfural and 5-HMF as the by-products of hydrolysis was not detected till 160 °C, but there was a subsequent increase in the production of 5-HMF and furfural from 180 to 220 °C, and the maximum 5-HMF (5.77 mg.g^{-1}) and furfural (10.93 mg.g^{-1}) was observed at 220 °C (**Fig. 6.4**). The release of maltopentaose (maximum of 3.71 mg.g^{-1}) was less compared to maltohexaose (maximum of 54.10 mg.g^{-1}), but follows the same trend as maltohexaose. After 180 °C cellobiose disintegrates into glucose while producing maximum glucose at 200 °C. Glucose production increases with temperature from 140 °C to 200 °C and drops down at 220 °C due to its conversion to erythrose and HMF. Maximum glucose of 97.04 mg.g^{-1} was obtained at 200 °C. On the other hand, hemicellulose sugars such as xylose, arabinose etc. also follows the same behaviour as glucose, which degraded to furfural at higher temperature. Maximum xylose (109.33 mg.g^{-1}) was obtained at 180 °C. The effect of varying hydrolysis temperature (140, 160, 180, 200 and 220 °C) and time (5, 10, 15, 20, 25, 30, 35 and 40 min) on the release of sugars are shown in **Fig. 6.2**.

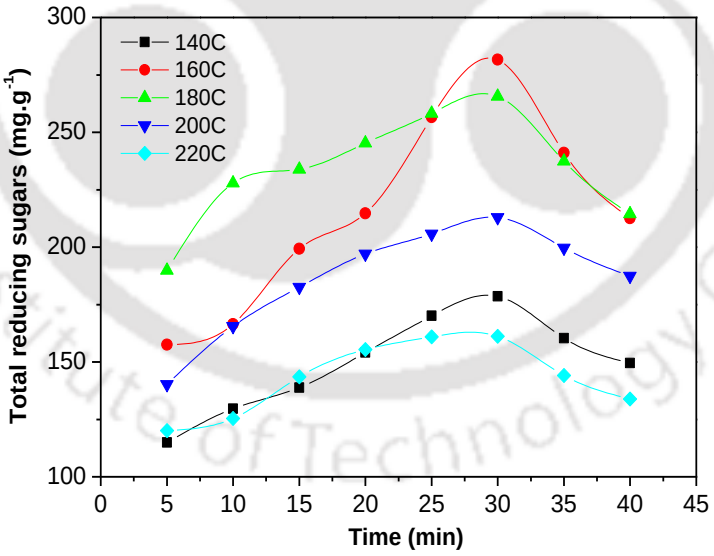


Fig. 6.2: Concentration of total reducing sugars at 3 wt% biomass of citronella.

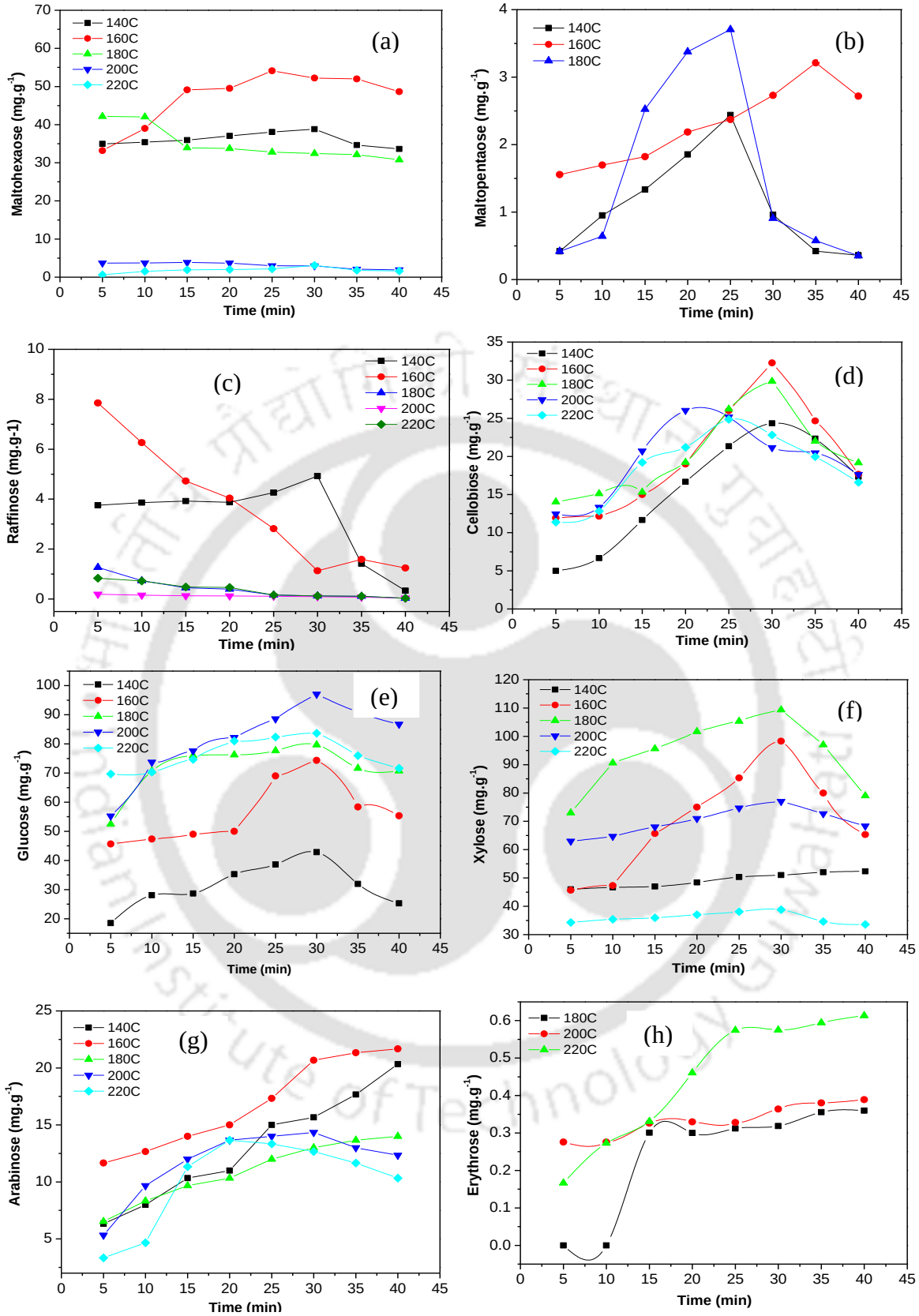


Fig. 6.3: Concentration of different sugars at varied reaction temperature and time, (a) Maltohexaose; (b) Maltopentaose; (c) Raffinose; (d) Cellobiose; (e) Glucose (f) Xylose; (g) Arabinose; and (h) Erythrose from citronella.

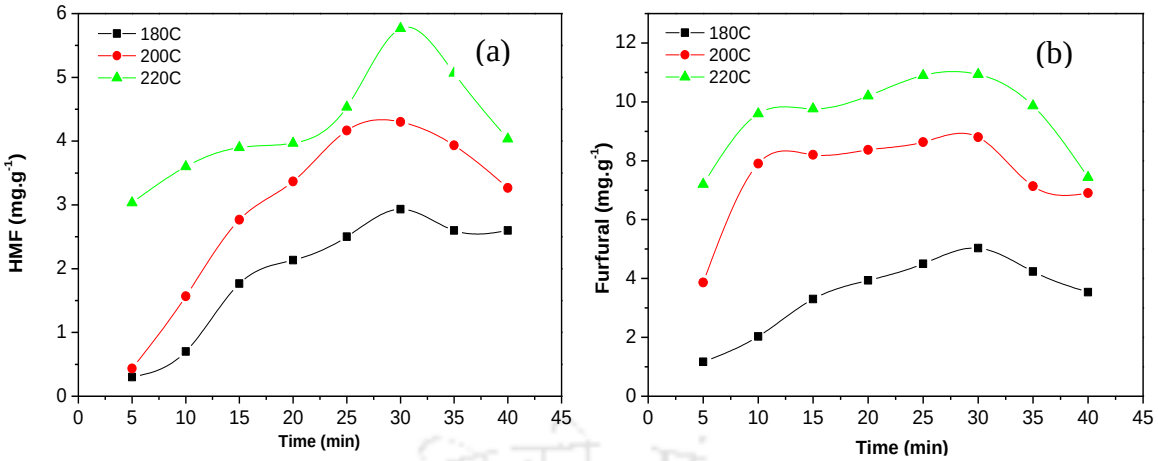


Fig. 6.4: Concentration of inhibitory products at different reaction temperature and time, (a) 5-HMF and (b) Furfural, at 3 wt% biomass of citronella.

On the other hand, in case of bagasse, the maximum sugars obtained was 325.09 mg.g^{-1} at 180 °C and 30 min, which includes 30.57 mg.g^{-1} of maltopentaose, 0.64 mg.g^{-1} of raffinose, 15.33 mg.g^{-1} of cellobiose, 62.63 mg.g^{-1}of sucrose, 112.13 mg.g^{-1} of glucose, 92.89 mg.g^{-1} of xylose, 7.96 mg.g^{-1} of arabinose and 2.93 mg.g^{-1} of fructose. While at the same condition, significant concentration of 5-HMF and furfural, 14.41 mg.g^{-1} and 3.07 mg.g^{-1}, respectively was also produced. On the other hand, the total sugars obtained for different temperature at 30 min was 200.64 mg.g^{-1}, 298.43 mg.g^{-1}, 238.42 mg.g^{-1} and 183.53 mg.g^{-1}, for 140 °C, 160 °C, 200 °C and 220 °C, respectively (**Fig. 6.5** and **Fig. 6.6**). The concentration of degraded products increased with an increase in the temperature and attained 46.3 mg.g^{-1} of 5-HMF and 4.5 mg.g^{-1} of furfural at 220 °C and 40 min for bagasse (**Fig. 6.7**).

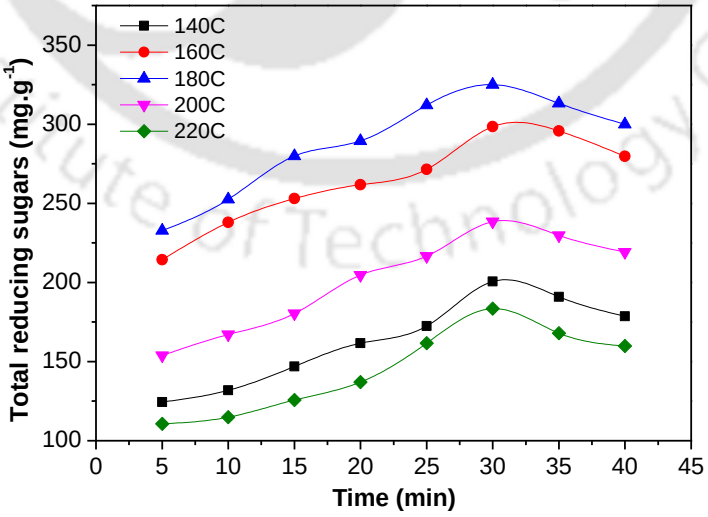


Fig. 6.5: Concentration of total reducing sugars at 3 wt% biomass of bagasse.

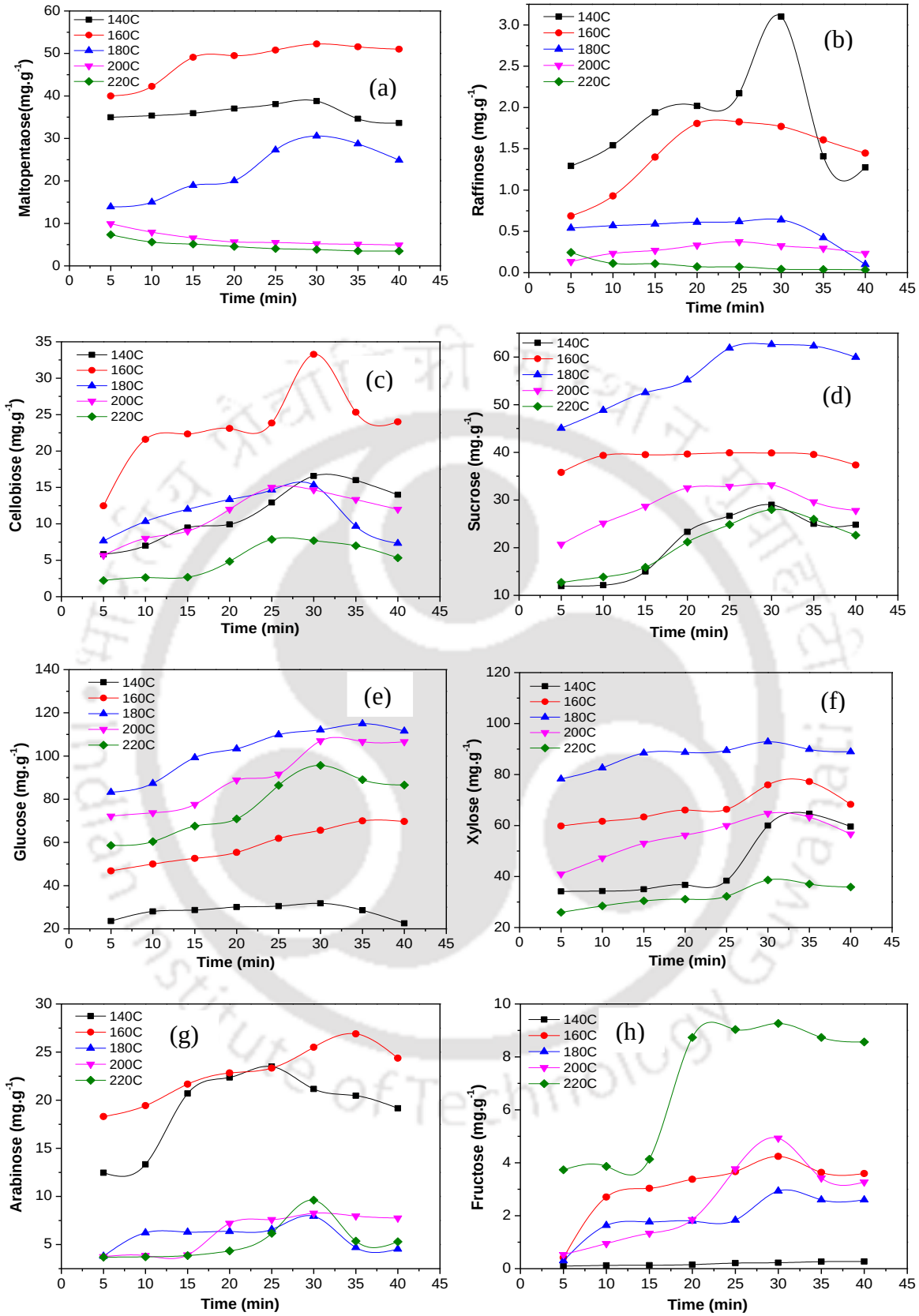


Fig. 6.6: Concentration of different sugars at varied reaction temperature and time,(a) Maltopentaose; (b) Raffinose; (c) Cellobiose; (d) Sucrose; (e) Glucose; (f) Xylose; (g) Arabinose; and (h) Fructose from bagasse.

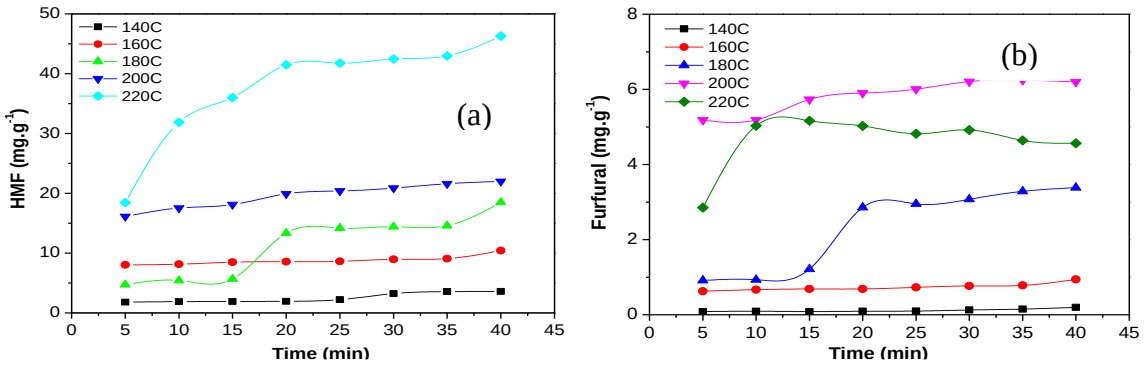


Fig. 6.7: Concentration of inhibitory products at different reaction temperature and time, (a) 5-HMF and (b) Furfural, at 3 wt% biomass of bagasse.

The sugar yield obtained in this study was higher than those from other reports such as in the study by Prado et al. (2014) reported SCW hydrolysis of pressed palm fiber, grape seed and coconut husk to produce fermentable sugars using a 50 ml semi-batch reactor at 208-257 °C for 30 min. They observed increased sugar yield with increase in the temperature for all the feedstocks and obtain the maximum sugar yield of 11.9 %, 6.4 % and 11.7 % from pressed palm fibre, grape seed and coconut husk, respectively. The results obtained in this study have been compared with the other reports in terms of sugar yield in **Table 6.1**.

Table 6.1: Literature review on SCW hydrolysis of different lignocellulosic biomass.

Sl. No.	Type of biomass and reactor	Operating condition	Reducing sugars Conc.	Reference
1.	Rice straw and batch reactor	280 °C, 20 MPa, and 8 min	TRS Conc. = 346 $mg.g^{-1}$	Lin et al., (2015) Bioresource Technology 186, 8–14
2.	Sugarcane straw and Semi-continuous flow reactor	200 °C, 10 MPa, and 8.87 min	TRS Conc. = 320 $mg.g^{-1}$ (32 %)	Lachos-Perez et al., (2017) Bioresource Technology 243, 1069–1077
3.	Sugarcane bagasse pith and batch type reactor	200 °C, 40:1 L/S, and 40 min with 1 MPa CO_2	TRS yield = 402 $mg.g^{-1}$ (40.2 %)	Liang et al., (2017) Bioresource Technology 228, 147–155
4.	Sugarcane bagasse and Semi-continuous flow reactor	200 °C, 15 MPa and 20 min	TRS yield = 15.5 %	Lachos-Perez et al., (2016) J. of Supercritical Fluids 108, 69–78
5.	Sugarcane bagasse pith and batch type reactor	200 °C, 30:1 L/S, 50 min and with 2 MPa CO_2	TRS yield = 43.6 %	Liang et al., (2016) RSC Adv., 6, 99322
6.	Rice straw and batch reactor	190 °C and 10 min	TRS yield = 21.5 %	Pourali et al., (2010) Chemical Engineering Journal, 160, 259–266

The release of total sugars initially found to increase with time and temperature. Further, increase in both reaction time and temperature beyond certain value unexpectedly produced low amount of sugars. This is mainly attributed to the fact that oligosaccharides and monosaccharides degrades at much faster rate than cellulose/hemicellulose or lignin (Khajenoori et al., 2009). Thus, before achieving the complete holocellulose conversion into total sugars, the formed oligosaccharides or monosaccharides disintegrate to other degradation products (Zakaria and Kamal, 2015). The overall degradation profile can be explained as – Polysaccharides (i.e cellulose and hemicellulose) first degrade into oligosaccharides (Maltohexaose, Maltopentaose, Raffinose etc.) and monosaccharides (Glucose, xylose, Arabinose etc.). Subsequently with increase in the temperature, oligosaccharides degrade into di, tri or monosaccharides. Further increase in reaction temperature and time degrades monosaccharides to sugar inhibitors (5-HMF, Furfural etc.). Tolonen et al. (2013) evaluated the cellulose degradation using subcritical and supercritical water at 280-380 °C and obtained water-soluble oligosaccharides, insoluble oligosaccharides, glucose, fructose, 5-HMF and furfural. Maximum, 25 % oligosaccharide was obtained at 380 °C and 0.25 sec. The release of hemicellulose sugars (i.e. xylose and arabinose) was maximum at around 180 °C, because of the fast degradation of hemicellulose as described by Mohan et al. (2015). Norsyabilah et al. (2013) obtained maximum xylose yield from hemicellulose under SCW condition at 190 °C and also observed insignificant change in xylose production at 200 °C, whereas Cardenas-Toro et al. (2014) reported complete depolymerisation of hemicellulose at ≤ 200 °C. On the other hand, the release of cellulose fractions viz. glucose and fructose requires higher temperature to disintegrate due to the crystalline nature of cellulose. Therefore, in order to achieve maximal glucose and fructose release in the hydrolysate biomass need to be treated at temperature around 200 °C under subcritical condition. Because, the ionisation constant of water which increases with temperature destroys the glycosidic bond of cellulose and releases monomeric sugars at high temperature. But, beyond 220 °C, glucose production decreases which might be due to its decomposition to 5-HMF (Khajenoori et al., 2009). From **Fig. 6.1**, it can be observed that the release of 5-HMF and furfural increases with increasing reaction temperature and time. For citronella, maximum 5-HMF (5.77 $mg.g^{-1}$) and furfural (10.93 $mg.g^{-1}$) was obtained at 30 min and 220 °C while, for bagasse maximum 5-HMF of 46.29 $mg.g^{-1}$ and 4.57 $mg.g^{-1}$ of furfural was achieved at 220 °C and 40 min hydrolysis time. Hence, study on reaction kinetics and optimisation of SCW hydrolysis process is necessary in order to obtained maximum content

of desired sugar or total reducing sugars with minimum release of degraded products. This reaction kinetics is important for better understanding of the hydrolysis of biomass under SCW conditions.

6.2.2 Kinetics of reaction mechanism in SCW hydrolysis

Hydrolysis of lignocellulosic biomass is a very complex process and leads to the formation of various decomposed products. Therefore, a simplified reaction kinetic model is important to evaluate its decomposition kinetics. The reaction mechanism for the formation and degradation of saccharides in SCW hydrolysis of citronella and sugarcane bagasse can be simplified based on the irreversible series and consecutive first-order reaction as shown in the following **Eq. (6.1)** (Zhao et al., 2009; Lin et al., 2015; Mohan et al., 2015).

$$\text{Biomass (A)} \xrightarrow{k_1} \text{Saccharides (B)} \xrightarrow{k_2} \text{Degradation products (C)} \quad \textbf{(6.1)}$$

Where, k_1 and k_2 are the first order reaction rate constants of saccharides formation and degradation respectively. For the saccharides formation, reaction rate equation and its integral form is given in **Eq. (6.2)** and **Eq. (6.3),** respectively.

$$r_A = \frac{dC_A}{dt} = -k_1 C_A \quad \textbf{(6.2)}$$

$$C_A = C_{A0} \exp(-k_1 t) \quad \textbf{(6.3)}$$

Where C_{A0} is the initial concentration of biomass ($mg.g^{-1}$) and "t" is the time (min) under reaction conditions. Further, **Eq. (6.3)** can be simplified according to **Eq. (6.4)**. A linear fit can be plotted between $-\ln(1-X_A)$ and "t" which gives the slope as k_1 (min^{-1}).

$$\ln\left(\frac{C_A}{C_{A0}}\right) = \ln(1-X_A) = -k_1 t \quad \textbf{(6.4)}$$

Similarly, the reaction rate constant for the saccharides degradation reaction (k_2) can be obtained by simplifying the rate equation for second series reaction of **Eq. (6.1)**. The simplified rate equation for saccharides degradation can be demonstrated as **Eq. (6.5)**.

$$r_B = \frac{dC_B}{dt} = k_1 C_A - k_2 C_B \quad \textbf{(6.5)}$$

Where, C_B is the concentration of saccharides ($mg.g^{-1}$). Considering the boundary conditions i.e. $C_{B0} = 0$, $t = 0$ and combining **Eq. (6.3)** and **Eq. (6.5)**, we get **Eq. (6.6)**.

$$\frac{dC_B}{dt} = k_1 C_{A0} \exp(-k_1 t) - k_2 C_B \quad \textbf{(6.6)}$$

Now, **Eq. (6.6)** can be further simplified to **Eq. (6.7)** to obtain rate constant for saccharides degradation (k_2).

$$C_B = \frac{k_1 C_{A0}}{k_2 - k_1} (\exp(-k_1 t) - \exp(-k_2 t)) \quad \textbf{(6.7)}$$

The other kinetics parameters such as activation energy and pre-exponential factor can be obtained from Arrhenius equation, which is the common expression to determine the temperature dependence on the rate constants. The simplified form of Arrhenius equation can be expressed as **Eq. (6.8)**. The slope and intercept obtained from the plot between –ln(k) and the reciprocal of temperature (T) gives the activation energy and pre-exponential factor, respectively.

$$-\ln(k) = \frac{E_a}{RT} - \ln(A) \quad \textbf{(6.8)}$$

Where E_a is the activation energy ($kJ.mol^{-1}$), R is the universal gas constant ($J.mol^{-1}.K^{-1}$), T is the temperature of reaction (K), A is the pre-exponential factor (min^{-1}) and k is the rate constant (min^{-1}).

6.2.2.1 Determination of reaction kinetic parameters

The reaction rate constant for the saccharides formation (k_1) at different reaction temperature was obtained from the slope of the plot of $-\ln(1-X_A)$ and t, which gives a linear fit as shown in **Fig. 6.8** and **Fig. 6.9.** Slope of the line indicates the reaction rate constant of reducing sugars formation at corresponding temperature and reaction time of 30 min for citronella and bagasse, respectively. The differential equations resulting from the model were fit to experimental data to obtain kinetic rate constants. By using Arrhenius plot, the activation energy as well as the pre-exponential factor was determined.

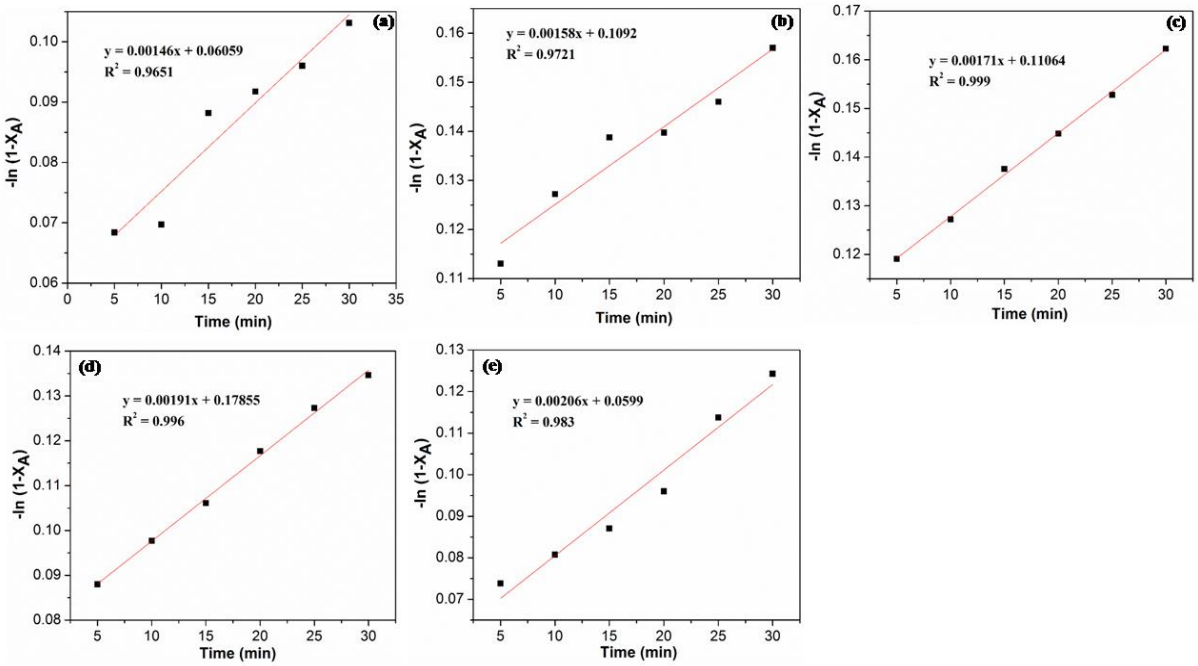


Fig. 6.8: Fitting curves of reaction rate equation at 30 min and different temperatures (a) 140 °C; (b) 160 °C; (c) 180 °C;(d) 200 °C; (e) 220 °C for Citronella.

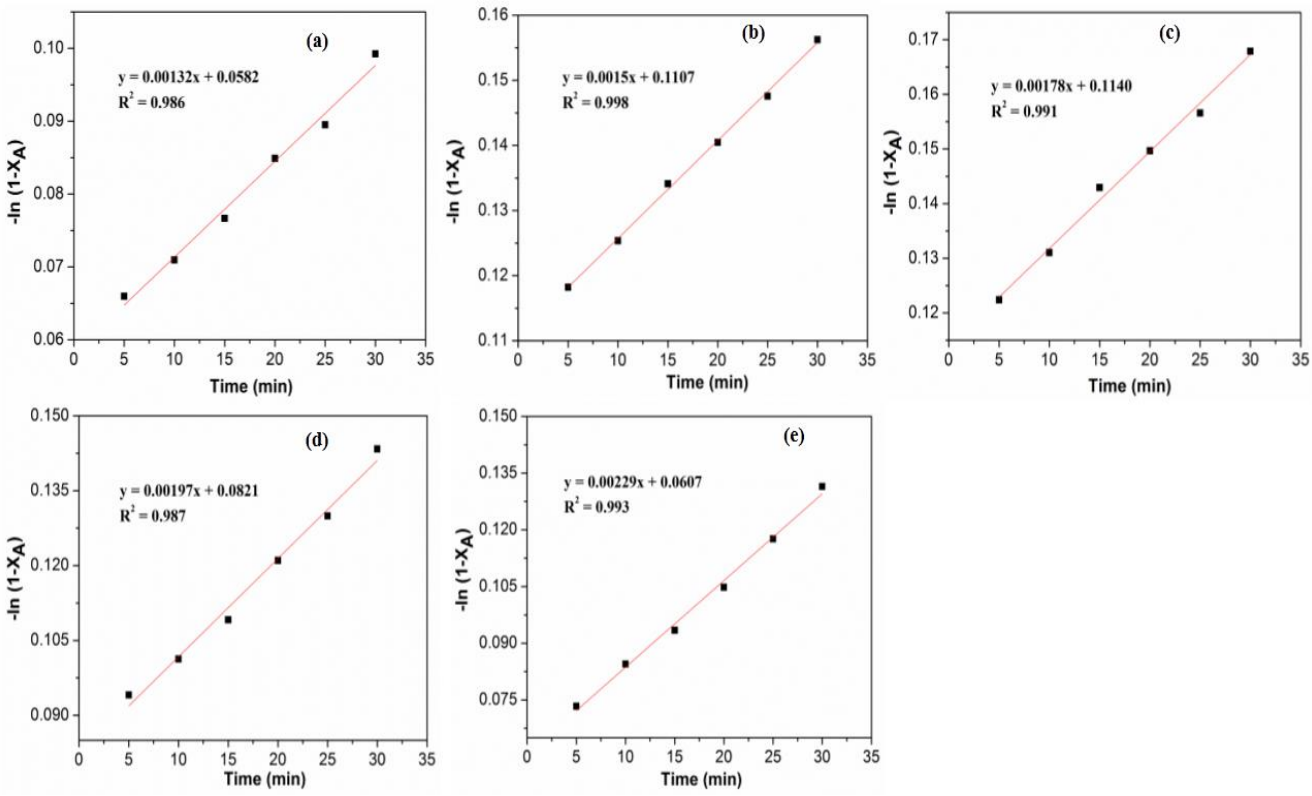


Fig. 6.9: Fitting curves of reaction rate equation at 30 min and different temperatures (a) 140 °C; (b) 160 °C; (c) 180 °C;(d) 200 °C; (e) 220 °C for bagasse.

On the other hand, **Fig. 6.10** and **Fig. 6.11** shows a linear fit between –ln (1-X_A) and "t", at 40 min and different temperatures for citronella and bagasse, respectively.

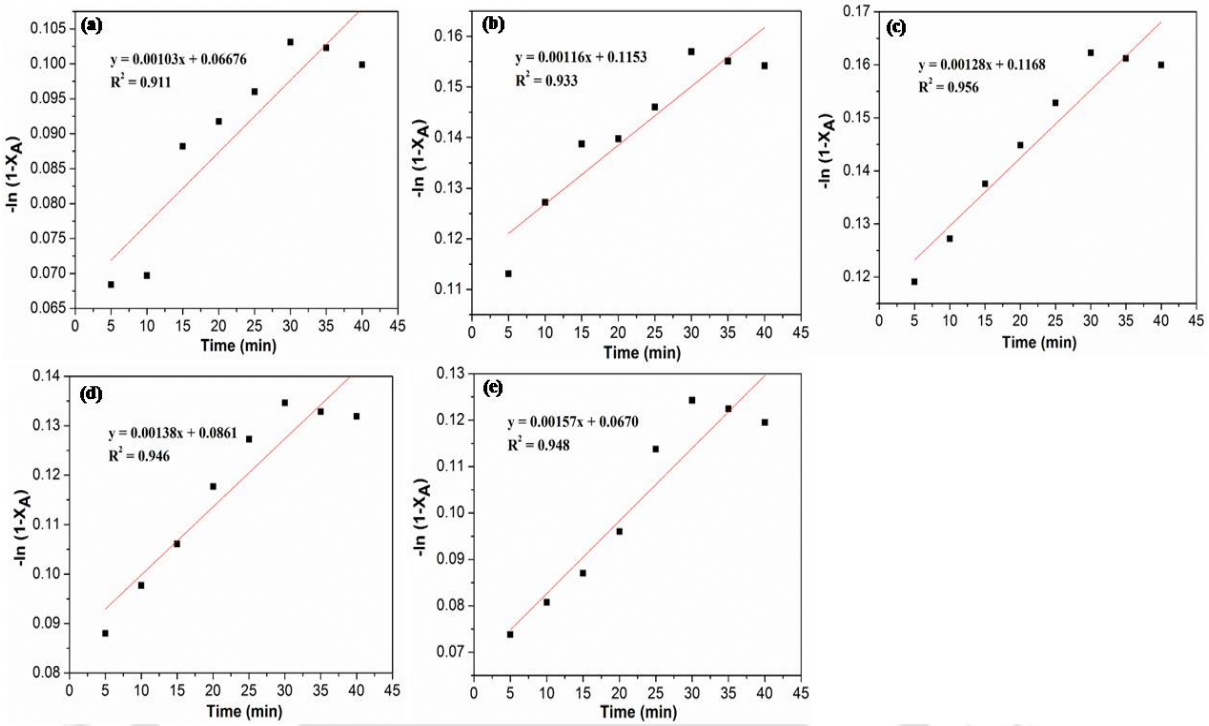


Fig. 6.10: Fitting curves of reaction rate equation at 40 min and different temperatures, (a) 140 °C; (b) 160 °C; (c) 180 °C;(d) 200 °C; (e) 220 °C for citronella.

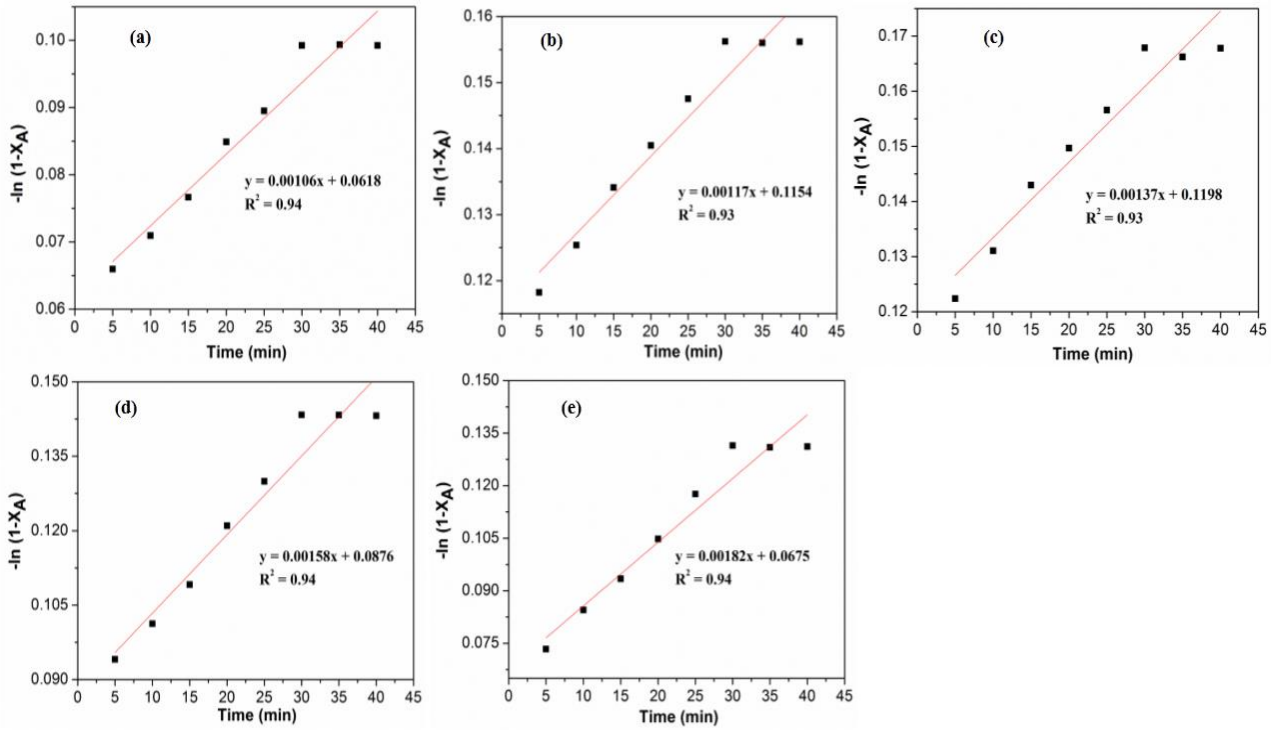


Fig. 6.11: Fitting curves of reaction rate equation at 40 min and different temperatures, (a) 140 °C; (b) 160 °C; (c) 180 °C;(d) 200 °C; (e) 220 °C for bagasse.

In this study, fitting of the experimental data to the kinetic equation was performed in two different ways. In the first case, fitting was carried out considering maximum reducing sugar production with minimal degraded products at an optimum reaction time (i.e., 30 min, 180 °C). While, in the second case, entire reaction time (40 min) (i.e., sugar formation and decomposition) was considered for the model fitting. From the study, it was observed that the fitting for saccharides formation at 30 min and 40 min follows the same pattern. The increase in the rate constant values for both the case revealed that the reaction temperature has a positive influence on the rate constant. A good agreement between the simplified model and the experimental data was obtained. Rogalinski et al. (2008) and Mohan et al. (2015) also obtained same curve fitting during their study on SCW hydrolysis of biopolymers and bamboo, respectively. In the present work, reaction rate constant shows some interesting trend with reaction temperature. It was noticed that the rate constant values of TRS formation and degradation increases with an increase in temperature. In case of citronella, with rise of temperature from 140 °C to 220 °C at 30 min, the rate constant k_1 increases from 0.00146 to 0.00206 min^{-1} whereas k_2 increases from 0.06215 to 0.07482 min^{-1}. Similarly, the curve fitting upto total reaction time of 40 min showed same trend as that of 30 min i.e., with increase in the temperature rate constant value increases. Interestingly, similar trend was noticed for bagasse at 30 and 40 min reaction time (**Table 6.2)**. The increase in rate constants might be due to increase in the concentration of H^+ in SCW which could break the glycosidic bonds for the hydrolysis of holocellulose (Lin et al., 2015). The values of the rate constant obtained for the saccharides formation and degradation at optimum reaction time (30 min) and entire reaction time (40 min) has been represented in **Table 6.2** and **Table 6.3** for citronella and bagasse, respectively.

Table 6.2: Rate constants of sugars fromation and degradation as function of temperature at 30 min and 40 min for citronella.

Temp. (°C)	Rate Constants (min^{-1})			
	30 min		40 min	
	k_1	k_2	k_1	k_2
140	0.00146	0.06215	0.00103	0.05487
160	0.00158	0.06330	0.00116	0.05567
180	0.00171	0.06578	0.00128	0.05813
200	0.00191	0.07107	0.00138	0.06131
220	0.00206	0.07482	0.00157	0.06583

Table 6.3: Rate constants of sugars fromation and degradation as function of temperature at 30 min and 40 min for bagasse.

Temp. (°C)	Rate Constants (min^{-1})			
	30 min		40 min	
	k_1	k_2	k_1	k_2
140	0.00130	0.06239	0.00106	0.05675
160	0.00150	0.06327	0.00117	0.05695
180	0.00180	0.06808	0.00137	0.06063
200	0.00200	0.07345	0.00158	0.06590
220	0.00230	0.08138	0.00182	0.07261

The Arrhenius plot for the saccharides formation and degradation with respective kinetic parameters for both the biomass are represented in **Fig. 6.12** and **Fig. 6.13**, respectively. In case of citronella, the activation (E_a) energy and pre-exponential factor (A) for maximum saccharides formation at 30 min of reaction time were 7.42 kJ. mol^{-1} and 0.013 min^{-1}, respectively; and for entire reaction time (i.e. 40 min) were 8.60 kJ. mol^{-1} and 0.0013 min^{-1}, respectively. While, E_a and A for saccharides degradation at (30 min) were 4.08 kJ. mol^{-1} and 0.196 min^{-1} respectively; and for entire reaction time (i.e. 40 min) were 3.85 kJ. mol^{-1} and 0.162 min^{-1}, respectively. On the other hand, for bagasse, the E_a and A for maximum saccharides formation at reaction time of (30 min) were 12.11 kJ. mol^{-1} and 0.045 min^{-1} respectively; and for 40 min reaction time were 11.65 kJ. mol^{-1} and 0.031 min^{-1}, respectively. Similarly, E_a and A for the saccharides degradation at 30 min were 5.68 kJ. mol^{-1} and 0.308 min^{-1}, respectively; and for entire reaction time (40 min) were 5.32 kJ. mol^{-1} and 0.249 min^{-1}, respectively. The lower activation energy of saccharides degradation compared to saccharides formation reveals that the rate of saccharides decomposition was faster than the formation of sugars.

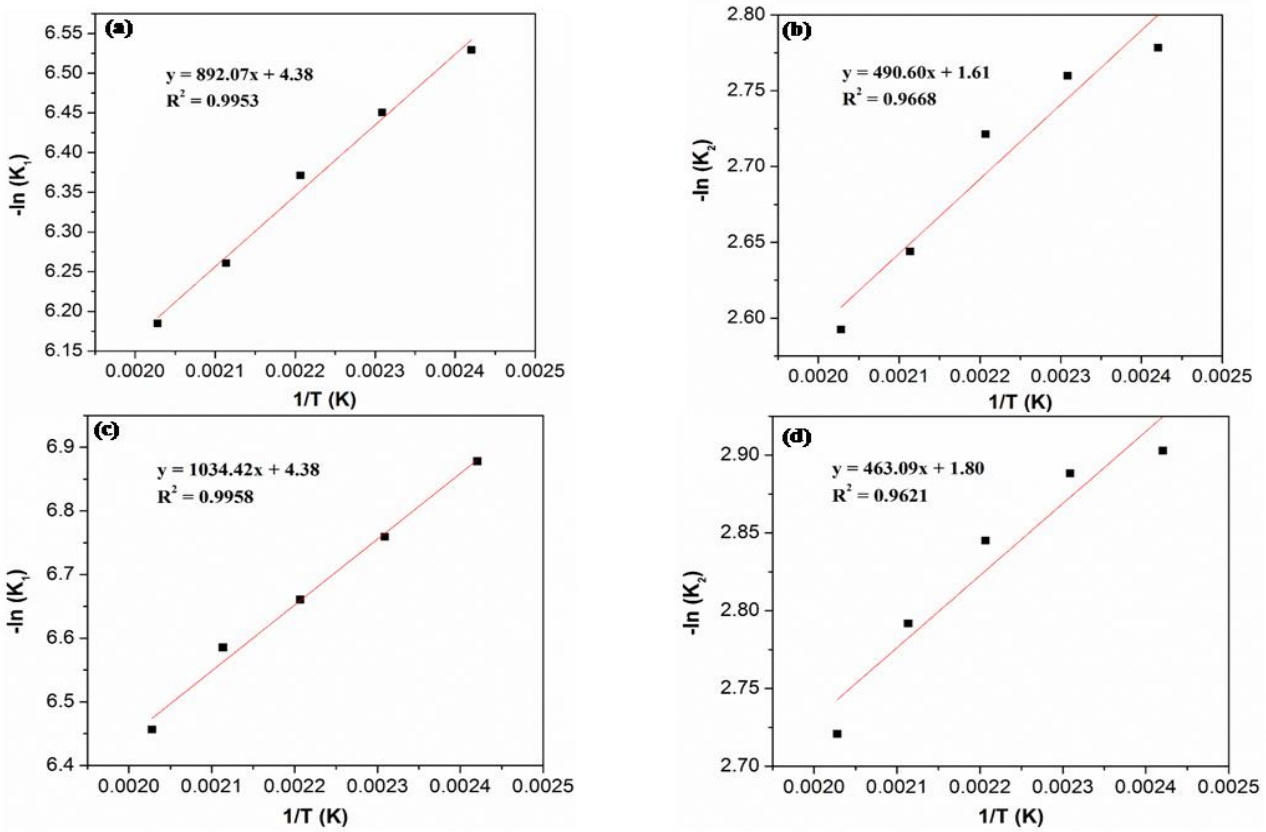


Fig. 6.12: Arrhenius plot for the production (k_1) and decomposition (k_2) of TRS at 30 min ((a) and (b)); and 40 min ((c) and d)) respectively for citronella.

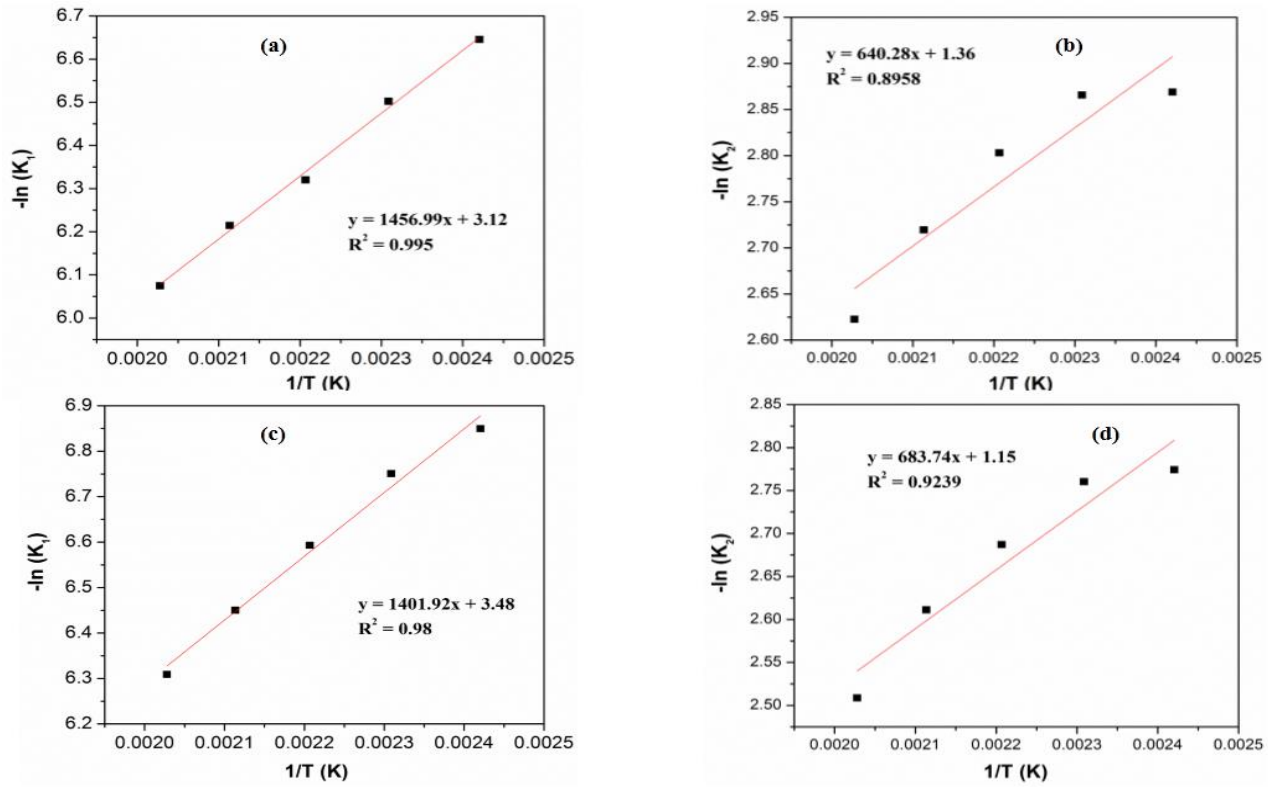


Fig. 6.13: Arrhenius plot for the production (k_1) and decomposition (k_2) of total reducing sugars at 30 min ((a) and (b)); and 40 min ((c) and d)) respectively for bagasse.

Further, in this study, we have tried to establish the proper optimisation of the SCW hydrolysis process which helps in maximising the reducing sugar production with minimal degraded products. Optimisation of SCW hydrolysis process was carried out using CCD technique. This essentially helps in understanding the significance of the individual effect of the optimisation variable and the magnitude of interaction between them.

6.2.3 Central Composite Design (CCD)

The CCD experimental design in the present study comprised of three significant parameters identified based on our preliminary experiments. The following experimental parameters (or independent variables) have been chosen for optimisation: temperature (A), reaction time (B), biomass loading (C). The experiments were conducted in batch reactor, based on our initial experiments. The exact experimental design formulated using design expert software, indicating range and levels of the independent variables for both citronella and bagasse, respectively are shown in **Table 6.4** and **Table 6.5**.

Table 6.4: Factors and coded levels of CCD for SCW hydrolysis of citronella.

Factor		Low	Medium	High
A	Temperature (°C)	140	160	180
B	Reaction Time (min)	20	30	40
C	Biomass Loading (B.L.) (wt %)	3	4	5

Table 6.5: Factors and coded levels of CCD for for SCW hydrolysis of bagasse.

Factor		Low	Medium	High
A	Temperature (°C)	160	180	200
B	Reaction Time (min)	20	30	40
C	Biomass Loading (B.L.) (wt %)	3	4	5

The design matrix and their corresponding experimental and predicted values are shown in **Table 6.6** and **Table 6.7** for citronella and bagasse, respectively.

Table 6.6: CCD matrix of the factors and its corrresponding actual and predicted responses for SCW hydrolysis of citronella.

Std. Order	Run Order	Temp. (°C)	B.L. (wt%)	Time (min)	Conc. of reducing sugars ($mg.g^{-1}$)	
					Actual	Predicted
1	13	140	3	20	188.35	188.21
2	7	180	3	20	205.54	205.63
3	17	140	3	40	171.29	171.24
4	6	180	3	40	196.49	196.51
5	5	140	5	20	255.15	255.11
6	4	180	5	20	267.86	267.89
7	11	140	5	40	221.51	221.40
8	10	180	5	40	241.92	242.03
9	16	126.36	4	30	189.55	189.74
10	20	193.64	4	30	221.89	221.73
11	2	160	4	13.18	240.78	240.81
12	15	160	4	46.82	204.79	204.79
13	3	160	2.32	30	185.07	185.11
14	12	160	5.68	30	279.64	279.64
15	19	160	4	30	249.64	249.98
16	1	160	4	30	249.37	249.98
17	9	160	4	30	250.64	249.98
18	18	160	4	30	250.21	249.98
19	8	160	4	30	249.79	249.98
20	14	160	4	30	250.21	249.98

Table 6.7: CCD matrix of the factors and its corrresponding actual and predicted responses for SCW hydrolysis of bagasse.

Std. Order	Run Order	Temp. (°C)	B.L. (wt%)	Time (min)	Conc. of reducing sugars ($mg.g^{-1}$)	
					Actual	Predicted
1	17	160	3	20	238.16	232.83
2	9	200	3	20	189.37	186.08
3	20	160	5	20	269.14	270.63
4	3	200	5	20	238.51	238.48
5	1	160	3	40	261.63	257.99
6	6	200	3	40	223.65	218.49
7	13	160	5	40	266.16	265.77
8	12	200	5	40	239.21	240.87
9	2	146.36	4	30	241.49	244.40
10	18	213.64	4	30	181.87	184.15
11	11	180	2.32	30	255.54	264.13
12	14	180	5.68	30	318.12	314.73
13	7	180	4	13.18	211.67	214.16
14	5	180	4	46.82	234.61	237.32
15	10	180	4	30	314.31	313.33
16	8	180	4	30	309.45	313.33
17	4	180	4	30	310.45	313.33
18	19	180	4	30	316.45	313.33
19	16	180	4	30	312.75	313.33
20	15	180	4	30	317.45	313.33

6.2.3.1 Analysis of variance (ANOVA) of quadratic model and Model Validation

The parameters affecting the release of sugars from biomass can be evaluated from *F*-test of ANOVA as represented in **Table 6.8** and **Table 6.9** for citronella and bagasse, respectively. The significance of each coefficient was determined by *F*-values and *p*-values. The large *F*-value and corresponding low *p*-value depicts that the variable has a significant effect in the process. If the value of probability (*p*) function is greater than 0.05, which indicates that process or model does not have a significant effect on the response variable (Montgomery, 2005; Singh and Sharma, 2012; Joglekar and May, 1987; Guan and Yao, 2008).

Table 6.8: Analysis of variances for the quadratic model for SCW hydrolysis of citronella.

Source	Sum of Squares	DF	Mean Square	*F*-value	*P*-Value
Model	18467.78	9	2051.98	17330.93	< 0.0001
A-Temperature (°C)	1235.56	1	1235.56	10435.47	< 0.0001
B-Time (min)	1565.49	1	1565.49	13222.06	< 0.0001
C- B.L. (wt%)	10786.94	1	10786.94	91106.20	< 0.0001
AB	30.85	1	30.85	260.56	< 0.0001
AC	10.74	1	10.74	90.72	< 0.0001
BC	140.03	1	140.03	1182.69	< 0.0001
A^2	3525.67	1	3525.67	29777.70	< 0.0001
B^2	1330.30	1	1330.30	11235.66	< 0.0001
C^2	558.32	1	558.32	4715.53	< 0.0001

Table 6.9: Analysis of variances for the quadratic model for SCW hydrolysis of bagasse.

Source	Sum of Squares	DF	Mean Square	*F*-value	*P*-Value
Model	37467.01	9	4163.01	167.82	< 0.0001
A-Temperature (°C)	4381.55	1	4381.55	176.63	< 0.0001
B-Time (min)	3090.93	1	3090.93	124.60	< 0.0001
C- B.L.(wt%)	647.69	1	647.69	26.11	0.0005
AB	106.51	1	106.51	4.29	0.0651
AC	26.25	1	26.25	1.06	0.3279
BC	450.45	1	450.45	18.16	0.0017
A^2	17673.33	1	17673.33	712.45	< 0.0001
B^2	1028.95	1	1028.95	41.48	< 0.0001
C^2	13820.31	1	13820.31	557.13	< 0.0001

From **Table 6.8** and **Table 6.9**, it can be seen that the developed model is highly significant with *p*-value less than 0.0001 which indicates that these variables have a significant effect on the reducing sugar yield in the range of these parameters considered in the experiments. Further, the second-order factor such as temperature (A^2), time (B^2), and biomass loading (C^2); as well as the interaction parameters AB and BC found to be highly significant to the response. The quadratic factors such as A^2, B^2 and C^2 with *p*-value < 0.0001 reveals that the release of sugar increases with increasing temperature, time and biomass loading independently. The mutual interactions between the factors towards the response can be observed from the curvature of 3D response plot as shown in **Fig. 6.14** and **Fig. 6.15** for citronella and bagasse, respectively.

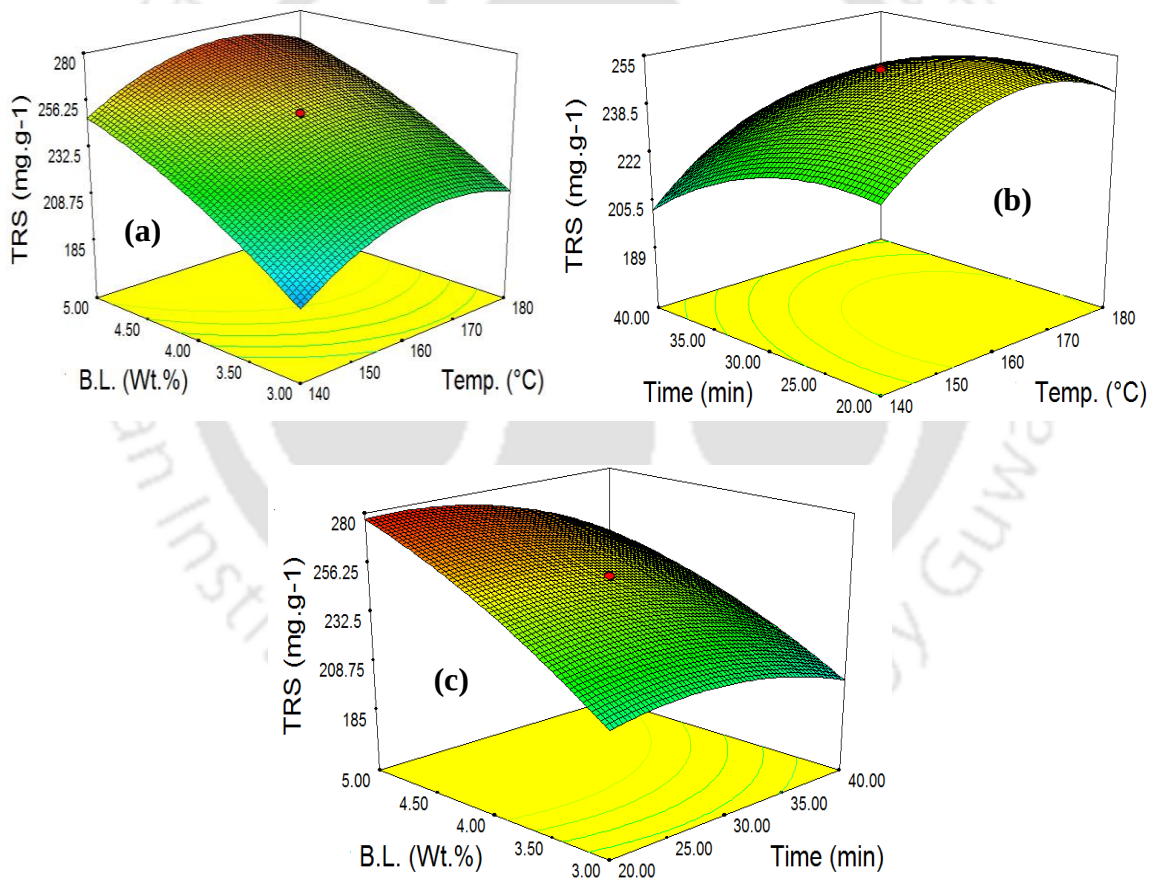


Fig. 6.14: 3D response surface plots representing the interaction effects between the process parameters: (a) temperature and biomass loading; (b) temperature and time; (c) biomass loading and time, for citronella.

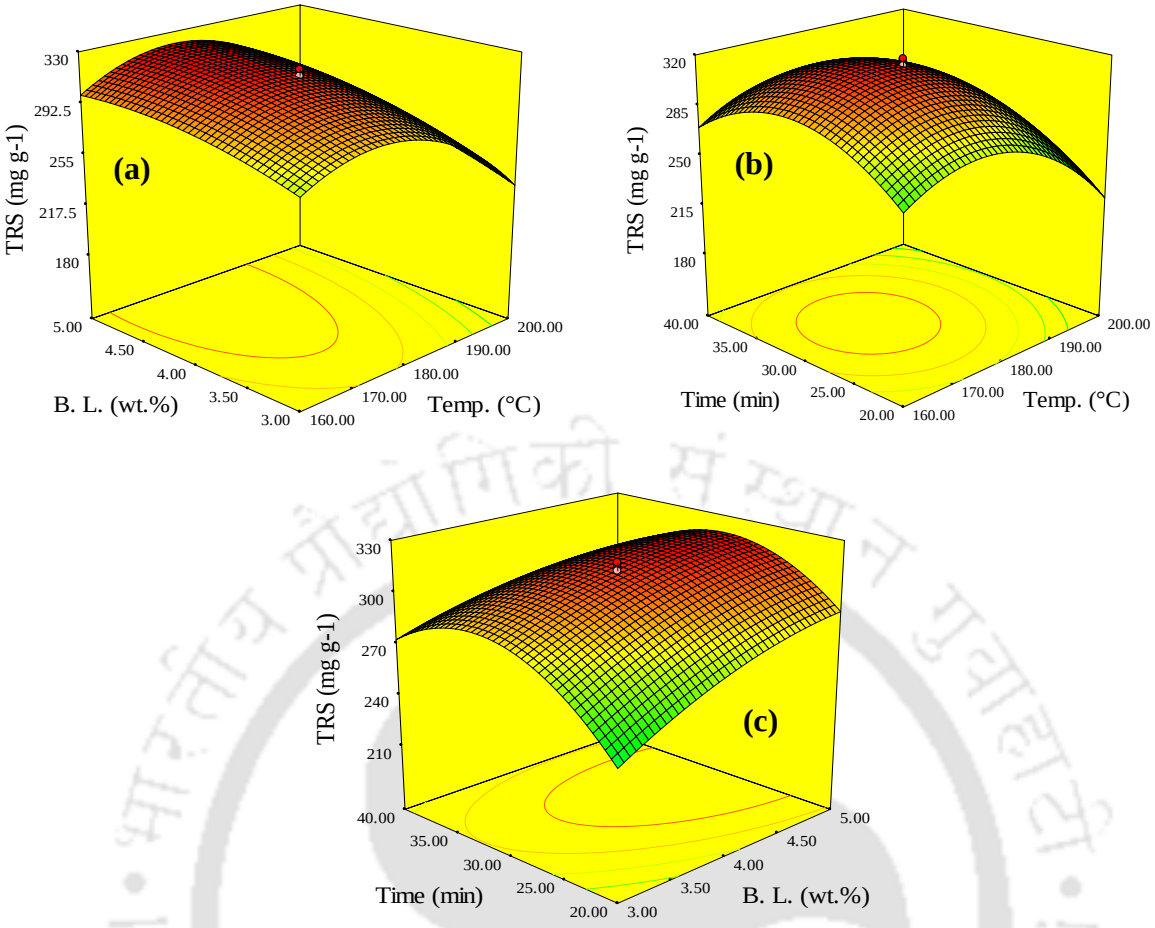


Fig. 6.15: 3D response surface plots representing the interaction effects between the process parameters: (a) temperature and biomass loading; (b) temperature and time; (c) biomass loading and time, for bagasse.

These 3D response surface plots are very helpful in understanding the interaction effects of two variables at a time, keeping the other factor at a fixed level (at zero level). From **Fig. 6.14(b)** and **Fig.6.15(b)**, changing the levels of temperature and time (other than zero level) reveals a decreasing trend of reducing sugars concentration. **Fig. 6.14(a)** and **Fig. 6.14(c);** and **Fig. 6.15(a)** and **Fig. 6.15(c)** shows the interactive effect of biomass loading with temperature and biomass loading with time on the reducing sugars. As can be seen from the figures, varying the biomass loading (any level) at a fixed zero level of the temperature and time enhances the reducing sugars concentration. While varying the levels of affecting variables such as temperature and time (high or low) at a fixed zero level of the biomass loading, leads to a decrease in the sugars concentration. This may be due to the formation of degraded products at a higher level. The model equation developed for the extraction of sugar from citronella and bagasse, respectively using SCW technique in terms of coded

values are given in **Eq. (6.9)** and **Eq. (6.10)**.

$$Y_1 = 249.98 + 9.51A - 10.71B + 28.10C + 1.96AB - 1.16AC - 4.18BC - 15.64A^2 - 9.61B^2 - 6.22C^2 \quad (6.9)$$

$$Y_2 = 313.33 - 17.91A + 15.04B + 6.89C + 3.65AB + 1.81AC - 7.50BC - 35.02A^2 - 8.45B^2 - 30.97C^2 \quad (6.10)$$

Where, Y_1 and Y_2 are the predicted response (TRS) for citronella biomass and bagasse, respectively.

The optimum condition for the maximum production of reducing sugars for the process variables is generated as: T = 164.38 °C, t = 22.46 min and B.L. = 5.00 wt% for citronella; and T = 170.3 °C, t = 30.7 min, and B.L. = 4.18 wt% for bagasse. At the optimal condition experiments were performed in triplicate and average values are reported. The experimental reducing sugar concentration (279.64 mg.g^{-1} and 320.50 mg.g^{-1}) was close to the predicted value (278.38 mg.g^{-1} and 319.05 mg.g^{-1}) for citronella and bagasse, respectively, which indicates that model fits very well to the data. The significance of the model is checked by the determination of the regression coefficient (R^2) value. For a good model fit, R^2 value should be in the range of 0.80 (Joglekar and May, 1987; Guan and Yao, 2008). The statistical parameters of the developed model such as: R^2 = 0.99 and large *F*-value of 17330.93 with low probability (p<0.0001) for citronella shows that the applied model was highly significant, the same has been observed for bagasse, with R^2 = 0.99 and high *F*-value of 212.18 with low probability (p<0.0001). The comparison of the experimental and predicted value of the response (i.e., total reducing sugars) as presented in **Fig. 6.16** and **Fig. 6.17** demonstrates a good fit model (R^2 = 0.99).

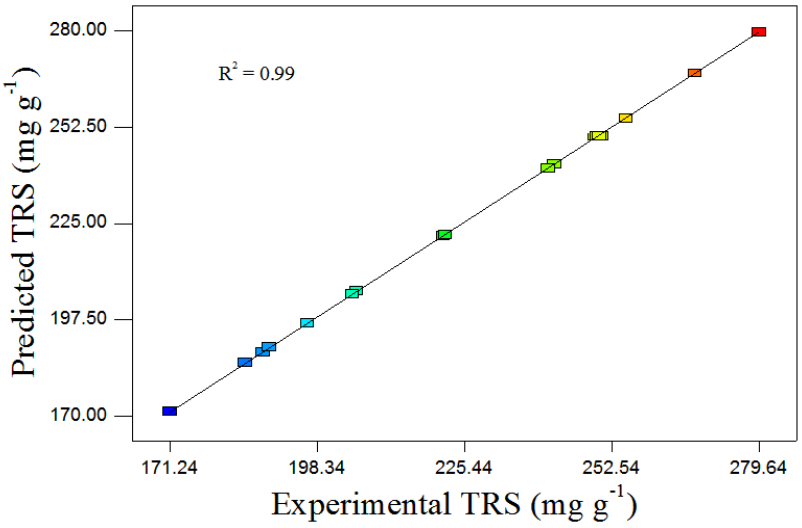


Fig. 6.16: Comparison plot between predicted and actual values of TRS concentration for citronella.

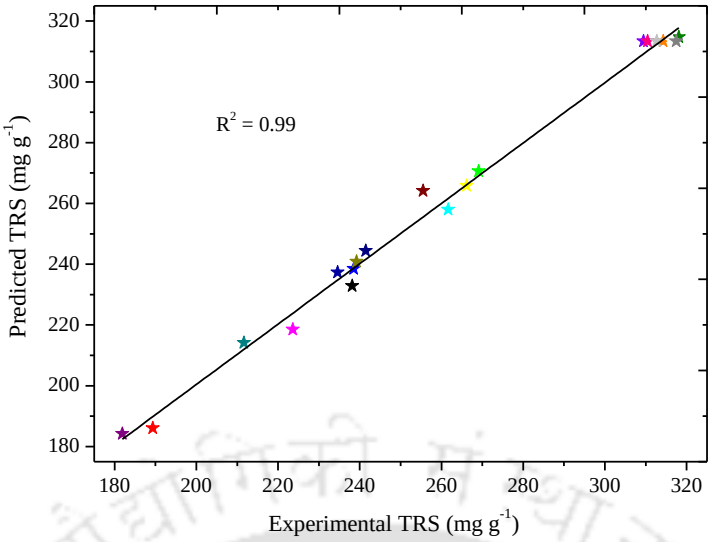


Fig. 6.17: Comparison plot between predicted and actual values of TRS concentration for bagasse.

6.2.4 Characterisation of residual biomass

6.2.4.1 X-ray diffraction (XRD) analysis

The estimation of crystallinity of biomass helps to determine the degradation of biomass (mostly cellulose). The crystalline structure of biomass is mainly because of the strong inter- and intra-molecular hydrogen bonding of hydroxyl groups of cellulose chains and Van der Waals forces of glucose molecules in the hemicellulose and cellulose (Naik et al., 2010). The degradation of hemicellulose and amorphous cellulose occurs at low temperature followed by higher degradation of crystalline cellulose at higher temperature (Quintana et al., 2015). The crystallinity index of the spent citronella biomass was 34.91 %. After SCW treatment, the crystallinity index of residual biomass increased from 35.31 % to 52.68 % with increase in the temperature from 140 °C to 180 °C and crystallinity index value was slightly decreased from 29.57 % to 20.15 % with further increase in temperature from 200 °C to 220 °C. Increase in the crystallinity index of treated sample indicates that the hydrolysis effect on amorphous zone is more compared to crystalline zone. The decrease in crystallinity index at 220 °C implies that the treated sample is associated with cellulose decrystallisation, which might be due to phase change that took place between 210 °C and 220 °C. On the other hand, the crystallinity index of untreated bagasse was 37.41 %. After treatment, the crystallinity index of residual biomass increased from 54.03 % to 63.32 % with increase in the temperature from 140 °C to 160 °C and found to decrease from 59.96 % to 34.77 % with further increase in temperature from 180 °C to 220 °C. These result implies that the treated

biomass sample altered its structure and relatively become amorphous in nature at higher temperatures i.e., 220 °C (**Fig. 6.18** and **Fig. 6.19**).

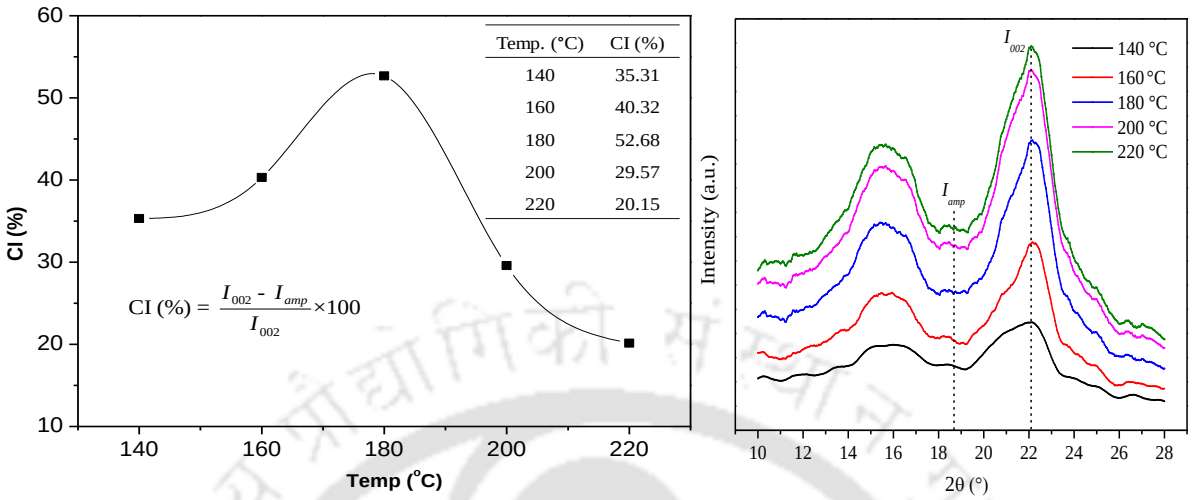


Fig. 6.18: XRD analysis of citronella after SCW treatment at different temperature.

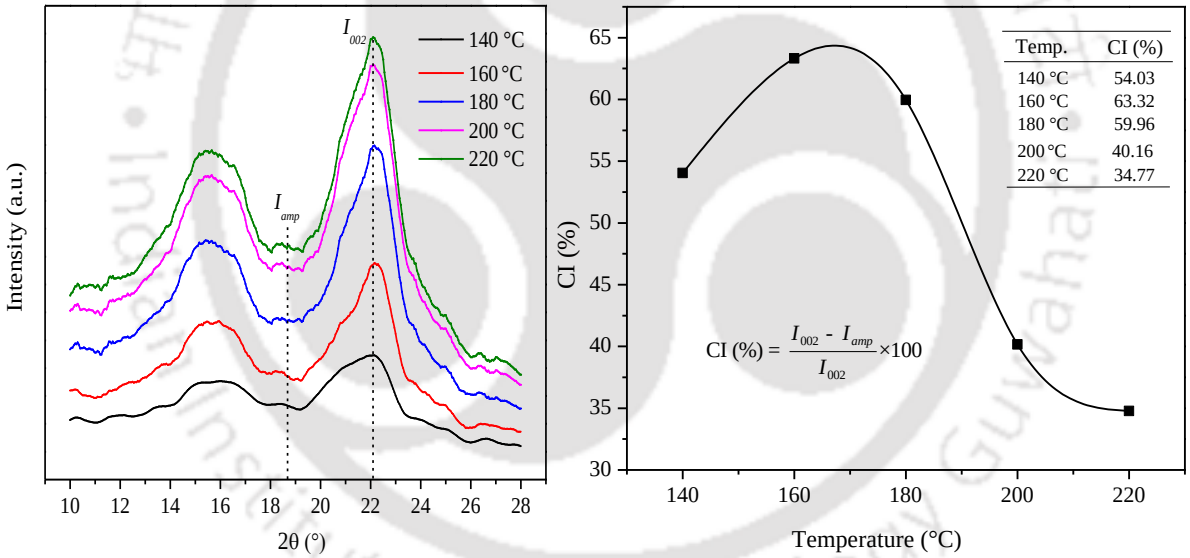


Fig. 6.19: XRD analysis of bagasse after SCW treatment at different temperature.

6.2.4.2 Fourier transforms infrared spectroscopy (FTIR) analysis

FTIR analysis of subcritical treated residual biomass at different temperature was performed to investigate the chemical changes occurred in the biomass structure before and after pretreatment and to understand the solubilisation process (**Fig. 6.20**).

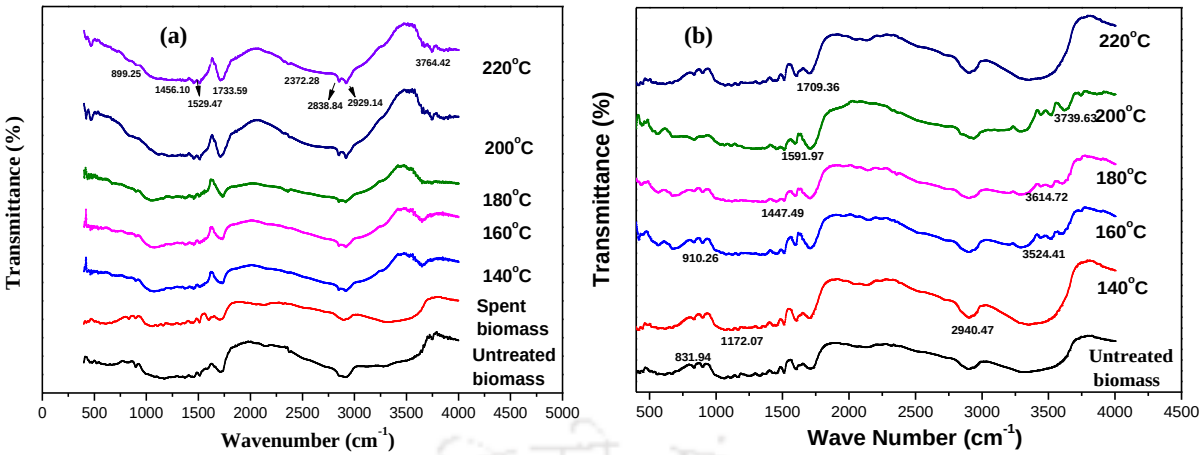


Fig. 6.20: FTIR spectra's of (a) citronella and (b) sugarcane bagasse before and after SCW treatment at different temperature.

An intense strong and sharp peak observed in all the biomass samples at around 3800-3200 cm^{-1} particularly at 3764.42 mostly correspond to polymeric free hydroxyl (O-H) stretch. Another intense and diverged peak at around 2935-2915 cm^{-1} represents aliphatic alkyl group C-H methyl and methylene asymmetric stretch. Appearance of peaks at around 2830 cm^{-1} represents terminal aldehydic C-H stretch. A characteristic medium peak was noticed at around 2372.28 cm^{-1} which may corresponds to certain cyanocarbons. Another distinct and sharp peak observed in the range of 1750-1705 cm^{-1} in all the samples signifies un-conjugated xylan as well as aldo, keto, estero and or acido (C=O) stretch. A medium peak at 1529.47 cm^{-1} corresponds to aromatic C=C ring and lignin ring stretch. Methylene C-H stretch (1485-1445 cm^{-1}) and methyl C-H symmetric bend in cellulose and hemicellulose (1380-1371 cm^{-1}) appeared in all the samples. Moreover, CH=CH trans-unsaturated functional group with sharp peaks also observed at 910-860 cm^{-1}. Boeriu et al. (2004) has reported most of the above stretches for lignin, which indicates that lignin is uniformly dispersed in the residual biomass. The peak at ~1450 and ~894 cm^{-1} represents the crystalline and amorphous fractions of cellulose, respectively (Sasmal et al., 2012). The changes in these band intensities indicate that the structure of biomass samples was delignified and solubilised after SCW treatment.

6.2.4.3 SEM analysis

SEM analysis of citronella plant sample showed significant morphological changes after subsequent treatment. The cells of the untreated plant material are intact and a smooth surface can be observed as shown in **Fig. 6.21(a)**.

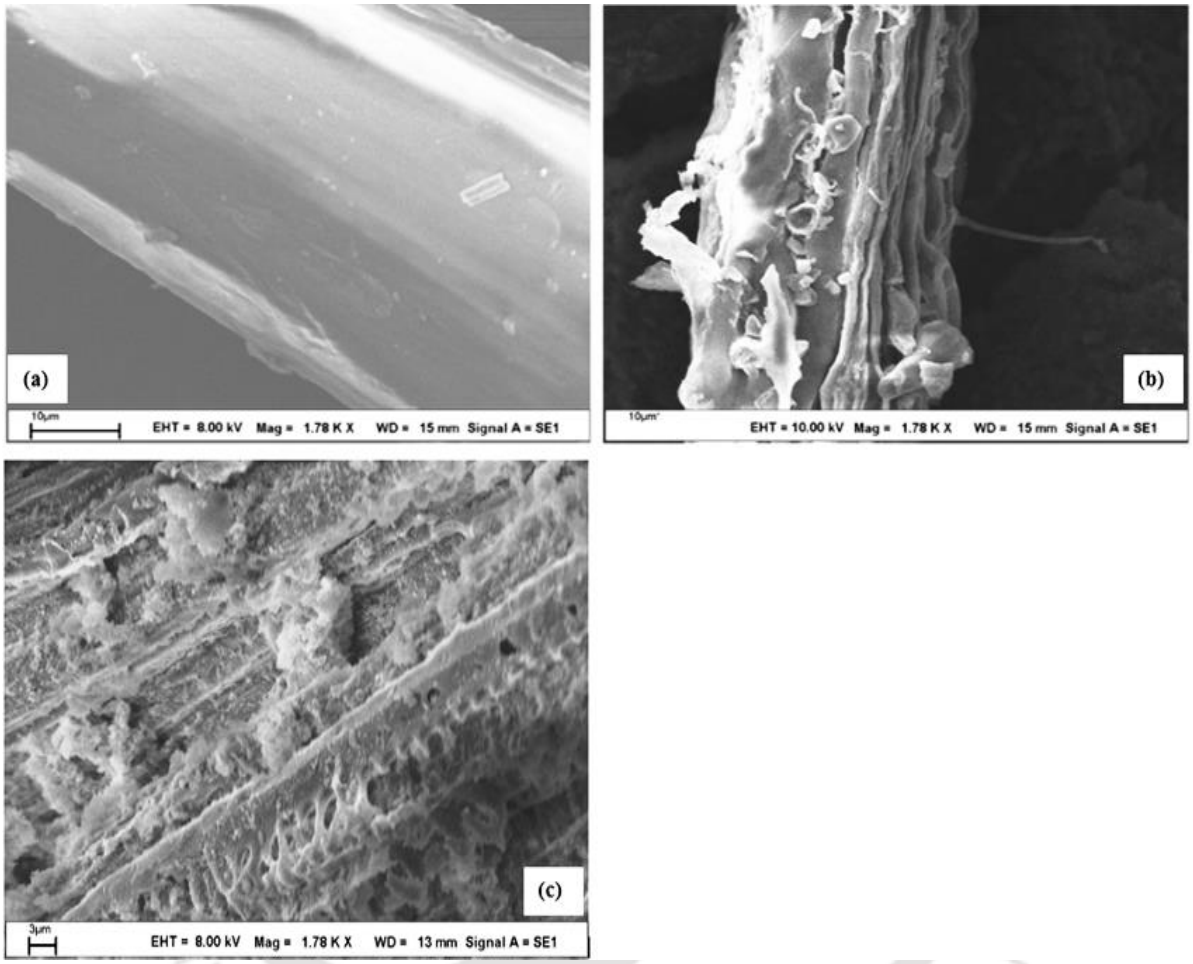


Fig. 6.21: SEM images of Citronella biomass; (a) untreated (b) spent biomass and (c) after SCW treatment.

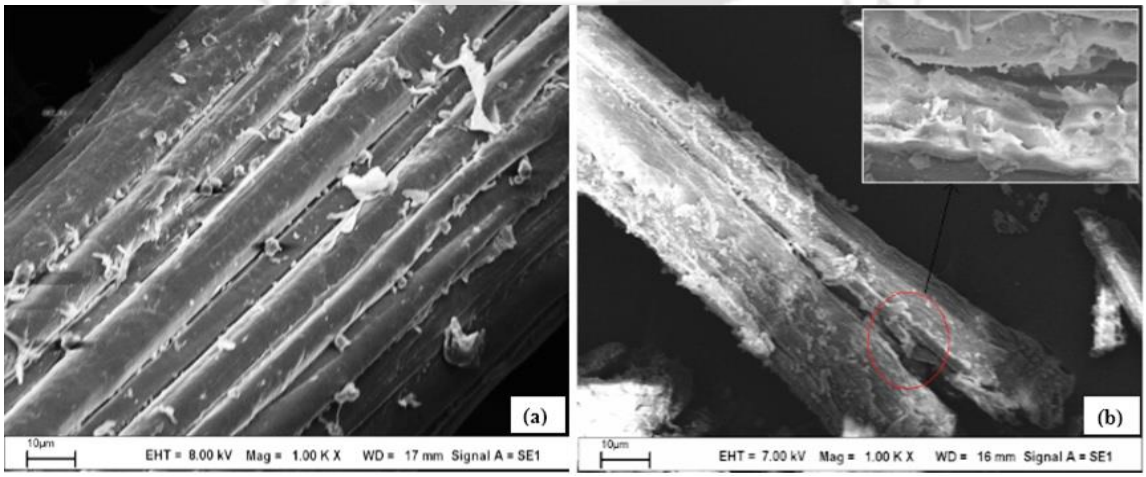


Fig. 6.22: SEM images of Bagasse ; (a) untreated biomass (b) after SCW treatment.

When the plant material is subjected to distillation the cellular content of the plant tissues gets distorted due to extensive thermal stress on the oil glands causing rapid expansion extending to disruption and releasing the oil content as represented in **Fig. 6.21(b)**. The structural changes in SCW treated sample with exposed pores revealed significant de-polymerisation of holocellulose content and removal of substantial amount of lignin (**Fig. 6.21(c))** (Giese et al., 2013). Similar, pattern of disruption was observed with bagasse as depicted in **Fig. 6.22 (a)** and **Fig. 6.22(b)**.

6.3 Comparison of TRS yield of the present work with literature

The results of current study has also been compared with available literature on different lignocellulosic biomass (**Table 6.1**). Lin et al. (2015) studied the hydrolysis of rice straw using batch-type reactor. They reported maximum TRS concentration of 346 mg g^{-1} at 280 °C, 20 MPa, and 8 min. Comparison of the results of present study with that of above reported value reveals TRS yield of 281.66 mg g^{-1} and 325.09 $mg.g^{-1}$ from citronella and bagasse are observed at relatively lower operating conditions. The difference in TRS yield and operating conditions may be due to difference in the composition and crystallinity of biomass. Recently, Liang et al. (2016) have reported the hydrolysis of sugarcane bagasse pith under subcritical CO_2-water in a batch reactor. The highest TRS yield of 43.6 % and 40.2 % was reported at 200 °C, 40:1 liquid/solid (L/S) ratio, and 40-50 min for 2 MPa and 1 MPa of CO_2, respectively. Whereas in the present study, 22.3 % of TRS yield was obtained at 200 °C, 30:1 L/S, and 40 min hydrolysis time. The difference in the TRS yield is much higher; which could be due to the addition of CO_2 and higher L/S ratio. It was reported that addition of CO_2 leads to acid-hydrolysed catalysis caused by the formation of carbonic acid. The strength of acidity increases with increase of CO_2 partial pressure which results in increased total reducing sugar yield. The present study also compared with the TRS produced using semi-continuous flow reactor. Considering the total holocellulose content of the biomass (60.37 wt% for citronella and 62.93 wt% for bagasse), TRS yield (ratio of final concentration of total reducing sugars to the initial concentration of holocellulose content in the biomass) of 46.57 % from citronella and 51.66 % from bagasse was obtained at an optimum condition (160 °C, 5 wt% biomass loading, and 30 min for citronella;180 °C, 5 wt% biomass loading, and 30 min for bagasse). Prado et al. (2014) reported 32 % of TRS yield with respect to holocellulose content of biomass during their study on hydrolysis of sugarcane bagasse using semi-batch reactor system. The TRS yield from semi-batch type reactor was leser than the

yield obtained in the present work as well as reported vlaues in literature. Therefore, from this comparison data, it was clear that the use of batch reactor could result higher TRS yield at lower operating conditions which is a safer and economically favorable operation.

6.4 Comparison of SCW hydrolysis with pretreatment methods.

Considering TRS yield as the main objective, SCW hydrolysis was compared with acid and alkali pretreatment followed by enzymatic hydrolysis, respectively. However, HPLC technique was used to quantify reducing sugars produced using SCW hydrolysis process whereas DNS assay was employed in the quantification of reducing sugar produced by chemical pretreatments (i.e. acid and alkali) and enzymatic hydrolysis. The total reducing sugars produced during SCW hydrolysis of citronella and bagasse was estimated to be 281.66 ± 0.18 $mg.g^{-1}$ and 325.09 ± 0.27 $mg.g^{-1}$, respectively. While, in the alkali pretreated enzymatic hydolysis of citronella and bagasse results in the TRS of 419.37 $mg.g^{-1}$ (73.80 $mg.g^{-1}$ in alkali pretreatment and 345.49 $mg.g^{-1}$ in subsequent enzymatic hydrolysis) and 441.92 $mg.g^{-1}$ (45.20 $mg.g^{-1}$in alkali treatment and 396.70 $mg.g^{-1}$ in subsequent enzymatic hydrolysis), respectively. On the other hand, the yield of reducing sugars obtained with only acid pretreatment of citronella and bagasse was 487.50 $mg.g^{-1}$ and 452.27 $mg.g^{-1}$, respectively. The subsequent enzymatic hydrolysis of the acid pretreated substrate released reducing sugar of 226.99 $mg.g^{-1}$ from citronella and 282.85 $mg.g^{-1}$ from bagasse (**Table 6.9**). It could be seen from data presented in **Table 6.9** that the total reducing sugars yield is higher in acid treated enzymatic hydrolysis of both the biomass i.e. 714.49 $mg.g^{-1}$ for citronella and 735.12 $mg.g^{-1}$ for bagasse. The TRS estimated in acid treated enzymatic hydrolysate was much higher than the actual sugars content of both the biomass (603.7 $mg.g^{-1}$ in citronella and 629.3 $mg.g^{-1}$ in bagasse). This possibly attributes to the reaction of furan (i.e. HMF and furfural) present in the substrate with DNS which eventually results in the generation of more colour intensity and hence estimating higher concentration of reducing sugars. Moreover, the presence of phenolic impurities during the preparation of DNS reagent might also contribute to increase the colour intensity (Teixeira et al., 2012; Wood et al., 2012; Saqib et al., 2011; Kamireddy et al., 2013). The above findings indicates that, it is not appropriate to use DNS assay in the estimation of reducing sugars. On the other hand, chemical pretreaments uses highly corrosive and dangerous chemicals which pose threat to the environment, and even their recovery also requires another operation. The chemical treatment process mostly targets only the hemicellulose fractions and crystalline cellulosic content of the biomass remains

unhydrolysed, which requires successive treatment with specific enzyme to degrade the crystalline structure, which is a time taking process leading to higher cost. In the enzymatic hydrolysis process of the chemically pretreated samples, the polymeric cellulose fractions are broken down into monomeric sugars (glucose). The whole sugar extraction process in chemical pretreatment requires several stages which takes longer time and more labour to extract maximum amount of TRS from the biomass. On the other hand, Although SCW hydrolysis requies high temperature and pressure, but it is a relatively simple, efficient and environment friendly process. It is a one-step auto hydrolysis process which requires less reaction time, cheap, non-toxic and non-corrosive. Water at subcritical condition not only targets the hemicellulose fractions of the biomass to solubilise and liberate pentose sugars, but also degrades the cellulose fractions to release hexose sugars while disrupting structure of lignin and cellulose crystallinity. Hence, SCW treatment process remains a good alternative for hydrolysis of lignocellulosic biomass.

Table 6.10: Comparative table for TRS yield in different treatment process.

Treatment process	**TRS Yield ($mg.g^{-1}$)**	
	Citronella	**Bagasse**
Dilute acid pretreatment	487.50 ± 0.83	452.27 ± 0.92
Enzymatic hydrolysis of acid pretreated substrate	226.99 ± 0.75	282.85 ± 0.89
Alkali pretreatment	73.88 ± 0.87	45.22 ± 0.93
Enzymatic hydrolysis of alkali pretreated substrate	345.49 ± 0.79	396.70 ± 0.84
SCW hydrolysis	281.66 ± 0.18	325.09 ± 0.27

6.5 Summary

Two major reactions are involved in the SCW hydrolysis process, which includes conversion of holocellulose to saccharides and degradation of saccharides. The release of oligosaccharides at lower temperature increased with reaction time but decreased at higher temperature as a result of its disintegration to monosaccharides. The monosaccharides further decompose to other degradation products (5-HMF and furfural) at higher temperatures. The lower activation energy obtained for saccharides degradation compared to saccharides formation revealed that the rate of decomposition of saccharides was faster than the

formation of sugars. The high regression coefficients of the response showed that the developed model showed good fit with the experimental data. The saccharides conversion on degree of cellulose crystallinity was evaluated using X-ray diffraction and the lower crystallinity obtained at higher temperature (220 °C) suggested maximum degradation of cellulose. SCW which is relatively a simple, efficient and environment friendly technique remains a promising process for the hydrolysis of lignocellulosic biomass.

Chapter 7

Levulinic acid production from bagasse and spent citronella biomass

The work in this chapter aimed at investigating the effect of process parameters on levulinic acid (LA) yield. Spent citronella biomass and sugarcane bagasse was used as a raw material for the production of LA. Initially, subcritical water hydrolysis (SCW) of the biomass was performed to produce reducing sugars (as discussed in chapter-6). For both the biomass, subcritical hydrolysate sample with maximum total reducing sugars (TRS) obtained at optimum condition was further processed to produce LA using homogeneous biodegradable methanesulfonic acid (MSA) as a catalyst. The process parameters affecting LA yield were optimised using response surface methodology to achieve maximum LA yield.

7.1 Introduction

Levulinic acid, also known as 4-oxopentanoic acid or γ-ketovaleric acid, is a C_5-chemical with a ketone and carboxylic group. These functional groups follow interesting patterns of reactivity resulting into a versatile building block for the synthesis of various biochemicals such as succinic acid, methyl tetrahydrofuran (MTH), levulinate esters etc. which has applications as organic solvent in industries, fuel additives and polymers (Girisuta et al., 2007; Wang et al., 2010; Mulder and Prakt, 1840). Moreover, it also acts as a precursor for ethyl levulinate, and MTH which can be blended with diesel or gasoline to generate a cleaner fuel. This versatile renewable platform molecule has also been identified by US Department of Energy as one of the 12 top value-added biochemicals (Werpy et al., 2004). LA is readily soluble in water, ethanol and many other organic solvents. It is a non-toxic (LD_{50} = 1850 $mg.kg^{-1}$) white crystalline solid organic biochemical that can be derived by degradation of HMF and hexose sugars and it also acts as a viable chemical bridge between biomass and petroleum processing. LA can be produced from pure glucose, fructose, starch, cellulose and lignocellulosic biomass in the presence of homogenous and heterogeneous catalyst (Girisuta, 2007). The acid-catalysed decomposition of C_6 sugar fragments leads to the formation of HMF as the intermediate product, which can be rehydrated to produce levulinic and formic

acids as final products (Zeng et al., 2012; Nazlina et al., 2012). The first synthesis of LA was reported by Dutch Professor G.J. Mulder in 1840's by heating sucrose with mineral acids at high temperature (Mulder and Prakt, 1840).

Java Citronella, like all other lignocellulosic biomass, is a complex material consisting of cellulose and hemicellulose polymers that are bound together by lignin. Cellulose and hemicellulose are both involved in the conversion of biomass to LA. Due to the action of acid catalysts polysaccharide cellulose fractions in the biomass gets hydrolysed into low molecular weight fractions such as di- or mono saccharides (e.g. glucose, fructose, sucrose etc.). Subsequently, monosaccharide glucose or fructose decomposed to HMF, which is further converted to yield LA. The conversion of HMF into LA involves the addition of H_2O molecule to C_2-C_3 bond of the furan ring. During this process due to number of side reactions, by-products (e.g. insoluble humin compounds) also produced, leading to a complex reaction network. The hydrolysis of hemicellulose results in the formation of glucose, galactose, xylose, and arabinose. The hexose sugars of hemicellulose fraction (glucose and galactose) also gets converted to HMF and subsequently to LA. Whereas, both C_5- sugars (xylose and arabinose) are anticipated to decompose into furfural (**Fig. 7.1**). Fructose is a known intermediate in the acid-catalysed decomposition of glucose. Dehydration of fructose to HMF is much faster than that of glucose. The lower LA yield occurs commonly due to the formation of undesired black insoluble-materials called humins.

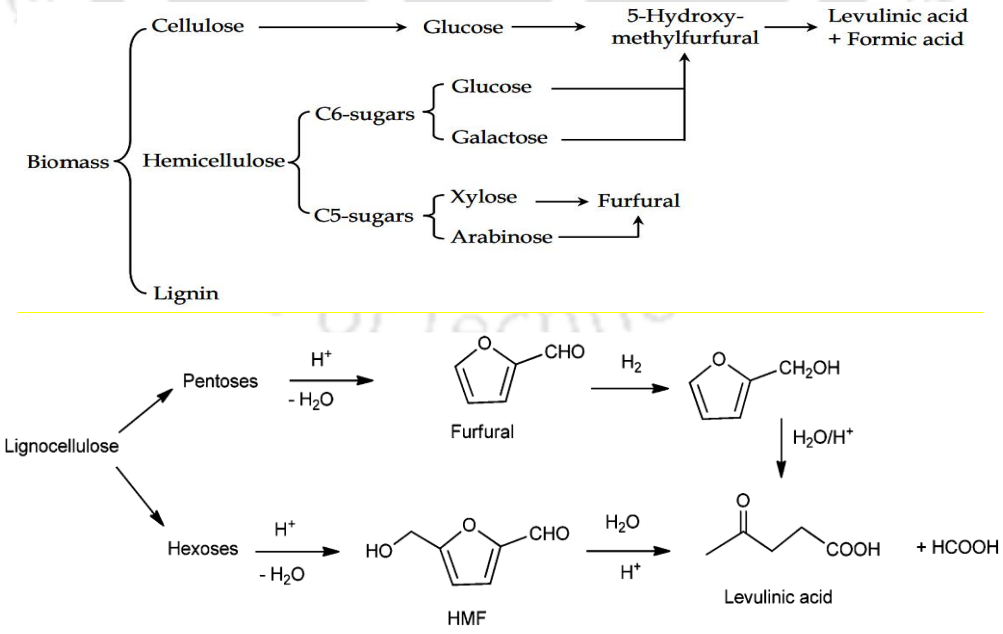


Fig. 7.1: Simplified reaction scheme for the conversion of lignocellulosic biomass to LA (Girisuta, 2007, Maria et al., 2014).

Several researchers have reported the conversion of biomass into levulinic acid using a dilute mineral acid, such as HCl and H_2SO_4 as catalysts (Efremov et al., 1998; Chang et al., 2007; Girisuta et al., 2007; Yan et al., 2008). H_2SO_4 and HCl are the preferred catalysts to achieve highest LA yield from glucose (Szabolcs et al., 2013; Cha et al., 2002; Lange et al., 2009). Szabolcs et al. (2013) studied the dehydration of cellobiose and cellulose in the concentration range of 0.1–2.5 M H_2SO_4 and obtained the optimum LA yield of 44 % and 34.2 % at conditions of 2 M H_2SO_4, 170 °C and 10 min; 2 M H_2SO_4, 170 °C and 30 min, for cellobiose and cellulose, respectively. Yan et al. (2008) have reported that lower HCl loading was ineffective in the hydrolysis of cellulose, because lower HCl loading results in the formation of lesser H^+ ions concentration thereby reduces hydrolysis rate. Peng et al. (2010) observed rapid increase in the LA yield with increased catalyst concentrations at optimum condition. Addition of excess catalyst showed insignificant effect on LA yield.

Some more literature on the use of HCl (Chang et al., 2007) and H_2SO_4 (Girisuta et al., 2008) have reported significant LA yield using these catalysts. However, some of the authors like, Yan et al. (2008), Chang et al. (2007), Girisuta et al. (2007), and Girisuta et al. (2008) reported that in presence of $CrCl_3$ (0.02 M), lower concentration of catalyst gives better yield compared to HCl (~1.2 M) and H_2SO_4 (0.1–1 M), respectively. Girisuta (2008) investigated various types of acids (homogeneous and heterogeneous Bronsted acids) and found that, for HMF to LA reaction, mineral acids such as sulphuric acid (H_2SO_4), hydrochloric acid (HCl) and hydrobromic acid (HBr) showed highest catalytic activities and LA yield. Although these hydrolysis reactions proceed smoothly, but LA yield from sugars suffers due to use of mineral acids as catalysts which are highly corrosive in nature. Moreover, the recovery of homogeneous catalysts from the reaction products is difficult (Lange et al., 2009). Thus, heterogeneous catalysts offer greater cost savings in terms of reducing corrosion and catalyst recycling, however suffer from lower product yield and inability to operate effectively at high biomass loading. Recently, number of strong sulfonic acids have been investigated as a catalyst for the reaction of simple sugars to LA and observed to have selectivity similar to sulfuric acid. Limited reports are available in literature on the use of environmentally friendly sulfonic acids as a catalyst for LA production. According to the available literature on methane sulfonic acid (MSA), the yield of levulinic acid obtained using MSA are comparable with sulphuric acid. Levulinic acid yield can be maximised at higher acid concentration, shorter reaction time and higher reaction temperature (Girisuta, 2007; Yan et al., 2008). Thus, in this thesis, MSA has been used as a catalyst with reducing sugars obtained through

subcritical water (SCW) hydrolysis of spent citronella biomass and sugarcane bagasse for LA production.

7.2 Results and Discussion

7.2.1 Preliminary experiments

The hydrolysate at which maximum total reducing sugars was obtained through SCW hydrolysis of sugarcane bagasse and citronella biomass (as studied in the previous chapter-6) was used to produce LA at different reaction conditions such as temperature: 200-240 °C; reaction time: 30-60 min; and MSA concentrations: 0.5-1 M. Results of the study revealed highest LA yield of 20.91 wt% and 11.95 wt% from sugarcane bagasse and citronella biomass, respectively at 220 °C, 1 M MSA and reaction time of 30 min (**Fig. 7.2, Fig. 7.3** and **Fig. 7.4**). The effect of various reaction parameters such as reaction time, temperature, and catalyst concentration on the conversion of LA are described in the following sub-sections. The emphasis was mainly towards selection of optimal reaction parameters to achieve maximum LA yield.

7.2.1.1 Effect of reaction temperature on LA yield

Effect of reaction temperature on the conversion of LA was studied over the temperature range of 200-240 °C keeping other parameters such as acid concentration, and reaction time as constant at 1 M MSA and 30 min, respectively. At elevated condition it was difficult to obtain higher yield of LA due to competing reactions such as the formation of levulinic acid as well as by-product i.e. humin. In case of bagasse and citronella biomass, the highest LA yield (12.63 wt% and 6.10 wt%, respectively) was obtained at 220 °C (**Fig. 7.2**). Peng et al. (2010) evaluated the effect of temperature on LA conversion using cellulose at 180–220 °C. They found that increase in the reaction temperature above 200 °C, decreased LA yield. Although, higher temperature accelerates the rate of reaction but at the same time produces by-product, such as humins. The influence of reaction temperature on the yield of LA was extensively studied by different researchers using different biomass feedstock's, such as kernel grain sorghum (Fang et al., 2002), wheat straw (Chang et al., 2007; Galletti et al., 2010), water hyacinth (Girisuta et al., 2008), poplar sawdust, paper sludge, olive tree pruning (Galletti et al., 2010), bagasse and paddy straw (Yan et al., 2008), cellulose (Girisuta et al., 2007; Wang et al., 2010; Peng et al., 2010; Lai et al., 2011). Girisuta et al. (2006) obtained higher LA yield at higher temperature (200 °C) compared with reported by Fang et al. (2002) from kernel grain sorghum at 160 °C. Similarly, Yan et al. (2008) also achieved higher LA

yield at 220 °C from paddy straw and bagasse. The above analysis revealed that higher temperature is required due to the complex mixture of biomass hydrolysate particularly, presence of lignin, in order to maximise the LA yield although humins might originate from glucose and HMF at high temperature (Lai et al., 2011).

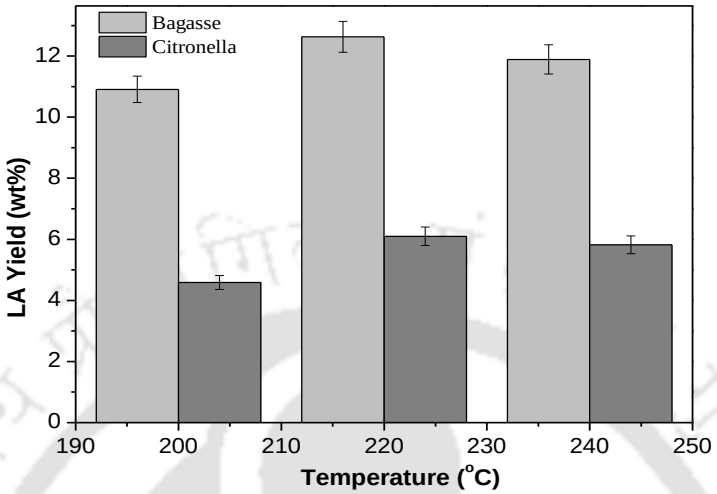


Fig. 7.2: Effect of temperature on LA yield for bagasse and citronella biomass.

7.2.1.2 Effect of catalyst (MSA) concentration on LA yield

The amount of LA is considerably lower at 0.5 M MSA compared to 1 M MSA, resulting due to reduced glucose formation from the cellulose fraction at low MSA concentration which might have occurred due to a slower rate of breakdown of glycosidic bonds in crystalline cellulose. In addition, subsequently the rate of reaction of glucose to HMF and LA might have decreased at low acid concentrations which results in lower LA yield.

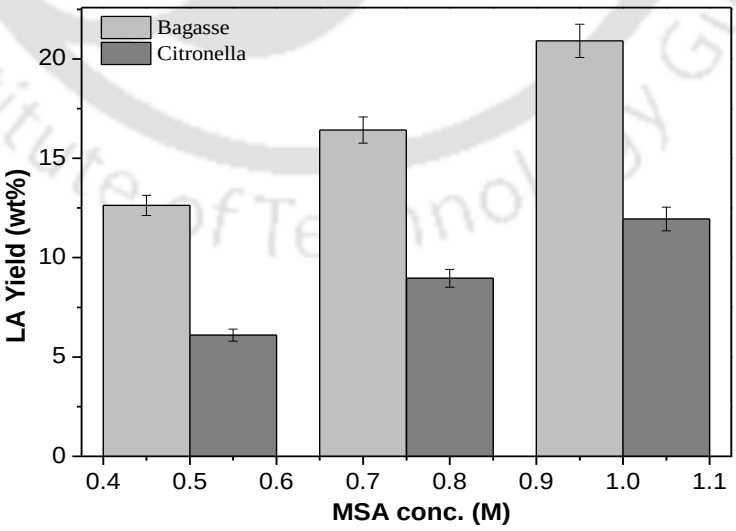


Fig. 7.3: Effect of MSA concentration on LA yield for bagasse and citronella biomass.

However, in case of bagasse, within the studied MSA concentration range, highest yield (20.91 wt%) was obtained at 1 M MSA keeping the reaction temperature, and time constant at 220 °C and 30 min, respectively. Further, decreasing the concentration to 0.75 M and 0.5 M MSA, the LA yield decreased to 16.42 wt% and 12.63 wt%, respectively. Similarly, in case of citronella, the highest yield (11.94 wt%) was attained at 1 M MSA under otherwise identical reaction conditions. Identical behaviour was noticed at lower acid loading 0.5 M and 0.75 M MSA.

7.2.1.3 Effect of reaction time on LA yield

Reaction time has a significant effect on LA yield, however it has a relative correlation with other process parameters like temperature, and catalyst concentration. The present work observed that, when hydrolysis is performed for longer duration, the LA yield decreases (**Fig. 7.4**).

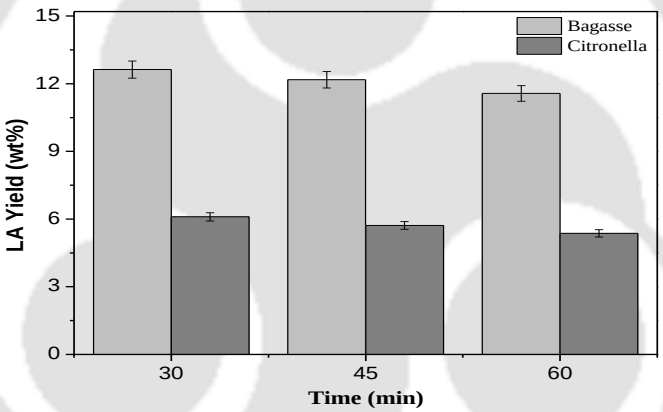


Fig. 7.4: Effect of reaction time on LA yield for bagasse and citronella biomass.

The increase in reaction time from 30 to 60 min decreased the LA yield from 12.63 wt% to 11.57 wt% keeping the reaction temperature, and MSA concentration constant at 220 °C, and 1 M MSA, respectively for bagasse. Whereas, for citronella LA yield decreased from 6.10 wt% to 5.37 wt% for same parameters. Cha et al. (2002) during their study on starch hydrolysis with 5 wt% H_2SO_4 reported that LA yield increased with an increase in reaction time from 20 to 60 min. However, LA yield does not change significantly with further increase in reaction time from 40 to 60 min. Yan et al. (2008) in their study during hydrolysis of bagasse observed that, hydrolysis rate was rapid during first 10 min and slower down during last 20 min, due to the prevailing side reactions at prolonged reaction time. On the other hand, hydrolysis rate was found highest after 25 min for paddy straw. These differences

in the hydrolysis rate occurred mainly due to the fact that kinetics differs according to the content of cellulose, type of cellulose and biomass composition. The results obtained in this study have been compared with the other reports in terms of LA yield in **Table 7.1**.

Table 7.1: Comparison of the results obtained in this study with published literature.

Sl. No.	Substrate and Loading (wt%)	Catalyst	Reaction conditions	Yield (wt%)	Ref.
1	1 % fructose	HCl	240 °C for 0.03 h	30	Salak et al., 2006
2	1 % fructose	H_3PO_4	240 °C for 0.03 h	4.5	Salak et al., 2006
3	15 % glucose	HCl	240 °C for 1 h	37.2	Deng et al., 2009
4	3 % Sucrose	$SO_4^{2-/}$	200 °C for 24 h	33.05	Jiang et al. 2010
5	20 % cellulose	$CuSO_4$	240 °C for 0.5 h	17.5	Cao et al., 2015
6	1 % water hyacinth	MSA	MW for 0.16 h	9.43	Huanran et al., 2011
7	7 % giant reed	HCl	200 °C for 1 h	15.8	Galletti et al., 2013
8	10 % whole kernel grain sorghum	H_2SO_4	200 °C for 0.67 h	32.6	Fang et al., 2002
9	6.4 % wheat straw	H_2SO_4	209.3 °C for 0.63 h	19.9	Chang et al., 2007
10	7 % wheat straw	HCl	200 °C for 1 h	19.3	Galletti et al., 2012
11	7 % wheat straw	HCl	MW, 200 °C for 0.25 h	20.6	Galletti et al., 2012
12	10.5 % Sugarcane bagasse	HCl	220 °C for 0.75 h	22.8	Yan et al., 2008
13	10.5 % paddy straw	HCl	220 °C for 0.75 h	23.7	Yan et al., 2008
14	7 % olive tree pruning	HCl	200 °C for 1 h	18.6	Galletti et al., 2012
15	7 % olive tree pruning	HCl	MW, 200 °C for 0.25 h	20.1	Galletti et al., 2012
16	7 % poplar sawdust	HCl	200 °C for 1 h	21.3	Galletti et al., 2012
17	7 % poplar sawdust	HCl	MW, 200 °C for 0.25 h	26.4	Galletti et al., 2012
18	7 % tobacco ships	HCl	200 °C for 1 h	5.2	Galletti et al., 2012
19	25 % chitosan	$SnCl_4$	210 °C for 0.5 h	27	Omari et al., 2012

The summary of comparative analysis of LA yield indicates that, preliminary results obtained in this work are more or less at par with that reported in literature. However, in order to reduce the total number of experiments and find the best combinations for optimisation of the process, central composite design (CCD) of response surface methodology (RSM) was employed. CCD has equal predictability in all directions from the centre and provides a better understanding of the effect of independent factor and their interaction among process parameters while influencing the response variable (LA yield). In addition, CCDs are optimised design for fitting quadratic models.

7.2.2 Design of experiment using central composite design (CCD) of RSM

RSM is usually used to determine the combined effects of various factors and to find the optimum conditions for multiple factor system. In the present study, CCD was used to optimise LA conversion process with the aim to maximise LA yield. To assure the statistical validity, the central points is replicated several times. A three level-three factor design was employed. Considering 6 central points, total of 20 experimental runs were generated consisting 7 factorial points and 7 axial points. After completing the experiments as per the designed matrix, a suitable mathematical model was developed to predict the response. Later complete analysis of variance (ANOVA) was done. Based on the preliminary results and literature data, the level of variables; temperature (A: 200–240 °C), reaction time (B: 30–60 minute), and MSA Conc. (M) (C: 0.5-1 M) was designed as represented in **Table 7.2**. The effect of reaction temperature, time and acid concentration have been investigated at different levels (low, medium and high).

Table 7.2: Real and coded values of independent variable.

Factor		Low	Medium	High
A	Temperature (°C)	200	220	240
B	Reaction Time (min)	30	45	60
C	MSA Concentration (M)	0.5	0.75	1

Table 7.3: CCD matrix of the factors and its corrresponding experimental and predicted responses for LA yield from bagasse and citronella.

Std. Order	Run Order	Temp. (°C)	Time (min)	MSA Conc. (M)	LA Yield (wt%) from bagasse		LA Yield (wt%) from citronella	
					Actual	Predicted	Actual	Predicted
1	13	200	30	0.5	20.25	20.30	10.41	10.44
2	7	240	30	0.5	20.63	20.62	10.35	10.36
3	17	200	60	0.5	19.15	19.07	9.91	9.92
4	6	240	60	0.5	18.55	18.59	9.86	9.91
5	5	200	30	1	22.89	22.91	11.35	11.31
6	4	240	30	1	22.19	22.32	11.71	11.73
7	11	200	60	1	22.64	22.69	9.71	9.72
8	10	240	60	1	21.28	21.29	10.20	10.18
9	16	186.36	45	0.75	20.41	20.40	9.88	9.87
10	20	253.64	45	0.75	19.58	19.50	10.22	10.20
11	2	220	20	0.75	22.25	22.16	11.96	11.97
12	15	220	70.23	0.75	20.26	20.27	10.28	10.25
13	3	220	45	0.33	19.98	20.01	9.89	9.83
14	12	220	45	1.17	24.58	24.48	10.76	10.79
15	19	220	45	0.75	23.75	23.82	11.14	11.16
16	1	220	45	0.75	23.87	23.82	11.18	11.16
17	9	220	45	0.75	23.81	23.82	11.15	11.16
18	18	220	45	0.75	23.81	23.82	11.14	11.16
19	8	220	45	0.75	23.87	23.82	11.18	11.16
20	14	220	45	0.75	23.87	23.82	11.18	11.16

The designed matrix as well as their corresponding predicted and experimental values for bagasse and citronella are presented in **Table 7.3**. From the results, it can be seen that experimentally highest LA yield (24.58 %) from bagasse was obtained at: 220 °C, 45 min and 1.17 M MSA. Whereas, lowest LA yield (18.55 %) was at 240 °C, 60 min and 0.5 M MSA. The above results revealed that higher acid concentration, optimum temperature and time is required to obtain higher LA yield from bagasse. On the other hand, highest (11.96%) and lowest (9.71 %) LA yield from citronella was obtained at: 220 °C, 20 min, 0.75 M MSA; and 200 °C, 60 min, 1.0 M MSA respectively. The influence of biomass composition

has significant effects on the LA yield due to its complex structure, for example, lignin results into variation of optimum condition. Also, it is difficult to distinguish the comparison based on LA yield, as the yield is mostly calculated based on the initial cellulose content of feedstock, C_6 content of holocellulose etc. High LA yield is usually obtained with high hexose sugars containing biomass at higher acid concentration, higher temperature and lesser reaction time. The LA yield obtained in this study with citronella biomass was relatively less compared to sugarcane bagasse. The lower LA yield attributed to relatively lesser content of hexose sugars in the citronella biomass compared to bagasse, as discussed in the previous chapters.

7.2.2.1 Analysis of variance (ANOVA) of quadratic model and model validation

The parameters affecting the LA yield from biomass can be evaluated from the *F*-test of ANOVA as presented in **Table 7.4** and **Table 7.5** for bagasse and citronella, respectively. The significance of each coefficient was determined by *F*-values and *p*-values. The large *F*-value and corresponding low *p*-value depicts that the variable has a higher effect in the process. If the value of probability (*p*) function is greater than 0.05, which indicates that process or model does not have a significant effect on the response of interest (Montgomery, 2005; Singh and Sharma, 2012; Joglekar and May, 1987; Guan and Yao, 2008).

Table 7.4: Analysis of variances for the quadratic model for LA yield from bagasse.

Source	Sum of Squares	DF	Mean Square	F-Value	p-value
Model	64.52	9	7.17	1003.03	< 0.0001
A-Temp. (°C)	0.98	1	0.98	137.77	< 0.0001
B-Time (min)	4.32	1	4.32	604.57	< 0.0001
C-MSA Conc.(M)	24.12	1	24.12	3374.49	< 0.0001
AB	0.03	1	0.03	46.47	< 0.0001
AC	0.42	1	0.42	58.72	< 0.0001
BC	0.51	1	0.51	70.9	< 0.0001
A^2	25.59	1	25.59	3580.07	< 0.0001
B^2	11.6	1	11.6	1623.14	< 0.0001
C^2	4.27	1	4.27	596.95	< 0.0001
Residual	0.064	9	0.007		
Lack of Fit	0.054	5	0.01	4.31	0.0911
Pure Error	0.01	4	0.003		
Cor Total	64.58	18			

Table 7.5: Analysis of variances for the quadratic model for LA yield from citronella.

Source	Sum of Squares	DF	Mean Square	F-Value	p-value
Model	8.54	9	0.95	656.02	< 0.0001
A-Temp. (°C)	0.13	1	0.13	88.3	< 0.0001
B-Time (min)	3.57	1	3.57	2468.11	< 0.0001
C-MSA Conc.(M)	1.11	1	1.11	770.83	< 0.0001
AB	0.0024	1	0.0024	1.65	0.2312
AC	0.11	1	0.11	79.36	< 0.0001
BC	0.58	1	0.58	404.1	< 0.0001
A^2	2.15	1	2.15	1483.65	< 0.0001
B^2	0.0045	1	0.0045	3.1	0.1123
C^2	1.22	1	1.22	843.3	< 0.0001
Residual	0.013	9	0.0015		
Lack of Fit	0.011	5	0.0023	5.4	0.0637
Pure Error	0.0017	4	0.004		
Cor Total	8.55	18			

From the regression analysis as shown in **Table 7.4**, it can be observed that developed model was highly significant with *p*-value less than 0.05. The independent variables such as temperature (A), time (B) and MSA concentration (C) have shown a substantial effect on the process of bagasse conversion to LA. Further, 2nd order effect of temperature (A^2), time (B^2), MSA concentration (C^2); as well as the interaction factors AB, AC, and BC also showed highly significant effect to the response. Similarly, in case of citronella conversion to LA as depicted in **Table 7.5**, it was seen that developed model was highly significant with a *p*-value less than 0.0001. Also, the independent variables {temperature (A), time (B) and MSA concentration (C)} and 2nd order parameter (temperature and MSA concentration) have shown a substantial effect. However, the interaction between parameter temperature (A) and time (B) and 2nd order effect of time (B) has relatively less significance to the response. The LA yield increased with reaction temperature up to the optimum temperature, 220 °C, beyond which the yield rapidly decreased with longer reaction time. The same trend was also reported by Chang et al. (2007). The interaction effects between reaction temperature and time on LA conversion has revealed that LA yield linearly increased with increasing reaction temperature. High reaction temperature increased the rate of chemical reaction. However, this condition might not be recommended as at the same time, undesired side reactions may also be formed. The yield of LA is generally reduced when performing the reaction at higher

temperatures due to the formation of humins. Therefore, higher temperature is unfavourable for the extent of reaction. The experimental results can be visualised in 3D response surface plots showing the correlation between various factors use in this study. The mutual interaction between the factors towards the response can be observed from the curvature in 3D response plot as shown in **Fig 7.5** and **Fig 7.6** for bagasse and citronella, respectively. 3D response surface plots are the graphical representation of the regression equation, and illustrate the independent and interactive effects of the factors. By using response surface plot the interaction between two variables and their optimum levels can be easily located and understood. The elliptical curves obtained showed a significant interaction between the independent factors.

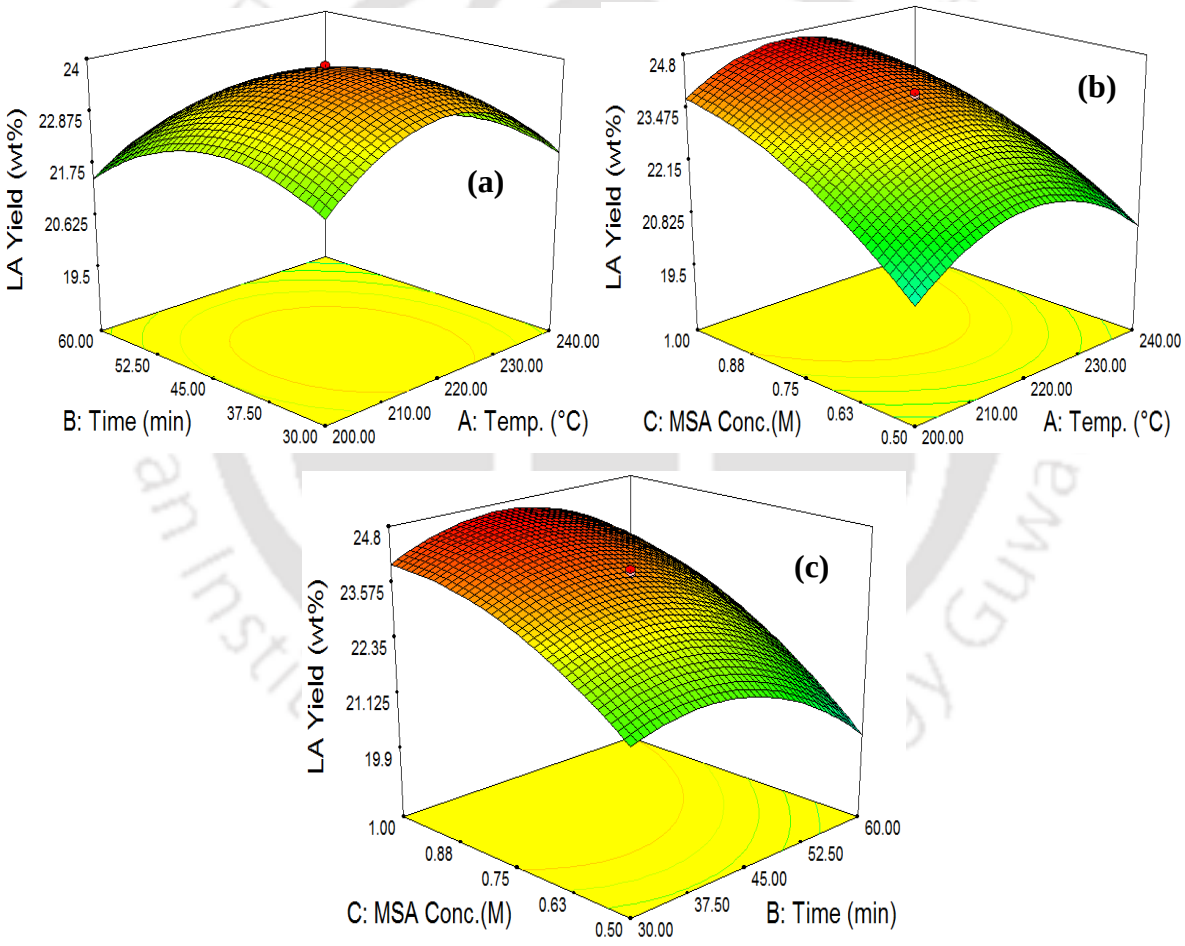


Fig. 7.5: 3D response surface plots representing the interaction effects between the process parameters: (a) AB; (b) AC; (c)BC, for bagasse.

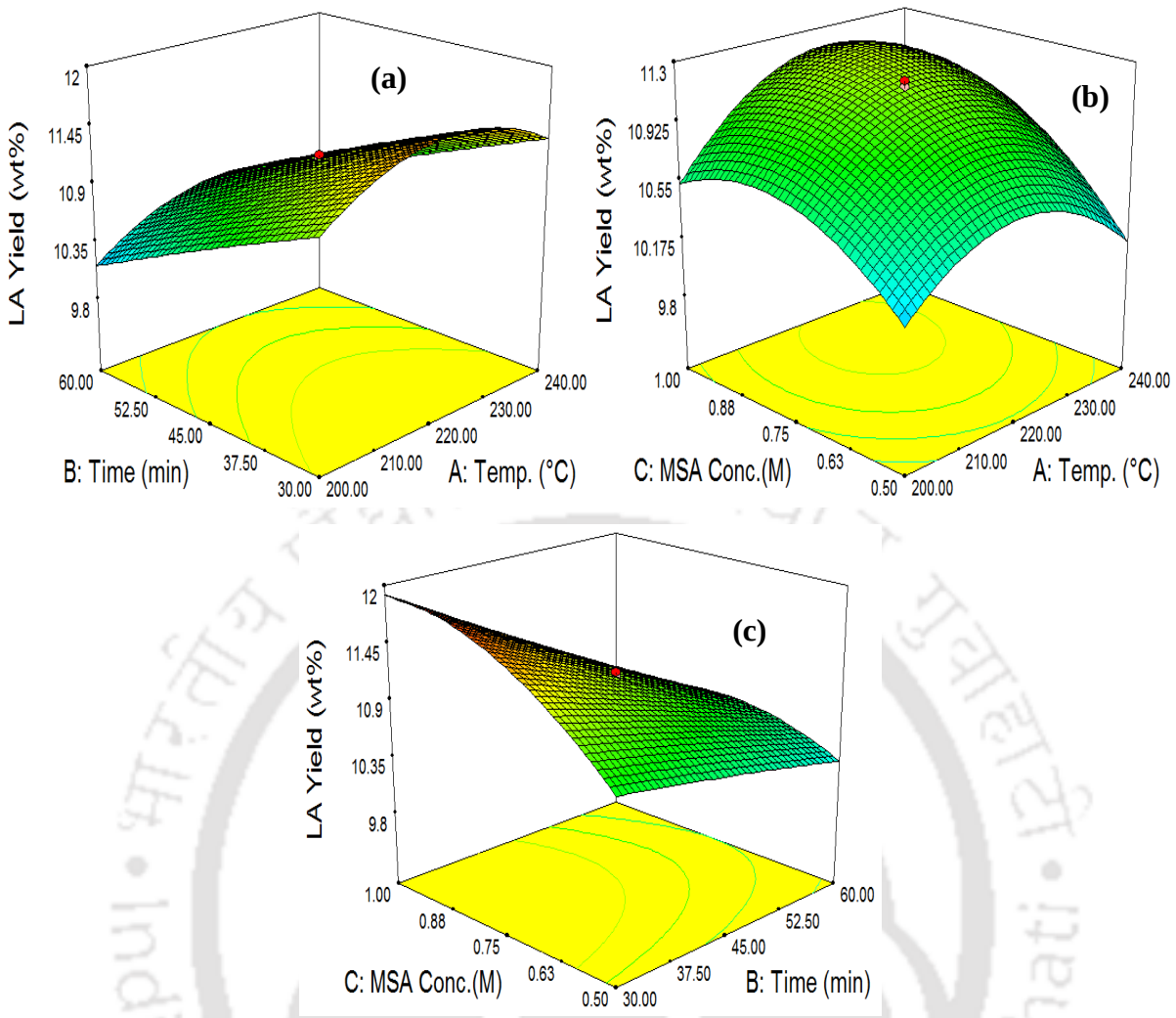


Fig. 7.6: 3D response surface plots representing the interaction effects between the process parameters: (a) AB; (b) AC; (c) BC, for citronella.

The main benefit of RSM is the graphic representations of the regression models in the form of 3D response surface plots to show the interactive effects between the independent variables. These 3D response surface plots are very helpful in understanding the interaction effects of two variables at a time, keeping other factors at a fixed level (at zero level). Maximum LA yield can be achieved across a range of variables as long as two out of the three conditions are met; high acid concentration, less reaction time or low temperature within the tested range, as shown by the plateaus in the **Fig. 7.5** and **Fig. 7.6** for bagasse and citronella, respectively.

The developed model equations for LA yield are expressed in terms of coded values **Eq.** (**7.1 and 7.2**) for bagasse and citronella, respectively.

$$Y_1 = 23.82 - 0.27A - 0.56B + 1.33C - 0.20AB - 0.23AC + 0.25BC - 1.37A^2 + 0.92B^2 - 0.56C^2 \quad \textbf{(7.1)}$$

$$Y_2 = 11.16 + 0.09A - 0.51B + 0.29C + 0.02AB + 0.12AC - 0.27BC - 0.40A^2 - 0.02B^2 - 0.30C^2 \quad \textbf{(7.2)}$$

Where, Y_1 and Y_2 are the predicted response (LA yield) for bagasse and citronella biomass, respectively.

Based on the model equation, LA yield within the range of variables of experimental design was predicted at optimum conditions in order to obtain high yield of Levulinic acid. The optimum condition for the maximum LA yield from the process is generated as: T = 203 °C, t = 44.55 min, and MSA concentration = 0.66 M for bagasse; and T = 231.86 °C, t = 38.44 min, and MSA concentration = 0.66 M for citronella. At the optimal condition, the experiments were performed in triplicate and average values were reported. The experimental LA yield obtained (22.73 wt% and 11.17 wt%) was close to the predicted value (22.51 wt% and 11.08 wt%) for bagasse and citronella, respectively, which suggests that there is less than 1.00 % error for both cases between the observed and predicted values and the model was fitted well to the data. The significance of the model is checked by the determination of regression coefficient (R^2) value. For a good model fit, R^2 value should be in the range of 0.80 (Joglekar and May, 1987; Guan and Yao, 2008). The statistical parameters of the developed model such as: R^2 = 0.99 and large *F*-value of 1048.65 with low probability (p < 0.0001) for bagasse and R^2 = 0.99 and *F*-value of 686.62 with low probability (p < 0.0001) for citronella, shows that the applied model was highly significant. The comparison of the experimental and predicted value of the response (i.e., levulinic acid yield) as presented in **Fig. 7.7** and **Fig. 7.8** also demonstrates a good fitness of the model (R^2 = 0.99). These results indicate that 99 % of the variability in the responses can be explained by the models and only ~1% of the total variability were not explained in the regression model. The high R^2 value can clarify that the model obtained is able to convince a good estimate of the response within the process conditions range.

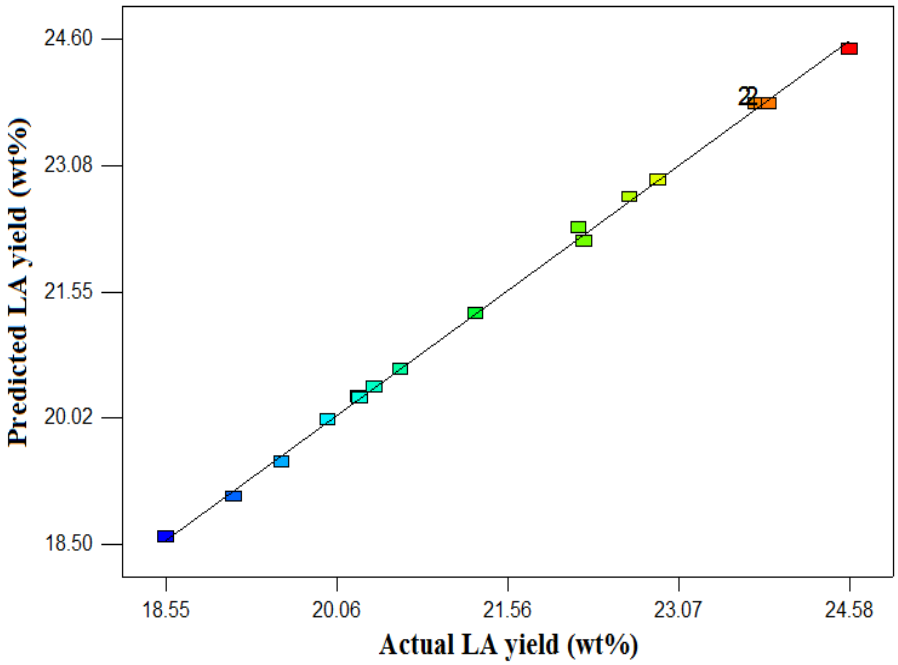


Fig. 7.7: Comparison plot between predicted and actual values of LA yield (wt%) for bagasse.

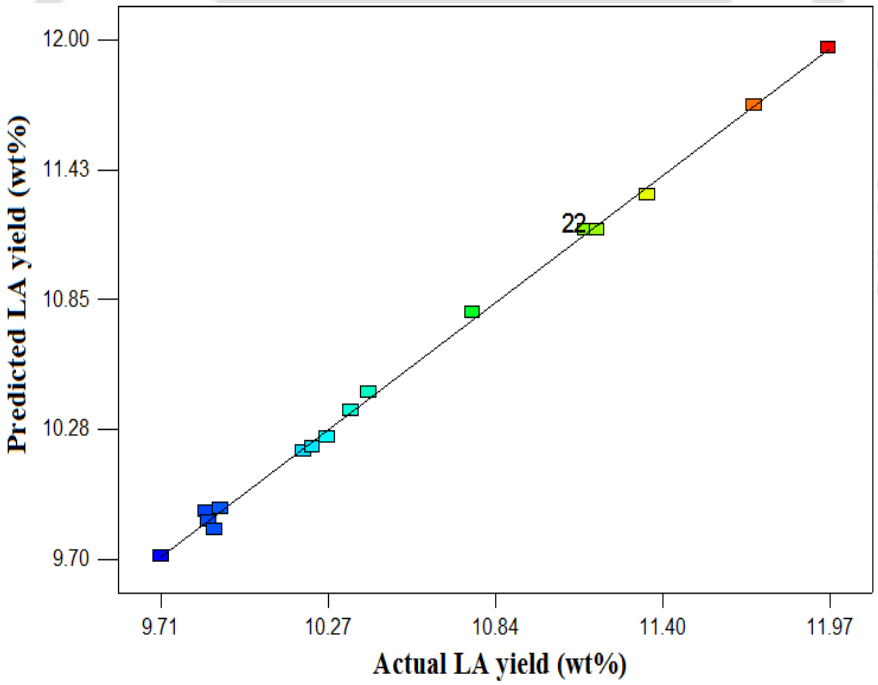


Fig. 7.8: Comparison plot between predicted and actual values of LA yield (wt%) for citronella.

7.3 Summary

Methane sulfonic acid-catalysed hydrolysis of bagasse and citronella biomass to LA has been studied (T: 200-240 °C; Reaction time: 30-60 min; MSA concentrations: 0.5-1 M). The yield of LA was considerably lower at 0.5 M MSA compared to 1 M MSA, which suggests that the amount of available monomeric hexose sugars in the reaction mixture is reduced considerably at lower acid catalyst concentrations. Fast heating rates allows maximal yield of LA at shorter reaction time however prolonged reaction time and increased temperature results to undesired side reactions forming by-products such as humin. A second order polynomial model was developed for the acid-catalysed hydrolysis of biomass to LA yield. A linear regression model was capable of predicting the Levulinic acid yield with respect to the effect of reaction time, temperature, and acid concentration. A good fit between the experimental data and the model was obtained (R^2=0.99). Comparative results with the literature depicted that sulfonic acid catalysts showed similar selectivity to mineral acids for the conversion of sugars to levulinic acid. Hence, MSA can be a preferred catalyst for the conversion of biomass to LA as compared to mineral acids.

Chapter 8

Conclusions and recommendations for future work

This chapter summarises the conclusions drawn from various chapters presented in this thesis. Also, some suggestions towards the scope for future research are also outlined.

8.1 Conclusions

The present study emphasis on unraveling the use of aromatic biomass for essential oil and levulinic acid production and mainly focuses on three aspects: 1) effective essential oil extraction process; 2) effective pretreatment process; and 3) production of levulinic acid. This is the first systematic report on characterisation of aromatic lignocellulosic biomass (Java citronella) available in the provinces of Northeast India. Java citronella has significant amount of essential oil which are of high industrial importance.

In this thesis, essential oil extraction from different parts of Java citronella (*Cymbopogon winterianus* Jowitt) was studied using distillation techniques. Hydro distillation (HD) process was compared with Steam distillation (SD), Ultrasonic assisted hydro distillation (UA-HD) and Ultrasonic assisted steam distillation (UA-SD) on the basis of essential oil yield and composition. In SD process, the obtained oil yield (2.63 %) was more than HD process (2.38 %). The ultrasonic pretreatment of 30 min to the plant material before distillation increases the yield of extracted essential oils to 2.85 % in UA-SD and 2.56 % in UA-HD process. Fresh citronella biomass found to contain more essential oil compared to dried plant showing the significant effect of moisture content on the oil yield. Comparison of the yield of extracted essential oils from various plant parts of citronella revealed that leaves contain maximum essential oil (yield of 2.38 % vw^{-1}), which is about 40 % more than stems and 17 % more than whole aerial parts. The compositional analysis of extracted citronella oil revealed the presence of about 95 % of commercially important compounds viz. citronellal (55.23 %), geraniol (26.29 %) and citronellol (13.41 %), indicating a superior quality of citronella oil. The high quality (in terms of main components of citronella oil such as citronellal, geraniol and citronellol) oil obtained from Java citronella reveals that the geographical location and environmental conditions of Karbi Anglong, Assam, India is suitable for the cultivation of Java citronella plant. The hydrosol obtained after distillation

process was found to contain sugar alcohols such as adonitol, erythritol and mannitol, which can be further utilised in nutraceutical and pharmaceutical industries.

The composition analysis of citronella biomass carried out using TGA revealed that citronella has lower cellulose content (31.70 % in fresh biomass and 28.15 % in spent biomass) than hemicellulose (32.66 % in fresh biomass and 32.22 % in spent biomass). XRD analysis of fresh biomass before pretreatment shows crystallinity index (CI) of 32.97%. And after hydro distillation crystallinity increased to 34.91% indicates the removal of water extractives in the process. Similarly, with the application of different pretreatment techniques like alkali (NaOH) (CI = 57.35 %), acidic (H_2SO_4) (CI = 56.18 %), and subcritical water (CI = 52.68 %), the CI value of the biomass increased, which indicates partial dissolution of hemicellulose fractions, removal of lignin and thereby exposing the crystallinity of cellulose fractions of the biomass. The high calorific value (17.0 $MJ.Kg^{-1}$), high content of carbon (42.55 %), hydrogen (5.83 %), oxygen (48.08 %) and high content of holocellulose (60.37 wt%) with low ash (0.57 %) and negligible sulfur content (0.1 %), suggests suitability of citronella biomass as an ideal feedstock for energy production.

Thus, besides extraction of essential oil, the spent biomass was pretreated using different pretreatment techniques like alkali, acid pretreatment and SCW hydrolysis techniques. Alkali pretreatment was found to be an efficient technique to delignify bagasse and spent citronella biomass and preserved the polysaccharide fraction. While, in case of dilute acid pretreatment, at optimum condition maximum TRS yield obtained was 71.87 % and 80.75 % for sugarcane bagasse and spent citronella biomass, respectively. Subsequent enzymatic hydrolysis of pretreated samples revealed decrease in CI of spent citronella biomass and bagasse to 34.16 % and 37.28 %, respectively indicating significant conversion of crystalline cellulose to glucose. Considering the high sugar yield, dilute acid pretreatment process using sulfuric acid could be considered as a good choice for the hydrolysis of spent citronella biomass and sugarcane bagasse. However, chemical pretreatments uses highly corrosive chemicals and requires neutralisation prior to its further utilisation, therefore a greener approach for utilising the biomass for the production of different value-added products is necessary. Hence, chemical pretreatments (dilute acid and alkali) was compared with subcritical water hydrolysis (a greener technique) on the basis of TRS yield. Considering the total holocellulose content of biomass (60.37 wt% for citronella and 62.93 wt% for bagasse), TRS yield (ratio of final concentration of total reducing sugars to the initial concentration of holocellulose content in the biomass) of SCW hydrolysis process at the optimum condition achieved was 46.66 % and 51.66 % for citronella and bagasse,

respectively. From the above results, it can be inferred that, SCW hydrolysis which is relatively simple, efficient and environment friendly technique remains a promising process for hydrolysis of lignocellulosic biomass.

The hydrolysate at which maximum total reducing sugars was obtained through SCW hydrolysis of sugarcane bagasse and citronella biomass was further used to produce LA. Highest levulinic acid yield of 22.73 wt% and 11.17 wt% was achieved at MSA concentration of 0.66 M for bagasse and citronella, respectively. Comparative analysis of the results obtained in the present work with that of literature depicts that sulfonic acid catalysts has similar selectivity to mineral acids for levulinic acid conversion.

Lack of knowledge about the indigenous tree and shrub species located in the forests at different provinces of Northeast India retards setting up biomass based essential oil and energy plants. Preliminary information on physico-chemical characterisation of biomass surely opens up an opportunity for its selection as a future candidate for biochemical or bioenergy production.

8.2 Recommendations for Future Work

In the present study, performance of various essential oil extraction process like hydro distillation, steam distillation, ultrasonic assisted distillation techniques and pretreatment techniques such as dilute acid, alkali, subcritical water hydrolysis, as well as levulinic acid production technique was thoroughly evaluated and optimised using response surface methodology. The key results of the research have been underlined in section 8.1. However, there are lots of scopes for further research on the developed processes. Some of the future scopes and suggestions for further studies are as follows:

- The efficacy of other greener pretreatment techniques such as Ionic liquid and Ozonolysis need to be investigated in the context of biomass hydrolysis and TRS production.
- In this study, detoxification of the pre-hydrolysate samples have not been carried out. These are extremely relevant for the further utilisation of the pre-hydrolysate for the synthesis of value added products. Future work shall include such investigations to enhance the applicability of low cost feedstock for bioenergy and value added chemical production.

- Levulinic acid production experiments can be conducted for heterogeneous catalysts such as ion exchange resins, zeolites, and acidic clays.
- All the experiments conducted in the thesis are at laboratory scale. These studies can also can be scaled up by using large scale extractors/reactors.

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APPENDIX - A

A.1. Calibration plots of standard sugars, sugar alcohols, furans and levulinic acid

The quantification of sugars in the unknown sample was done based on the calibration plots prepared from the known concentrations of the standard sugars, sugar alcohols, furans and levulinic acid using high performance liquid chromatography (HPLC). The peaks obtained in HPLC were identified from the retention time of standard sample and quantified using the built calibration plots standardised with at least 5 points of different known concentrations. The calibration plots of standard samples (**Maltohexaose; Maltopentaose; Raffinose; Cellobiose; Sucrose; Glucose; Galactose; Xylose; Arabinose; Fructose; Erythrose; Adonitol; Mannitol; Erythritol; Sorbitol; 5-HMF; Furfural; Levulinic acid)** are given in Fig. A1; A2 and A3. Similarly, A4 shows calibration plot of Glucose prepared using UV-vis.

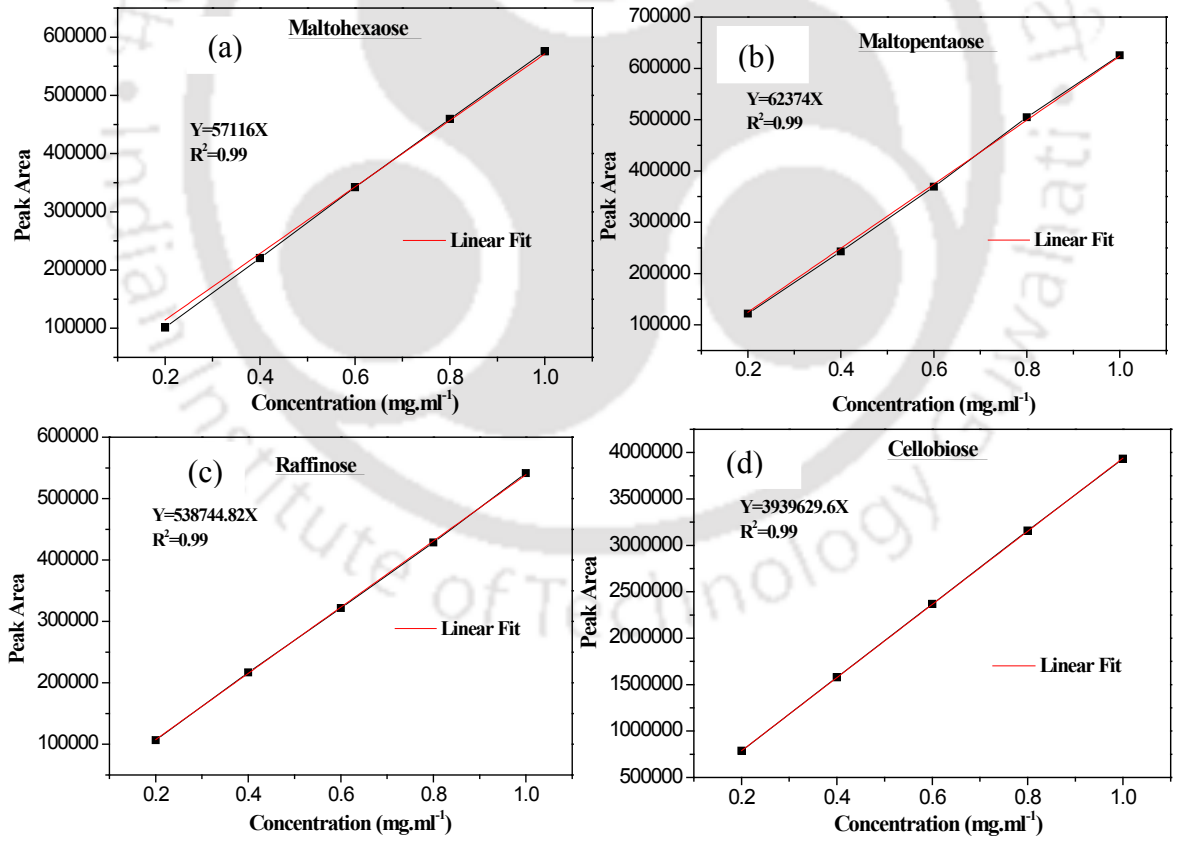

Fig. A1: Calibration plot of standard samples (>98% purity): (a) Maltohexaose; (b) Maltopentaose; (c) Raffinose; (d) Cellobiose.

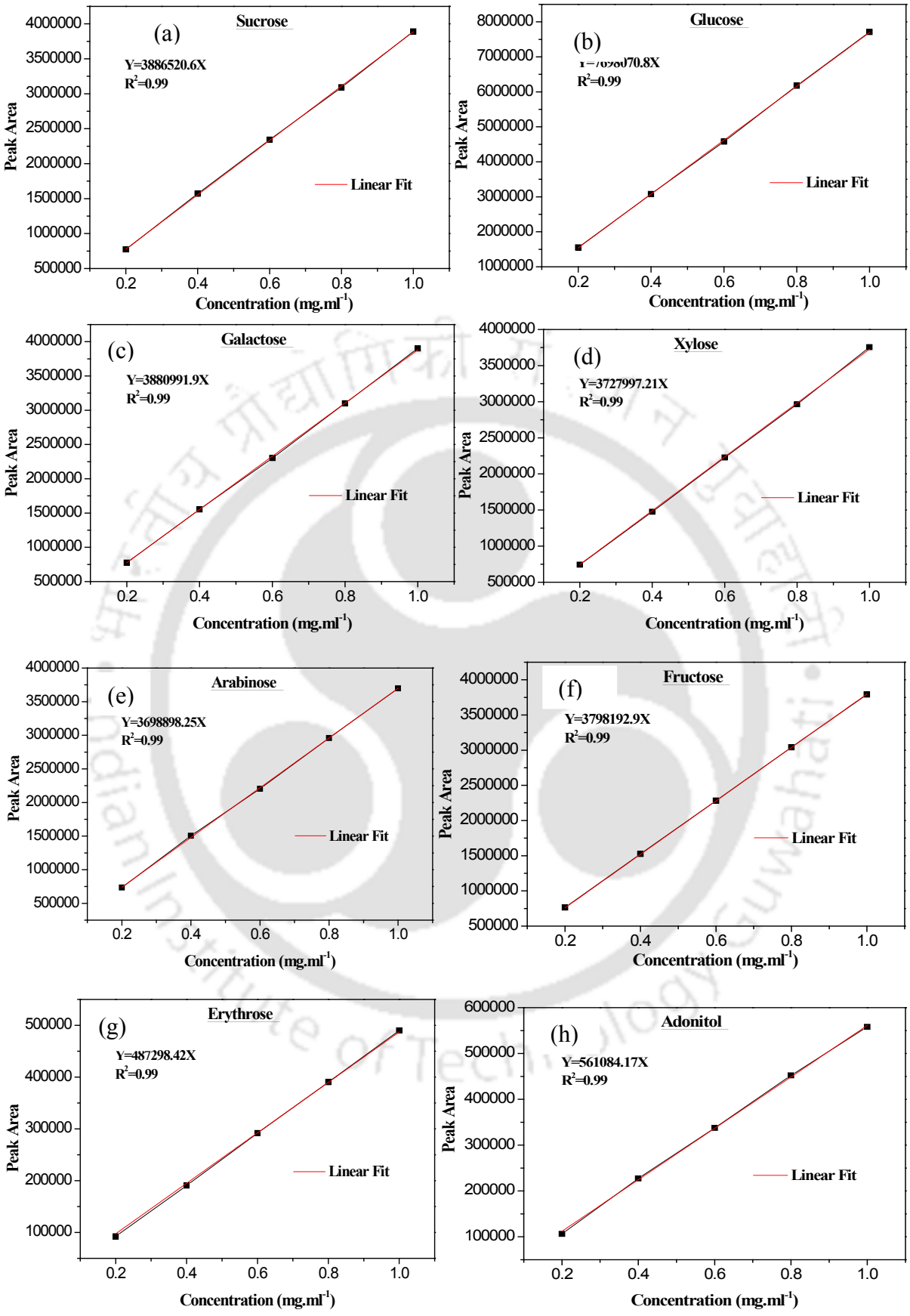

Fig. A2: Calibration plot of standard samples (>98% purity) : (a) Sucrose; (b) Glucose; (c) Galactose; (d) Xylose; (e) Arabinose; (f) Fructose; (g) Erythrose; (h) Adonitol.

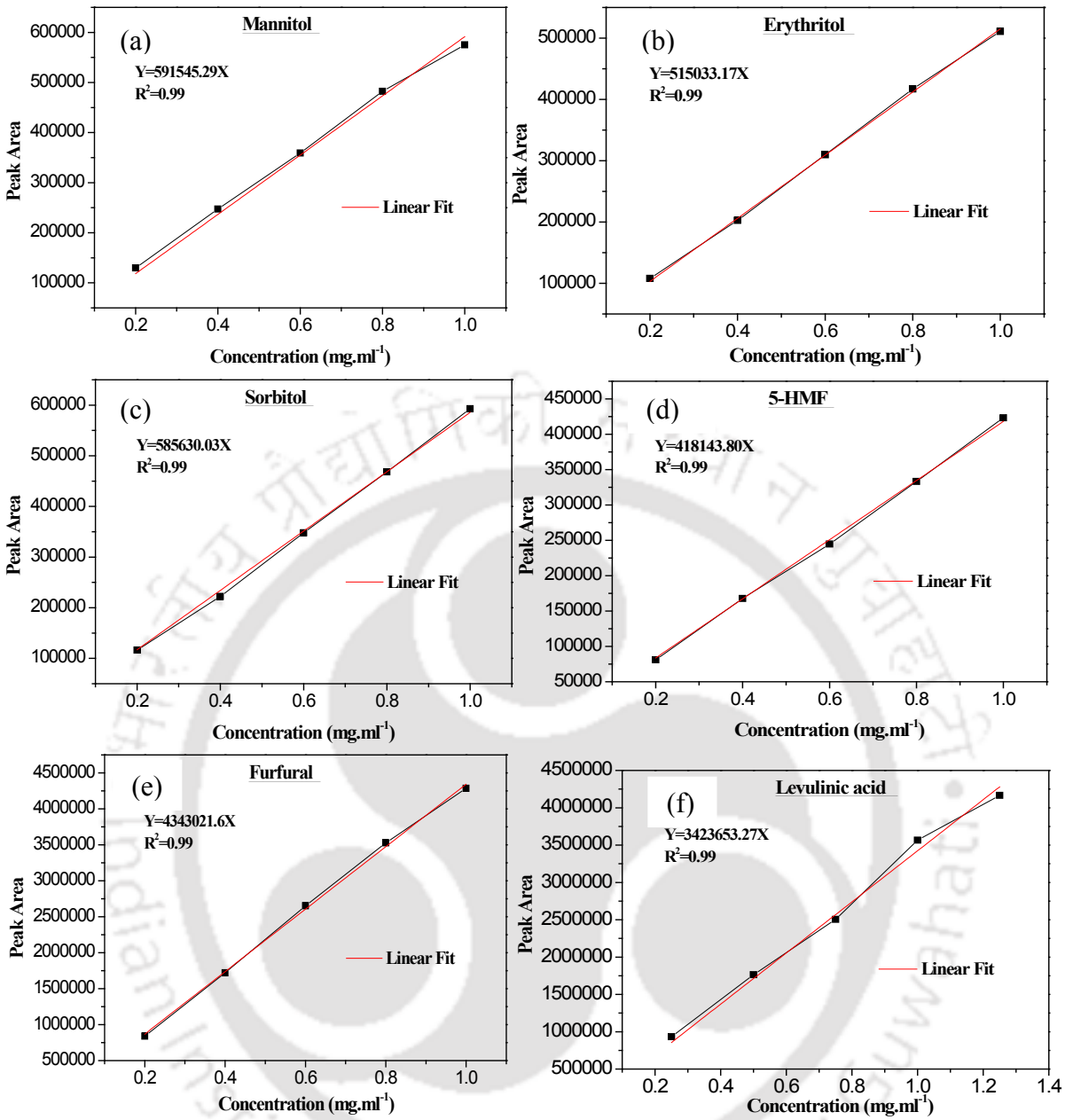

Fig. A3: Calibration plot of standard samples (>98% purity) : (a) Mannitol; (b) Erythritol; (c) Sorbitol; (d) 5-HMF; (e) Furfural; (f) Levulinic acid.

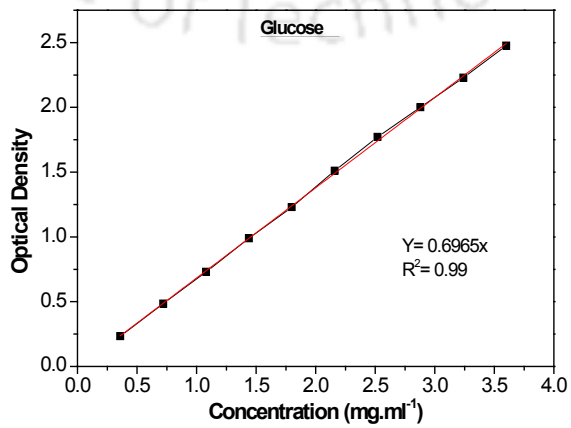

Fig. A4: Calibration plot of Glucose (>98% purity) prepared using UV-Vis.

APPENDIX – B

Error Analysis

B.1. Error is usually an "uncertainty" in scientific determination due to unavoidable imprecision in measurements. It does not signify a mistake. When experiments are repeated, then observation of the fluctuation in the results can be noticed. More often in laboratory, average data's are reported to indicate the actual value. Error analysis ascertains the calculations accuracy and reliability of the accounted data obtained during experimentation. Errors could be either systematic or random. Systematic errors influence in measurement which leads to the situation where the mean of separate measurements differs significantly from the actual value. All measurements are prone to systematic errors. Systematic error might occur due to imperfect calibration of measurement instruments (zero error); changes in the environment which interfere with the measurement process; and imperfect methods of observation. On the other hand, Random errors are inherently unpredictable errors in measurement that lead to measurable values being inconsistent when repeated measures of a constant attribute or quantity are taken. Random error is directly related to the precision of measuring instrument. Higher precision of the instrument will give smaller variability of fluctuations in its readings. The word random indicates that they have null expected value. They are scattered about the true value, and tend to have null arithmetic mean when a measurement is repeated several times with the same instrument.

B.2. Error during analysis

Throughout the study, different concentrations were measured with the help of UV-vis spectrophotometer and High Performance Liquid Chromatography (HPLC). For that, calibration curves were drawn from the known concentrations of the standard sample. Both the instrument provide measurement with three-digit precession after the decimal and during repetition, only fourth digit variation was observed. The coefficient of determination (R^2) for the calibration curves were varied between 0.99 (calibration in HPLC) to 1.0. Thus during the measurement of concentrations, maximum error occurred was 1.00 %.

APPENDIX-C

Table C.1. Sample Calculations for reaction kinetics in SCW hydrolysis of bagasse @140 °C.
Biomass: **Bagasse (3 wt%)**

	mg.g^{-1}	mg.g^{-1}		mg.g^{-1}	mg.g^{-1}		mg.g^{-1}	mg.g^{-1}	mg.3g^{-1}
Hemicellulose	**22.78**	**227.80**	**Cellulose**	**40.15**	**401.50**	**Holocellulose**	**62.93**	**629.30**	**1888 (C_{ao})**

Time (min)	Maltopentaose (mg.ml^{-1})	(mg.g^{-1})	Raffinose (mg.ml^{-1})	(mg.g^{-1})	Sucrose (mg.ml^{-1})	(mg.g^{-1})	Glucose (mg.ml^{-1})	(mg.g^{-1})	Xylose (mg.ml^{-1})	(mg.g^{-1})	Arabinose (mg.ml^{-1})	(mg.g^{-1})	Fructose (mg.ml^{-1})	(mg.g^{-1})	Total (mg.g^{-1}) "C_b"
5	**1.05**	34.98	**0.04**	1.29	**0.358**	11.94	**0.709**	23.63	**1.025**	34.15	**0.374**	12.47	**0.006**	0.21	**118.65**
10	**1.06**	35.38	**0.05**	1.54	**0.364**	12.15	**0.842**	28.08	**1.100**	36.67	**0.400**	13.33	**0.005**	0.16	**127.31**
15	**1.08**	35.94	**0.06**	1.94	**0.450**	15.00	**0.860**	28.66	**1.050**	35.00	**0.621**	20.70	**0.002**	0.06	**137.31**
20	**1.11**	37.03	**0.06**	2.02	**0.700**	23.33	**0.902**	30.06	**1.101**	36.70	**0.671**	22.37	**0.003**	0.09	**151.60**
25	**1.14**	38.08	**0.07**	2.17	**0.800**	26.67	**0.916**	30.53	**1.150**	38.33	**0.705**	23.50	**0.001**	0.04	**159.32**
30	**1.16**	38.81	**0.09**	3.10	**0.700**	23.33	**0.853**	28.44	**1.800**	60.00	**0.635**	21.17	**0.005**	0.16	**175.01**
35	**1.04**	34.63	**0.04**	1.41	**0.750**	25.00	**0.857**	28.58	**1.939**	64.63	**0.614**	20.47	**0.003**	0.10	**174.82**
40	**1.01**	33.62	**0.04**	1.26	**0.845**	28.17	**0.977**	32.56	**1.788**	59.60	**0.575**	19.17	**0.005**	0.17	**174.56**

Time (min)	5HMF(mg.ml^{-1})	(mg.g^{-1})	Furfural(mg.ml^{-1})	(mg.g^{-1})	Total (mg.g^{-1}) "C_c"
5	**0.054**	1.793	**0.003**	0.085	**1.878**
10	**0.057**	1.895	**0.003**	0.096	**1.991**
15	**0.057**	1.903	**0.003**	0.086	**1.989**
20	**0.058**	1.942	**0.003**	0.093	**2.036**
25	**0.066**	2.211	**0.003**	0.099	**2.311**
30	**0.097**	3.234	**0.004**	0.126	**3.359**
35	**0.107**	3.559	**0.005**	0.152	**3.711**
40	**0.108**	3.593	**0.006**	0.196	**3.789**

Time	Cao	Cb	Cc	Ca	Xa	Ln(1-X)
5	**1888**	**118.65**	**1.88**	**1767.37**	**0.06**	**0.07**
10	**1888**	**127.31**	**1.99**	**1758.60**	**0.07**	**0.07**
15	**1888**	**137.31**	**1.99**	**1748.60**	**0.07**	**0.08**
20	**1888**	**151.60**	**2.04**	**1734.26**	**0.08**	**0.08**
25	**1888**	**159.32**	**2.31**	**1726.27**	**0.09**	**0.09**
30	**1888**	**175.00**	**3.36**	**1709.54**	**0.09**	**0.10**
35	**1888**	**174.82**	**3.71**	**1709.37**	**0.09**	**0.10**
40	**1888**	**174.56**	**3.79**	**1709.56**	**0.09**	**0.10**

Publications

1. R.Timung, M.Mohan, B.Chilukoti, S.Sasmal, T.Banerjee, V.V.Goud, (2015). Optimisation of dilute acid and hot water pretreatment of different lignocellulosic biomass: A comparative study. **Biomass & Bioenergy**. 81, 9-18.
2. M.Mohan, R.Timung, N.N.Deshavath, T.Banerjee, V.V.Goud, V.V.Dasu, (2015). "Optimisation and Hydrolysis of Cellulose under SCW Treatment for the Production of Total Reducing Sugars". **Royal Society of Chemistry Advances**. 5, 103265-103275.
3. R.Timung, C.R. Barik, S.Purohit, V.V.Goud, (2016). Composition and anti-bacterial activity analysis of citronella oil obtained by hydro distillation: Process optimisation study. **Industrial Crops and Products**. 94, 178-188.
4. R.Timung, N.N.Deshavath, V.V.Goud, V.V.Dasu, (2016). Effect of subsequent dilute acid and enzymatic hydrolysis on reducing sugar production from sugarcane bagasse and spent citronella biomass. **Journal of Energy**. Article ID-8506214, 1-12.
5. R.Timung, V.V.Goud, (2018). Subcritical Water hydrolysis of spent Java citronella for the production of reducing sugar. **Materials Today**. Proceedings 5(11), 23128-23135.

Research articles under Review

1. R.Timung, N.N.Deshavath, V.V.Goud, V.V.Dasu, (2016). Comparative study on the enzymatic hydrolysis of alkaline pretreated biomass of sugarcane bagasse and spent citronella for reducing sugar production.
2. R.Timung, M.Mohan, V.V.Goud, (2017). Optimisation and reaction kinetics study on SCW hydrolysis of sugarcane bagasse for production of reducing sugar.
3. R.Timung, V.V.Goud, (2017). Extensive characterisation of Java citronella (*Cymbopogon winterianus* Jowitt) biomass for feasible feedstock towards value added production.

International/National conferences

International Conference

1. R.Timung, N.N. Deshavath, V.V.Goud, V.V.Dasu, Hydrolysis of sugarcane bagasse to produce reducing sugar for bioethanol production, Indo-US Conference on "Advanced Lignocellulosic Biofuels (Indo-US CALB- 2014)", 10-11 November, 2014, CSIR-IICT, Hyderabad, India.

2. R.Timung, V.V.Goud, SCW hydrolysis of spent Java Citronella biomass for production of reducing sugar, 5th International Conference of Advances in Energy Research, 15-17 December, 2015, IIT Bombay, Mumbai, India.

3. R.Timung, M. Mohan, T. Banerjee, V. V. Goud, Production of reducing sugars from sugarcane bagasse by SCW hydrolysis, 1st International Conference on Bioscience and Biotechnology " Molecular Life Sciences for the Development in the 21st Century", BioTech-2016, The International Institute of Knowledge Management (TIIKM), 12-13 January, 2016, Colombo, Srilanka.

4. R.Timung, N.N.Deshavath, V.V.Goud, V.V.Dasu, Comparative study on the enzymatic hydrolysis of alkaline pretreated biomass of sugarcane bagasse and spent citronella for reducing sugar production, International Conference on waste management, "RECYCLE 2016", 1-2 April, 2016, Indian Institute of Technology Guwahati, Guwahati, Assam, India.

5. R.Timung, C.R. Barik, S.Purohit, V.V.Goud, Anti-bacterial properties of hydrodistilled Java citronella (*Cymbopogon winterianus* Jowitt) essential oil. 3rd International Conference on Natural Products Utilisation - From Plants to Pharmacy Shelf, 18-21 October, 2017, Bansko, Bulgaria.

6. R.Timung, V.V.Goud, (2018). Comparison of essential oil extraction from Java citronella using Hydro distillation, Steam distillation, Ultrasonic assisted hydro and steam distillation. BIODIVERSE 2018; International symposium on biodiversity and biobanking, 27-29 January, 2018, IIT Guwahati, Assam, India.

7. R.Timung, V.V.Goud, (2020). Optimisation of Levulinic acid production from sugarcane bagasse and spent citronella biomass. International E-Conference on Recent Transformations in Chemical & Textile Technology, 24-26 August, 2020,

University Institute of Chemical Technology K. B. C. North Maharashtra University, Jalgaon in association with Uttar Pradesh Textile Technology Institute, Kanpur.

National Conference

1. C.R.Barik, R.Timung, L. Sahoo, V.V.Goud, Promising Monocot Plants of North Eastern India for Biomass Energy Production: A Solution to the Energy Crisis, Reflux 2014, 29-30 March, 2014, IIT Guwahati, Guwahati, India.

2. N.N.Deshavath, R.Timung, V.V.Goud, V.V.Dasu, Dilute acid pretreatment of bamboo for the production of fermentable sugars, National Conference on "Sustainable Development of Environmental Systems (NCOSDOES- 2014)", 20-21 June, 2014, IIT Guwahati, Guwahati, India.

3. R.Timung, V.V.Goud, Essential oil extraction from java citronella (*Cymbopogon winterianus Jowitt*) using hydro distillation", International Conference on "Chemical Engineering- Emerging Dimensions and Challenges Ahead & Indo Japanese Symposium on Separation Technology for Green Environment", in Indian Chemical Engineering Congress (CHEMCON- 2014), 27-30 December, University Institute of Chemical Engineering & Technology, Panjab University, Chandigarh, India.

4. R.Timung, V.V.Goud, Subcritical water treatment of sugarcane Bagasse for production of fermentable sugars, Frontier Energy Research with Industry Acamedia Pathnership. 20-21 March, 2015, IIT Guwahati, Guwahati, India.

5. R.Timung, M.Mohan, B.Chilukoti, S.Sasmal, T.Banerjee, V.V.Goud, Dilute acid pretreatment of bamboo (*Bambusa cacharensis*) for production of reducing sugar, National Seminar on "Science, Technology and Innovation: Its impact on communities of N.E India", 10-11 September, 2015, Gauhati University, Guwahati, India.

6. R. Timung, V. V. Goud, Estimation of Java Citronella (*Cymbopogon winterianus* Jowitt) composition using Thermo Gravimetric Analysis, 68th Annual Session of Indian Institute of Chemical Engineers, Indian Chemical Engineering Congress (CHEMCON- 2015), 27-30 December, 2015, Indian Institute of Technology Guwahati, Guwahati, Assam, India.

7. R.Timung, V.V.Goud, (2020). Optimisation of a Lignocellulosic biomass pretreatment using Response Surface Methodology. First National Conference on "Application of Mathematical Tools in Social Sciences and Sciences (Online)", 17-18 October, 2020, Zakir Husain Delhi College, University of Delhi.

Workshops attended

1. Participated in Workshop on **"Basic Statistics Using Software"** conducted by Applied Statistics Unit of Indian Statistical Institute, Kolkata, 14-18th August, 2014. NIT Silchar, Assam, India.
2. Participated in National workshop on **"Technical Writing"** conducted under Technical Education Quality Improvement Programme sponsored by the ministry of Human Resource Development, Government of India held on 6-7th December, 2014, IIT Guwahati, Guwahati, India.
3. Participated in Workshop on **"X-ray diffraction systems and related applications"** containing XRD basics, experimental techniques & data analysis using High score plus software conducted by PAN analytical, 11-12th September, 2014, IIT Guwahati, Guwahati, India.
4. Participated in Online One Day Workshop on '**Research Methodology**", organised on 29th October 2020, by Department of Commerce and Economics, Vinayakrao Patil Mahavidyalaya, Vaijapur, Dist. Aurangabad, Under UGC Scheme STRIDE Component – I (Research Capacity Building).
5. Participated in a workshop titled "**FUEL CELL TECHNOLOGY FOR SUSTAINABLE DEVELOPMENT**", conducted on 2nd October, 2020 in Collaboration with IEEE, IIE and University of Lahore.
6. Participated in 2-Day National Workshop on **Citations, References and Research Ethics** conducted held on 13-15th September 2020, by Central Library, Banwarilal Bhalotia College, Asansol in collaboration with Internal Quality Assurance Cell, Asansol Girls College, Asansol.
7. Participated in national e-Workshop on "**Hand-On Practice on Different Tools Used in Chemistry**" on Dated 08th March, 2021, Organised by Shri Rajendra Govt. College, Sardarpur-Rajgarh, Dhar, Madhya Pradesh, India under the aegis of MPHEQIP (World Bank Project) and IQAC Cell.

8. Participated in One week Workshop on '**Research Methodology**", organised during 01-07 February 2021, by Govt. Auto. Girls P.G. college of Excellence, Sagar, Madhya Pradesh.
9. Participated in national Workshop on "**Skills and Employability**" on 08th March, 2021, Organised by Internal quality Assurance Cell (IQAC) and Training & Placement Cell, Smt. Radhadevi Goenka College for Women, Akola, Maharastra.
10. Participated in national Workshop on "**Mera Sapno Ka Bharat-Atmanirbhar Bharat"** on 12-13th February, 2021 organised by S.S. Jain SUBODH P.G. Autonomous College, Jaipur.
11. Participated in national Workshop on "**How to Plan Research"** on 03rd October, 2020 organised by Research and Development Cell of G.H. Raisoni University, Amravati.
12. Participated in One Day Workshop on '**Virtual Labs**", organised on 30th December 2020, by Government Degree College, Ganderbal, Jammu & Kashmir.
13. Participated in One Day Workshop on "**Technology Commercialisation**", organised on 15th February, 2021 organised by Turnip Innovations facilitated by Prof. Man Singh, Dean, and Central University of Gujarat.

Awards/Recognition

1. **Best Paper award** in the International Conference on waste management, "**RECYCLE 2016**", 1-2 April, 2016, Indian Institute of Technology Guwahati, Guwahati, Assam, India.

Certification

1. Participated in National Conference on **"Recent Advances in Cancer Biology and Therapeutics (RACBT)",** 5th December, 2014. IIT Guwahati, Guwahati, India.
2. Participated in National School on sustainable polymers & First symposium on **Advances in Sustainable Polymers (ASP-14)**, 6-11 January, 2014, IIT Guwahati, Guwahati, India.
3. Participated in the "**IIT G-KIT Joint Symposium on Biobased Materials**", 20th January, 2015 IIT Guwahati, Guwahati, India.
4. Participated in Second symposium on **Advances in Sustainable Polymers (ASP-14)**, 21-22 January, 2015, IIT Guwahati, Guwahati, India.
5. Participated in IIT G Entrepreneurship Summit organised by EDC-IIT Guwahati, 13-15 March, 2015.

6. Participated in the National Symposium on "IPR **in Innovation and Entrepreneurship**" conducted under Technical Education Quality Improvement Programme sponsored by the ministry of Human Resource Development, Government of India, held on 16th March, 2015. IIT Guwahati, Guwahati, India.
7. Participated in the "**Research Conclave**" organised by the PhD council of the Students' Academic Board (SAB), 23-26 March, 2015, IIT Guwahati, Guwahati, India.
8. Participated in "**Research Scholars Congress-2015**" held on 23-24 May, 2015, organised by Research Scholars Forum-EEE, IIT Guwahati and conducted under Technical Education Quality Improvement Programme sponsored by the ministry of Human Resource Development, Government of India.
9. Participated in "**Research Wave Competition**", held on October 11th, 2015, organised by Association of Civil Engineering in association with Student Alumni Interaction Linkage cell (SAIL), IIT Guwahati.
10. Participated in the Webinar Session "**Application of Statistics**", held on 18th August, 2020, organised by Department of Mathematics, Michael Job College of Arts and Science for Women, Coimbatore, Tamil Nadu.
11. Participated in the Webinar Session "**MOLECULAR MARKERS BASED VARIATIONS IN PLANTS: SNPs METHOD**", held on 21st August, 2020, organised by Department of Zoology, Michael Job College of Arts and Science for Women, Coimbatore, Tamil Nadu.
12. Participated in the Webinar on "**How to crack GATE exam**", held on 22nd August, 2020, organised by Department of Mathematics, Nehru Institute of Technology, Coimbatore, Tamilnadu.
13. Participated in webinar on "**Basics of GCMS, Instrumentations and Applications**", held on 22nd August, 2020, organised by Post Graduate and Research Department of Chemistry, Maharaja's College, Ernakulam, Kerala.
14. Participated in the International Webinar Session "**Approaches to Online Teaching, Learning and Evaluation during COVID 19**", held on 26th August, 2020, organised by IQAC Cell, Michael Job College of Arts and Science for Women, Coimbatore, Tamil Nadu.
15. Participated in "**Patent Protection for Innovation Driven Educational Institutions**", held on 29th August, 2020, organised by Turnip Innovations, in association with The Frontiers Legal.

16. Participated in **National Level E-Conference on "Nutrition and physical fitness for healthy life",** organised on 15th September 2020, by the College of Horticulture, UHS Campus, GKVK post, Bengaluru, Karnataka, India.
17. Participated in the **One Day International Webinar on the Topic "Advances in Material Physics "**, held on 23rd January 2021, organised by RYAN publishers.
18. Participated in National Technical meet on "**Micro and Nano fabrication and characterisation techniques**", held on 9th November 2020, organised by Department of Physics, Kamaraj College, Tamil Nadu.
19. Participated in the One Day National Level Webinar on "**Contemporary research: Navigating the maze**" organised on 31st October 2020, by the Faculty of Humanities and Science, Adayalampattu Phase – II campus, Tamil Nadu.
20. Participated in the International online webinar titled "**DATA ANALYTICS FOR NON-CODERS**", on 31st October 2020 Organised by Department of Commerce Manipal Academy of Higher Education, Manipal.
21. Participated in the one day National webinar entitled "**Intellectual Property Rights and Patent Process**" held on 24th October 2020, Organised by G. H. Raisoni Institute of Business Management, Jalgaon in Collaboration with R, GNIIPM.
22. Participated in the one day International Webinar on "**Advances in water and transport intensive industry for sustainable development**" on 23rd October 2020, organised by International research and Collaboration Cell (IRCC) of Shri Venkateshwara Group of Institutions.
23. Participated in the invited lecture series SIAS Cognise Logue: 2020-21 on "**Biomass based (bio) refineries for renewable biofuels and chemicals**", Organised on 24th October 2020, by the Department of Microbiology in association with IQAC, SAFI Institute of Advanced study, Malappuram.
24. Participated in One Day National Level Webinar on "**Importance of Statistics in Today's Scenario**" an IQAC Initiative, Organised on 20th October, 2020, by the Department of Statistics, Smt. Bangaramma Sajjan Arts, Commerce & Science College for Women, Vijayapur, Karnataka.
25. Participated in One Day International Webinar on "**Research paper Publication in Scopus Indexed Journals**" held on the 17th of October, 2020, organised by RYAN publishers.

26. Participated in National Level Webinar on "**Recent Trends in Materials Chemistry**" Organised on 15-17th of October, 2020, by the Department of Chemistry RGUKT, R K Valley.
27. Attended the Rayat-Bahra Connect Webinar Series 2020 on "**Characterization Techniques for Nanoparticles & Data Analysis**" on 13-14th October, 2020.
28. Participated in National e-Seminar on Advances in "**Physical and Mathematical Science**s" on 10th October 2020, organised by Manoharbhai Shikshan Prasarak Mandal, Armori, Gadchiroli, Maharastra.
29. Participated in International Webinar on "**Innovative Research on Chemical Science for Sustainable Development**", Organised on 24th September 2020, in collaboration with Department of Chemistry and IQAC of KRM, Deoni and IQUAC of KMC College, Khopoli.
30. Participated in National Webinar on "**Access E-Resources for Academic and Research Excellence**" organised on 9th October 2020, by IQAC & Library and Information Centre at JSS College for Women, Saraswathipuram, Mysuru, Karnataka.
31. Participated in the seminar on "**Converters for Renewable Energy System**" organised on 7th October 2020, by the Department of Electrical and Electronics Engineering, Arasu Engineering College, Kumbakonam through online pedagogy.
32. Participated in One Day National Webinar on "**How to Write Technical Research Paper**" Organised on 29th September 2020, by IQAC and Department of Commerce and Management ATNCC Shivamogga,.
33. Participated in the Webinar on "**Recent Problems in Energy & Environment: Their Solutions**", on 22nd September 2020, sponsored by UD TEQIP-III and organised by University Department of Electronics Engineering, Rajasthan Technical University, Kota.
34. Participated in one day National Webinar on "**Spectroscopic Techniques**", organised on 13th September 2020, by Department of Chemistry, Yogeshwari Mahavidyalaya, Ambajogai.
35. Participated in International Webinar on '**Mathematical Applications in Human Cognition and Neuroscience**" organised on 16th September, 2020, by Department of Mathematics, Assam Don Bosco University, Guwahati, Assam, India

36. Participated One Day National Webinar on "**Recent Advances in Carbon Nanomaterials**" held on 16th September, 2020, organised by Department of Physics, Dr. Ambedkar Institute of Technology, Bengaluru-56.
37. Participated in International Webinar on "**Analytical Instrumentation on Bio Chemical Studies and Research**" held on 11-12th September, 2020, organised jointly by Gulf Bio Analytical Group of Companies, Dubai and Life Sciences and Chemical Sciences Department, St. Thomas College, Bhilai, C.G. India.
38. Participated in International Webinar on "**Advances in Material Physics**" held on 23rd January, 2021, organised by RYAN publishers.
39. Participated in International Webinar on "**Crisis of Security Challenges: A Global Concern**" held on 15-16th January, 2021, organised by Department of Strategic studies, Tuljaram Chturchand College of Arts, Science and Commerce, Baramati, Maharastra.
40. Participated in the International Webinar on "**Enhancing Effective Communications Skills**", March 8, 2021, organised by Balaji Institute of Management Sciences, NARSAMPET, Warangal, Telangana.
41. Participated in International e-conference on "**Emerging Trends & Challenges in Chemical Sciences**" held on 10th March, 2021, organised by Department of Chemistry, Baburaoji Adaskar Mahavidyalaya Kaij, Beed (MS), India.
42. Participated in National e-conference on "**Advanced Research in Materials Sciences**" held during 22-23rd February, 2021, organised by Department of Physics (SF), Kamaraj College-628003, Thoothukudi, Tamilnadu.
43. Participated in International e-conference on "**Emerging Trends in Life Sciences**" held on 27th February, 2021, organised by Department of Botany, Baburaoji Adaskar Mahavidyalaya Kaij, Beed (MS), in Association with Microbiologist Society, India.
44. Participated in UGC-CPE sponsored one-day national symposium on "**Nano Science & Nano Technology**" held on 31st January, 2021, organised by Department of Physics, Science College, Nanded, Maharastra, India.
45. Participated in AICTE sponsored one week Short Term Training Program (Part I) on the "**Pedagogic Approach for Effective Teaching Learning through Outcome Based Education**" held during 01-06th February, 2021, organised by Department of Mechanical Engineering, Anjuman College of Engineering and Technology, Sadar Nagpur, Maharastra.