PRODUCTION OF LEVULINIC ACID FROM SUGARCANE BAGASSE

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By

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PREFACE

The work described in this thesis was performed by the author under the supervision of Professor N. Deenadayalu at Durban University of Technology, Durban, South Africa, from 2014-2015. The study presents original work by the author and has not been submitted in any form to another university. Where use is made of the work of others, it has been clearly stated in the text.

Signed: Date:

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Prof. N. Deenadayalu (Supervisor)

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DEDICATION

This thesis is dedicated to my late father Mr G.C. Mthembu, thank you for being a good father. My mother Mrs C.O. Mthembu, thank you for being the best mama, encouraging and supporting me and lastly but not least my brother Mr Z.S. Mthembu, thank you for being a good example and for encouragement. I love you all

"Humble yourselves therefore under the mighty hand of God, that he may exalt you in due time." _1 Peter 5:6

ABSTRACT

The main aim of this work was to produce levulinic acid (LA) from sugarcane bagasse (SB) and since there is approximately 3 000 000 tons of bagasse produced per annum by 16 factories that are located on the north coast of Kwa-Zulu Natal, after the extraction of sugar.

For this project fructose was firstly used for the production of LA, thereafter SB was used to produce LA. Cellulose was extracted from sugarcane bagasse using two types of pre-treatments namely (i) acid-alkali pre-treatment and (ii) liquid hot water (LHW). In the latter method acid hydrolysis and enzymatic hydrolysis was used to hydrolyse cellulose to glucose.

For the acid-alkali pre-treatment work, two types of bagasse was used namely (i) mill-run bagasse and (ii) depithed bagasse and for the LHW a mill-run bagasse (pellets form) was used.

In both pre-treatment methods the glucose solution was then acid catalysed by two different acids (i) an environment friendly acid, methanesulfonic acid (MSA) and (ii) sulphuric acid, producing levulinic acid. The results showed that MSA and sulphuric acid produced almost the same yield of LA but, MSA is preferred for the production of LA since it is less toxic and less corrosive than sulphuric acid.

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^{o}C	-	Degrees
^{1}H NMR	-	Proton nuclei magnetic resonance
5-HMF	-	5-(Hydroxymethyl) furfural
CAGR	-	Compound annual growth rate
DNS	-	3,5-dinitrosalicylic acid
DSC	-	Differential scanning calorimetry
FPU/ ml	-	Filter paper unit per milliter
FTIR	-	Fourier transforms infrared spectroscopy
g	-	Grams
GC-MS	-	Gas chromatography mass spectroscopy
h	-	Hour
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
k	-	Kelvin
Kg	-	kilogram
LA	-	Levulinic acid
Meq	-	Milliequivalent
Min	-	Minute
SB	-	Sugarcane bagasse

SEM	-	Scanning electron microscopy
TGA	-	Thermogravimetric analysis
XRD	-	X-ray diffraction
DoE	-	Design of Experiment
gds	-	grams dry substrate

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 Introduction

There are numerous companies that utilize sugarcane to produce sugar such as Huletts, Illovo, and TSB etc. These sugar industries produce tons of sugarcane bagasse per annum; the sugarcane bagasse is already used for power in sugar mills and also in paper industries. Currently, intensive research on bagasse is based on the important products which can be produced from sugarcane bagasse such as levulinic acid (LA). What makes LA more interesting compared to other products that can be produced from sugarcane bagasse is that it is very highly reactive. LA can react with other compounds to produce products for pharmaceutical, biological active material, polymers, adsorbents, personal care products, batteries, coatings, electronics, photography, plasticisers, flavouring agents, corrosion inhibitors, anti-freeze agents, antifouling compounds, fuels, herbicides and solvents (Bozell *et al*. 2000; Kingler and Wolfgang. 2005; Rackemann and Doherty. 2011).

The main purpose of this project was to produce Levulinic acid (LA) (4-oxopentanoic acid) also known as 3-acetylpropionic acid is a linear C5-alkyl carbon chain. Levulinic acid is a widely used industrial chemical with one carbonyl, one carboxyl and α-H in its inner structure (fig. 1.1), which belongs to short chain and non-volatile fatty acid (Dautzenberg *et al*. 2011). Some of the relevant physical properties of LA are given in table 1.1.

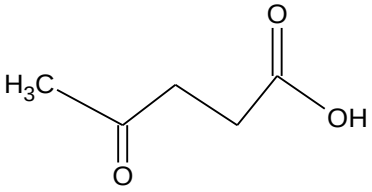


Figure 1.1 The molecular structure of levulinic acid

When analysing the structure of LA, it can be seen that the carbon-oxygen double bond from carbonyl group has a strong polarity, while oxygen atom has a stronger attracting electron ability compared to the carbon atom, so the π electron will transfer into the greater electronegative oxygen, thus leading to the formation of positive charge centre in carbon atom. The electrophilic center of the carbon atom plays an important role when the carbonyl group performs chemical reaction.

Table 1.1: Physical properties of levulinic acid (Zhang *et al*. 2012; Peng *et al*. 2009)

Molecular weight	**Refractive index[a]**	**Density (kgm^{-3})[a]**	**Pka**	**Melting point (K)**	**Boiling point (K)**
116.2	1.4796	1140	4.5	306-308	518-519

[a] Value given for 293 K

Owing to the relatively strong electron receptor effect of the oxygen atom of the carbonyl group, LA has higher dissociation constants than a common saturated acid, so that its corresponding acidity is stronger (Zhang *et al*. 2012).

LA belongs to a group of compounds, which have several highly active sites, because of that it can be used as a platform chemical for preparing many other high value added products (Zhang *et al.* 2012). Since LA is highly reactive, different kinds of products can be produced from it using different types of chemical reaction such as esterification, halogenation, hydrogenation, oxydehydrogenation, condensation and other chemical reactions.

For example, in the pharmaceutical industry, calcium levulinate which is produced from LA is a calcium supplement that can be made into pills, capsules, or injections (Xue *et al*. 2001); it also serves as the food nutrition enhancer that enhances bone formation and muscular excitability. A certain study was conducted which revealed that calcium levulinate could be subjected to high temperature to form significantly deoxygenated and dense energy products, which have great research value in the study of biomass conversion (Schwartz *et al*. 2010). Non-steroidal anti-inflammatory drugs and medical levulinate also can be made from LA.

In agriculture they use LA for the production of δ-aminolevulinic acid (DALA) which is a photoactivation weedicide of high environmental compatibility, selectivity, biodegradability; it is harmless to crops and human health (Zhang *et al*. 2000, Yang *et al*. 1997). DALA can also be used as defoliant for the treatment of fallen leaves before picking cotton and apples.

The levulinate potash has the advantage of cold resistance, drought resistance, and insect resistance, so it is a highly effective fertilizer. 2-methyl-3-indoleacetic acid, produced from levulinic acid, is a common plant growth hormone that can promote the growth of root and stem (Chang *et al*. 2005).

Levulinates, α-angelica lactone, and gamma-valerolactone (GVL) are made from LA and they can all be used as perfume material and food additives. Levulinates are mainly used for removing nicotine and keeping fruits fresh (He *et al*. 2001); α-angelica lactone is a flavor and cigarette additive (Shieh *et al*. 1973), which gives off a sweet smell by mixing smoke incense, caramel aroma, and chocolate aroma. GVL is widely used as an edible essence and in tobacco flavors, for its soft and lingering fresh fruit aroma, medical aroma and sweet smell (Liu *et al*. 1999).

The diphenolic acid (DPA) is produced from LA, it is a high polymer material and it has a wide practical use and extensive application value in polymeride and other materials (Kitano *et al*. 1975). It can serve as feedstock for the production of water soluble resins that are used in industrial Millipore filter and oil filter paper (Chang *et al*. 2007).

LA and its derivatives have many other applications such as plasticizer, surfactant, softener, cleanser and emulsifier (Bozell *et al*. 2000).

LA can also be used to prepare potential biofuels like methyltetrahydrofuran, valerolactone, ethyl levulinate and also dehydration of levulinic acid produces angelic lactone (George *et al*. 2006).

In recent years, the need for sustainability has constantly driven the chemical industry towards exploring renewable chemistry and biotechnology to develop products from bio-based sources. These products are developed with an objective of using them as "platforms" for manufacturing a host of industrial and specialty chemicals to replace their synthetic counterparts.

LA has long since been identified as such a platform chemical, as a result of this, in spite of its relatively small market size, it has been the subject of significant R&D to act as a precursor to specialty chemicals including fuel additives such as Methyltetrahydrofuran (MTHF), pesticides such as D-amino levulinic acid (DALA), Diphenolic Acid (DPA) and so on.

The technology to produce bio-based levulinic acid is not very complicated, with the most widely used approach being dehydrative treatment of biomass or carbohydrates with acid. Other key technologies include hydrolysis of acetyl succinate esters, acid hydrolysis of furfuryl alcohol and by oxidation of ketones with ozone. Presently, levulinic acid finds applications in pharmaceuticals, pesticides, cosmetics, food additives and minor uses in nylons, synthetic rubbers and plastics (www.grandviewresearch.com, last accessed in March 2015).

LA is used in combination with photodynamic therapy (PDT; special blue light) to treat actinic keratoses, it is a small crusty or scaly bumps or horns on or under the skin that result from exposure to sunlight and can develop into skin cancer of the face or scalp. Aminolevulinic acid is a photosensitizing agent. When aminolevulinic acid is activated by light, it damages the cells of actinic keratosis lesions (AHFS Consumer Medication Information. 2014. The American Society of Health-System Pharmacists, Inc., 7272 Wisconsin Avenue, Bethesda, Maryland, 2015).

A topically administered metabolic precursor of protoporphyrin IX, after topical administration aminolevulinic acid hydrochloride (ALA HCl) is converted to protoporphyrin IX (PpIX), which is a photosensitizer. When the proper wavelength of light activates protoporphyrin IX, singlet oxygen is produced, resulting in a local cytotoxic effect (U.S. Department of Health and Human Services, National Institutes of Health, and National Cancer Institute, 2015).

Sodium levulinate is the sodium salt of levulinic acid. It is used as a preservative and skin conditioning agent in cosmetics and personal care products. Sodium levulinate is also used as a preservative in food, especially fresh meats. It was found that sodium levulinate also inhibit the growth of aerobic microorganisms during storage, while not affecting color or pH levels. It is likely to perform similarly in cosmetics and other skin care formulas, protecting products from the growth of microorganisms without significantly altering the integrity of the other ingredients (https://www.truthinaging.com/ingredients/sodium-levulinate, last accessed in April 2015). Fig. 1.2 shows how LA can be used as a starting material for the production of gamma-valerolactone (GVL), pentanoic acid (PA), 1-butene and hydrocarbon fuels.

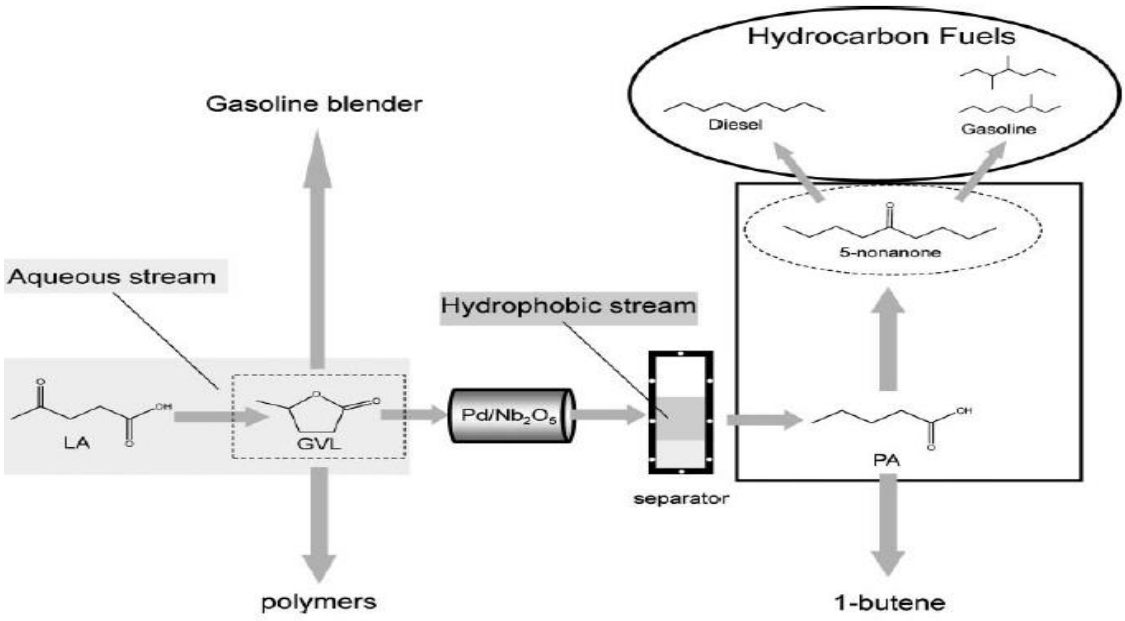


Figure 1.2 Schematic diagram of the applications for the catalytic upgrading of levulinic acid to fuels and chemicals (Serrano-Ruiz *et al*. 2010)

The sugarcane is mainly used for sugar and alcohol production, when the sugarcane stems are milled to obtain the cane juice, which is used for the sugar production. The residual fraction that is left after the extraction of juice is called sugarcane bagasse (SB). The SB consists of components such as: cellulose, lignin, hemicellulose, ash *etc*, as shown in table 1.2. The components of sugarcane bagasse form a very complex structure to provide it resistance from chemical and/or microbial degradation (Gupta *et al*. 2011). By virtue of this strong packaging of the biomass structural polymers, an effective pretreatment method is required to break the lignin and hemicellulose seal and to expose the cellulose for hydrolysis (Gupta *et al*. 2009; 2011; Manzoor *et al*. 2012). Bagasse is an extremely inhomogeneous material comprising around 30 – 40 % of "pith" fibre, which is derived from the core of the plant and is mainly parenchyma material. There are two types of bagasse namely (i) mill-run bagasse which is the original bagasse which still contains the pith whereas the (ii) depithed bagasse is the mill-run bagasse in which the pith is removed. The waste in sugar industries is minimized by using bagasse to form chemical products that are very useful in different areas.

Cellulose is the major constituent of all plants materials including wood, cotton, flax, hemp, jute, ramie, cereal straws, rice straws and sugarcane bagasse. It forms about half to one-third

of plant tissues, it is the most abundant and renewable natural resource on earth (Mandal and Chaknabarty. 2011). For this project the cellulose will be used for the production of levulinic acid from mill-run, depithed and mill-run (pellets form) sugarcane bagasse, fig. 1.3 shows the production of LA from sugarcane bagasse.

Table 1.2: Chemical composition of sugarcane bagasse (% w/w, dry basis)

Components (%)	**Reference**					
	Rainey 2009	da Silva *et al*. 2010	Rocha *et al*. 2011	Rabelo *et al*. 2011	Canilha *et al*. 2012	Chandel *et al*. 2014
Cellulose	47.0	38.8	45.5	38.4	45.0	39.53
Hemicellulose	27.0	26.0	27.0	23.2	25.8	25.63
Lignin	23.0	32.4	21.1	25	19.1	30.36
Ash	1.0	2.8	2.2	1.5	1.0	1.44
Extractives	-	-	4.6	-	9.1	2.90

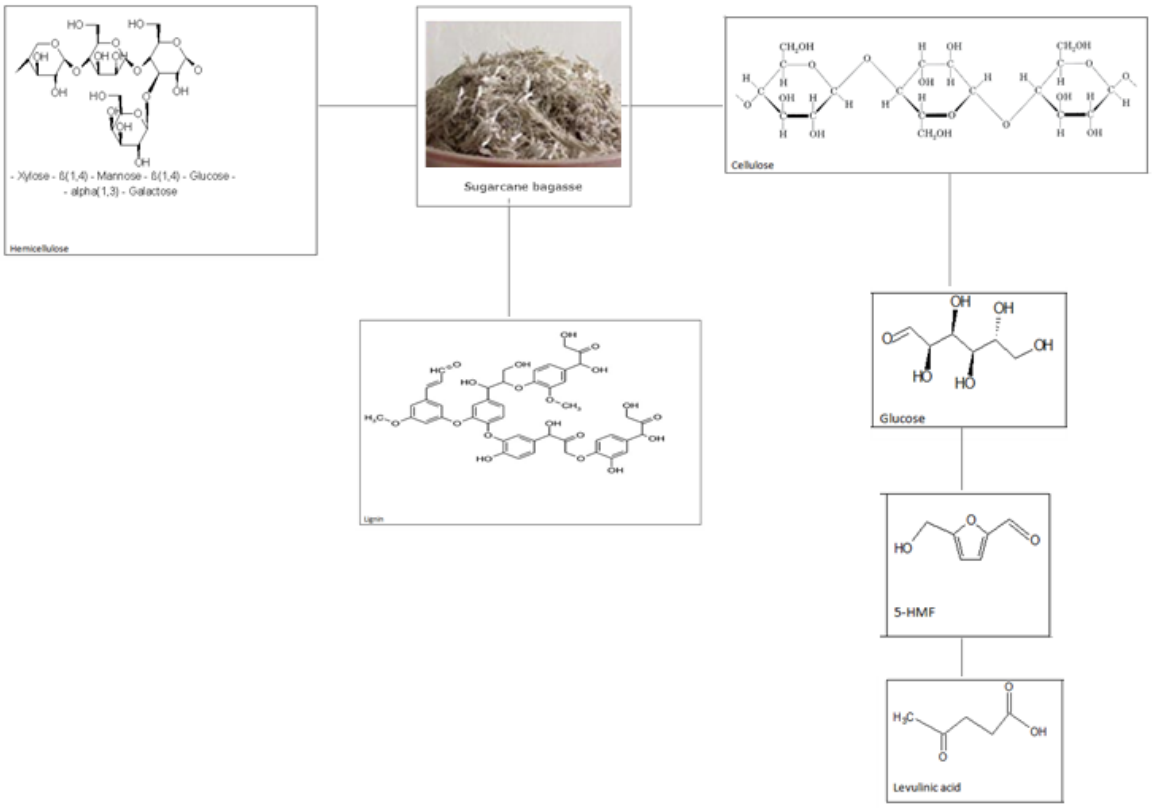


Figure 1.3 Production of LA from sugarcane bagasse

There are many methods that are used for the extraction of cellulose such as:

- Liquid-solid extraction
- Liquid-liquid extraction
- Partitioning
- Acid-base extractions
- Ultrasound extraction (UE)
- Microwave assisted extraction (MAE)
- Solvent extraction
- Enzyme-assisted extraction

However, these extraction methods have drawbacks to some degree. The choice of extraction procedure depends on the nature of the natural material and the components to be isolated. But for SB, alkaline extraction is popular (Wiggins. 1949; Saunders and Blume. 1981).

1.2 Objectives

The main purpose of this work was to produce levulinic acid from sugarcane bagasse using an experimental design that complies with the principles of Green Chemistry. The sugarcane bagasse was pretreated to remove hemicellulose and lignin prior to conversion of cellulose to levulinic acid. Pretreatment that preferentially separate hemicellulose includes dilute acid, hot compressed water (HCW) and steam explosion methods (Girio *et al*. 2010). There are two methods that were used to extract cellulose and glucose from SB in this project:

- Acid-alkali pretreatment - Where acid was used to dissolve the hemicellulose in acid solution from the SB and the base was used to remove the lignin from the acid treated SB. After both pre-treatment, cellulose SB was produced.

- Liquid hot water and enzymatic hydrolysis - The hemicellulose was dissolved in water through LHW method; it only uses water and high temperature of 200 °C. The enzyme was used to remove the lignin and convert cellulose into glucose, from cellulignin produced from the LHW. After both LHW and enzymatic hydrolysis, a glucose solution was produced.

When cellulose or glucose solution is extracted from sugarcane bagasse it can be hydrolysed, usually sulphuric acid is used and hydrochloric acid but for this work both sulphuric acid and methanesulfonic acid were used. The purpose was to observe the difference between the LA yields that will be produced by the two different acids. Methanesulfonic acid was chosen because it is environmentally friendly and less corrosive compared to sulphuric acid.

The optimization of levulinic acid production was conducted using Stat-Ease Design-Expert 8.0.7.1, comparing parameters such as temperature, time and acid concentration for the production of LA to determine which parameters will produce a higher LA yield and also to observe the effect these parameters have on the LA yield. The optimization was done to determine the optimum conditions for the production of levulinic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 Production of LA from Biomass

2.1.1 Methods Used to produce LA from Biomass

There are several routes that have been described for the production of LA from biomass, such as (Mukherjee *et al*. 2015):

- Enzymatic or acid-catalysed conversion of polymeric carbohydrates to hexoses, hexoses (glucose) isomerises to fructose, which is then dehydrated using acid catalyst to HMF, which is then converted to LA.
- Hydrolysis of furfural alcohol.
- Acid treatment of pentoses followed by reduction.
- Ring cleavage of furfural.
- Ozonolysis of unsaturated hydrocarbons.
- Oxidation of 5-methylfurfural with hydrogen peroxide in presence of formic acid.
- Reduction of 4-(diphenylmethylsilyl)butyrolactone with the Grignard reagent (methylmagnesium iodide).
- Conjugate addition of nitroethane to acrolein to form 4-nitropentanal, which is subsequently oxidised to LA.

A large number of reaction systems have been investigated for the production of LA one of the main differences between the various reaction systems is the type of catalyst, solvent used and a range of different feedstocks.

2.1.2 Catalyst used for Production of LA from Biomass

For this work sulphuric acid and methanesulfonic acid were used as a catalyst.

2.1.2.1 Catalyst

Liquid catalysts

Liquid catalysts that are mostly used for the dehydration reaction of converting glucose into LA are:

- Sulphuric acid (H_2SO_4)
- Hydrochloric acid (HCl)
- Phosphoric acid (H_3PO_4)
- Methanesulfonic acid (CH_4O_3S)

H_2SO_4 and HCl are widely used because they are readily available, low cost and higher yield of LA can be produced from them but their disadvantage is that they are highly corrosive and that is not good for industrial use whereas the methanesulfonic acid is a strong acid, non-oxidising, biodegradable acid catalysts that is more environmental friendly, less corrosive and since it is non-oxidising it prevent unnecessary side reaction that can occur when using sulphuric acid and HCl.

Clarkson (1962) reported that HCl is the most preferable catalyst for converting carbohydrate into LA because of two factors: (i) HCl can be recovered and recycled easily and (ii) LA can be separated using simple vacuum distillation.

Volatile acid catalysts such as hydrochloric acid (HCl) provide the simplest LA recovery process and it involves filtering to remove solids and atmospheric/vacuum distillation and steam stripping. This process allows 90-95 % of the acid catalyst and water to be recycled to achieve LA of 95-97 % purity. Alternatively, the acid catalyst can be neutralized and removed as a salt but the ability to recycle catalysts is beneficial for cost-effective production processes. Less volatile acid catalysts such as sulphuric acid, the solvent extraction or reactive extraction separation methods have been used as to improve the separation and purification of LA (Rackemann and Doherty 2011). Ayoub (2008) reported that the reactive extraction methods using water-immiscible alcohols as both the esterifying agent and solvent for LA production removes the need for additional solvents, processing and allow the acid catalyst-water mixture to be easily recycled.

Simulated moving bed chromatography using multiple chromatographic columns has also been reported to recover LA in high purity (Rackemann and Doherty 2011).

2.1.3 Effect of Various reaction Conditions

Numerous investigations have been conducted to determine the effects of various processing conditions on reaction yields such as (Rackemann and Doherty 2011):

- Acid concentration – Reaction yields increase with acid concentration up to a critical concentration limit which depends on other processing conditions and the type of feedstock used. It is known that too aggressive conditions lead to various side reactions and re-polymerization.
- Temperature – The yield of LA from bagasse increases with increasing temperatures in the range 150-230 ^{o}C and many researchers have found the optimum temperature to be from 200-220 ^{o}C.
- Solvent concentration – The optimal concentration for the solvent was found to depend on acid concentration but generally it is less than 90 wt%.
- Residence time – The effect of residence time on yield was found to be dependent on the cellulose content of the biomass source.

2.1.4 Solvents

There are important parameters or factors that have to be considered when choosing a solvent, the parameters to consider are:

- Environmental impact of the solvent
- Mobility
- Acute toxicity for human
- Chronic toxicity for humans
- Acute toxicity for aquatic organisms
- Persistency in environment
- Bioaccumulation

Mukherjee *et al*. (2015) reported that only water is the solvent which is completely suitable in terms of the above parameters and water is the widely used solvent for the production of LA because of it physical and chemical characteristics, and it is environmentally friendly.

2.1.5 Feedstock for LA Production

There are different types of biomass and feedstock used for the production of LA but the biomass of interest for this project is sugarcane bagasse (SB). Table 2.1 shows the different kinds of feedstock that can be used for the production of LA, apparatus such as temperature and concentration. And yield of LA. From the table 2.1 it can be observed that, the bagasse produced the highest yield of LA of 82.7 % when using 4.5 % of HCl at 220 ^{o}C.

Table 2.1: LA yield from different biomass feedstock (Galleti *et al*. 2012; Yan *et al*. 2008; Fang and Hanna 2002; Girusuta *et al*. 2008)

Feedstock	**Cellulose content (%)**	**Acid (meq)**	**Temperature (°C)**	**Yield of LA based on theoretical yield (%)**	**References**
Poplar sawdust	57.6	HCl 37% (11.5)	200	51.8	Galleti *et al*. 2012
Paper sludge	57.1	HCl 37% (11.5)	200	77.0	Galleti *et al*. 2012
Olive tree pruning	39.4	HCl 37% (0.95)	200	66.0	Galleti *et al*. 2012
Wheat straw	39.2	HCl 37% (11.5)	200	68.9	Galleti *et al*. 2012
Tobacco chops	25	HCl 37% (11.5)	200	29.1	Galleti *et al*. 2012
Bagasse	42	HCl 4.5%	220	82.7	Yan *et al*. 2008
Sorghum grain	73.8	H_2SO_4 8%	200	45.6	Fang and Hanna 2002
Water hyacinth	26.3	H_2SO_4 10%	175	53	Girusuta *et al*. 2008

2.2 Sugarcane Bagasse Pretreatment

The sugarcane bagasse (SB) consist of cellulose (40-50%), hemicellulose (25-35%), lignin (15-20%), some extractives and ash (Alonso *et al*. 2010), fig. 2.1 shows the chemical structure of the main components of SB. The main purpose of this work was to produce LA from SB, producing value added product from what was known as a waste from sugarcane industries.

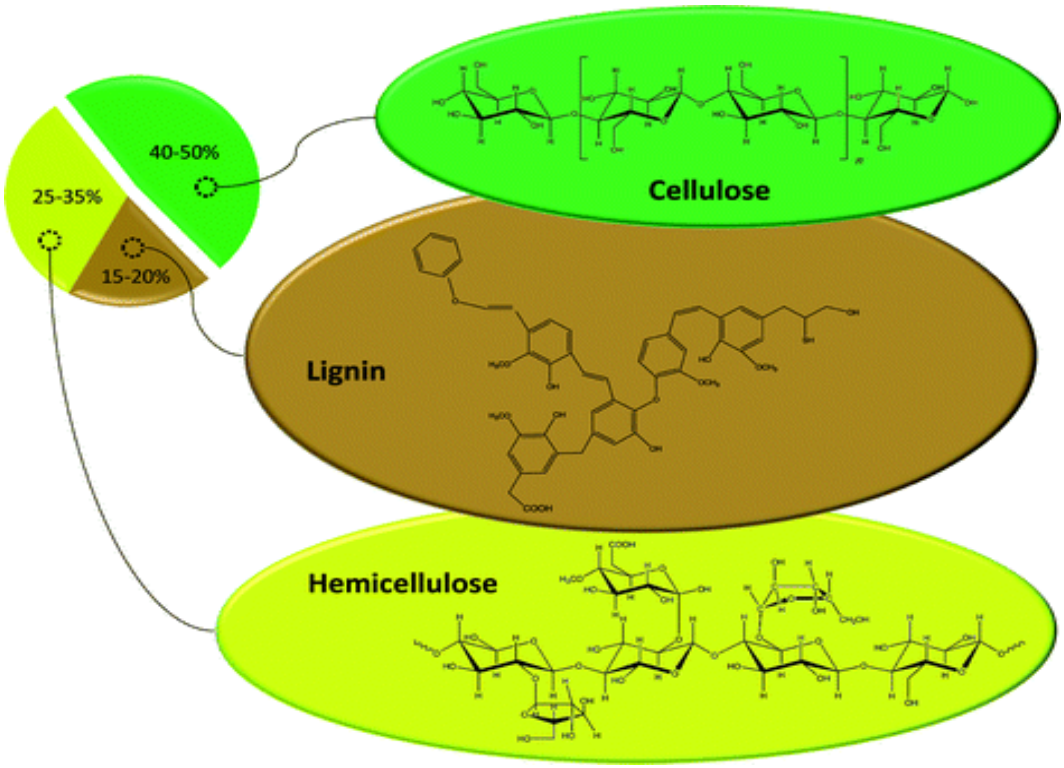


Figure 2.1 Sugarcane Bagasse Composition (Alonso *et al*. 2010).

In order to have access to cellulose which is used for the LA production, a pretreatment is required to remove hemicellulose and lignin (see fig. 2.2).

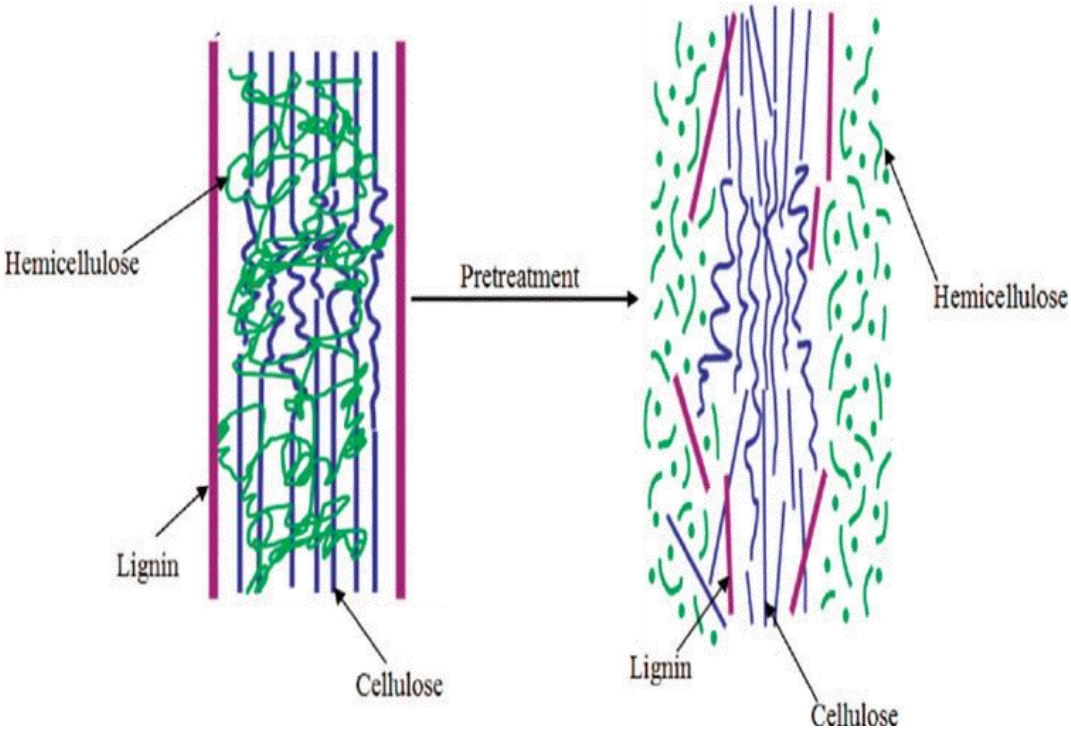


Figure 2.2 Impact of Pretreatment of SB (Kumar *et al*. 2009)

Cellulose is a high molecular weight linear polysaccharides of D-glucose linked by β-1.4 glycosidic bonds with a polymerization degree between 9000 and 15000 joined by a network of inter- and intra- molecular hydrogen bonding and van der Waals forces (Goring *et al*. 1962; Pettersen *et al*. 1984; Rackemann and Doherty 2011).

For the extraction of cellulose in SB, there are many extraction methods available such as: liquid-solid extraction, liquid-liquid extraction, partitioning, acid-base extractions, ultrasound extraction (UE), microwave assisted extraction (MAE). The capability of a number of extraction techniques have been investigated (Wiggins. 1949). For this work pretreatment that were used are (i) acid pretreatment, (ii) alkali pretreatment, (iii) LHW and (iv) enzymatic hydrolysis.

2.2.1 Acid Pretreatment

For the extraction of cellulose in SB, many different pretreatment methods have been used; acid pretreatment is the preferred and oldest method for the solubilization of hemicellulose in biomass. The most commonly used acid is sulphuric acid, which react with biomass to promote hemicellulose breakdown to xylose and other sugars (Mosier *et al*. 2005; Kuhad *et*

al. 2010). The process can be performed at a temperature range from 120-180 °C and residence times ranging from 15-60 min (Alvira *et al*. 2010). Acid pretreatment is preferred because the operation process is at low to medium temperature, resulting to lower energy costs (Girio *et al*. 2010). However, in high concentration the equipment corrosion and expensive costs of maintenance can occur (Alvira *et al*. 2010), therefore dilute acid is widely used for the acid pretreatment. The acid pretreatment products from SB are: (i) solid fraction which consists of cellulose and lignin (cellulignin) and (ii) liquid fraction which have hemicellulose.

2.2.2 Alkali Pretreatment

Alkali pretreatment is applied to the cellulignin from acid pretreatment for the removal of lignin. The alkali pretreatment causes swelling; which leads to an increase in internal surface area, decreasing the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure making cellulose available for enzymatic degradation or for acid hydrolysis (Sarkar *et al*. 2012). In alkali pretreatment different bases can be used such as: sodium hydroxide, calcium hydroxide, potassium hydroxide, ammonia hydroxide, and sodium hydroxide in combination with hydroxide peroxide (Zheng *et al*. 2009). Alkali process is known to cause less sugar degradation and many of the caustic salts can be recovered and/or regenerated. This process also utilizes lower temperature and pressures than other pretreatment technologies and for this work; sodium hydroxide was used as the base. The alkali pretreatment products are: solid fraction which is cellulose and liquid fraction which have lignin.

2.2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis is an ideal approach for degrading cellulose into reducing sugars, mild reaction conditions (pH between 4.8-5.0 and temperature between 45-50 °C) can be used; one of the enzymatic hydrolysis advantage is that it does not cause corrosion in the reactors and result in negligible by-products formation with high sugar yields. However, enzymatic hydrolysis depends on optimized conditions for maximal efficiency (hydrolysis temperature,

time, pH, enzyme loading and substrate concentration) and suffers from end-product inhibition and biomass structural restraints (Canilha *et al.* 2012*).*The enzyme that was used for this work is cellulase. Cellulases are composed of independent folding, structurally and functionally discrete units called domains or modules, making cellulases module. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Kuhad *et al*. 2011). Cellulases break down the cellulose molecule into monosaccharides (simple sugars) such as beta-glucose, or shorter polysaccharides and oligosaccharides. Enzymatic hydrolysis of cellulosic fraction requires three classes of cellulolytic enzymes (cellulases):

(i) Endo-β-1,4-glucanases (EG,E.C.3.2.1.4) which attacks regions of low crystallinity in the cellulose fiber, creating free chain ends.

(ii) Cellobiohydrolases or exoglucanase (CBH,E.C.3.2.1.91) which degrades the molecule further by removing cellobiose units the free chains-ends.

(iii) β-glucosidases (E.C.3.2.1.21) which hydrolyses cellobiose to produce glucose (Canilha *et al.* 2012). The products that were produced from the enzymatic hydrolysis after centrifuge are liquid fraction which has glucose and solid residue which is a waste since all the major components of SB has been removed.

The enzyme hydrolysis was also used for the removal of lignin and conversion of cellulose into glucose from the cellulignin produced from the LHW. The enzyme used for this work is cellic ctech 2. The products for this work are solid fraction which is lignin and liquid fraction which is consist of C6 sugars (glucose and xylose).

2.2.4 Surfactants

Surfactants are compounds that lower the surface tension (or interfacial tension) between two liquids or between a liquid and a solid. Surfactants enhance the enzymatic conversion of cellulose (Kumar *et al*. 2009). Polysorbate 80 is a non-ionic surfactant and emulsifier commonly used in foods and cosmetics. This synthetic compound is a viscous, water-soluble yellow liquid, commonly known by its brand name, tween 80. Polysorbate 80 is derived from polyethoxylated sorbitan and oleic acid. The hydrophilic groups in this compound are polyethers also known as polyoxyethylene groups; they are the polymers of ethylene oxide

(www.wikipedia.com, last accessed in March 2015). Chandel *et al*. (2014) used tween 20 for enzymatic hydrolysis to enhance specific surface area for better enzyme action. Tween 20 is also a non-ionic surfactant like tween 80.

For this work Tween 80 was used to study the effect of surfactant on the saccharification of pre-treated sugarcane bagasse. Tween and PEG are the mostly used non-ionic surfactants to enhance cellulose conversion and that is due to its ability to block non-productive adsorption of cellulase enzymes on lignin matrix hence efficiently increasing the enzymatic hydrolysis of pretreated lignocellulosic biomass. (Zhou *et al*. 2015; Borjesson *et al*. 2007). The role of a surfactant is to lower the surface tension between two liquids or between a solid and a liquid (in the case of this study between pre-treated bagasse and cellulase enzyme); they have both hydrophilic and hydrophobic properties and enhance hydrophobic substances withdrawal by decreasing the surface tension between the two liquids (Qing *et al*. 2010). Adsorption and orientation of the surfactant molecules at the solid-liquid interface should render the substrate readily wettable by the enzyme solution, bringing the substrate quickly into contact with the enzymes and allowing the enzymes to reach inaccessible substrate sites. Moreover, interactions between the sorbed surfactant molecules, the substrate, and the adsorbed enzymes might result in a decrease of the force with which the enzyme is held onto the substrate (Castanon and Wilke 1981). There are two primary mechanisms of surfactant effects that have been suggested: (1) surfactants adsorb to the lignin surface and reduce unproductive enzyme binding and (2) surfactants positively affect cellulase activity and enzymes stabilities (Yang *et al*. 2015).

2.2.5 Thermal Hydrolysis: Liquid Hot Water (LHW) Treatment

LHW is used as a thermal pretreatment of SB pellets. LHW uses only compressed hot water for the removal of hemicellulose and soluble lignin from the SB. Setting the pressure of the process above the vapour pressure of the water at operating temperature maintains the solvent in the liquid state (Mosier *et al*. 2005). The LHW products are: (i) liquid fraction which consist of hemicellulose and soluble lignin (ii) solid fraction which consist of cellulose and lignin (cellulignin). One of the LHW advantages is that it uses only water as a solvent and high temperature therefore it is environmentally friendly. Since LHW uses only water, this reduces the plant complexity as well as the necessity for waste-water treatment and recovery

of chemicals (Liu and Wyman. 2004). LHW has a high potential for a comprehensive utilization of all lignocellulose components. It enables high recoveries of C5-sugars from hemicellulose and lignin with well-preserved structure as well as high yields in the subsequent enzymatic saccharification (Zetzl *et al*. 2012). And there is another method similar to LHW it also uses water as a solvent, it is called hot compressed water (HCW) it is one of the thermochemical methods that has generated a widespread interest in recent years for conversion of bagasse when compared with other pre-treatment methods (Iryani *et al*. 2014).

2.2.6 Design of Experiments (DoE)

Real experiments are often time-consuming and expensive, especially if numerous parameters are used. The scattering of experimental results complicates differentiation between true and apparent effects. In many cases, more experiments are conducted which are more than necessary for reliable statistical analysis. Statistical DoE provides a set of powerful standardized tools for effective planning and evaluation of experimental designs by reducing the number of necessary experiments (Huppop. 2013).

2.3 Production of LA from Bagasse

For this work LA was produced from bagasse but in a process way (see fig. 2.3) firstly a pretreatment is applied for the removal of hemicellulose and lignin and cellulose is acid hydrolysed to glucose, some pretreatment produce glucose from cellulignin and from glucose acid hydrolysis is done which produce LA and formic acid.

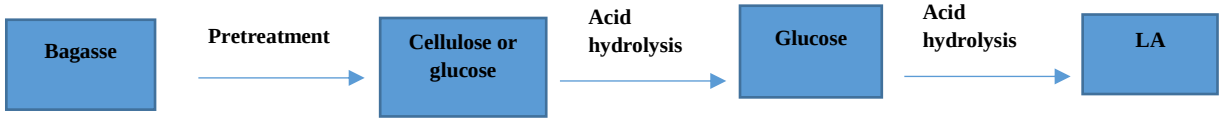


Figure 2.3 Process of producing LA from bagasse

Lifeng *et al.* (2008) produced LA from bagasse by liquefaction in the presence of 4.45 wt% of HCl in water. The effect of reaction temperature, reaction time and water to solid ratios was investigated and it was found that the levulinic acid yield is at a maximum when the temperature is 220 °C; reaction time is 45 min and a ratio of water to solid of 8.5 %. The maximum yield of LA that was obtained for bagasse was 22.8 % (Lifeng *et al*. 2008).

Iryani *et al*. (2013) produced 5-HMF and LA, directly from sugarcane bagasse under hot compressed water. 5-HMF is an intermediate in production of LA; they investigated the effect of temperature and reaction time. It was observed that the degradation of cellulose start at 240 °C (5 min) and it was completely degraded after 20 min at 270 °C, and after 10 min at 300 °C. Cellulose decomposed to form glucose, which further decomposes to form 5-HMF and organic acids (LA, acetic acid and formic acid).

2.4 Production of LA from Cellulose

Cellulose can be hydrolysed in pure water by the electrophilic hydrogen atoms of the H_2O molecule on the glycosidic oxygen, but this reaction is very slow due to cellulose being resistance to hydrolysis, this reaction can be speeded up by using elevated temperatures and pressures or can be catalysed by acids (concentrated or dilute), or by highly selective enzymes such as cellulases (Hayes *et al*. 2006). Cellulose is hydrolysed to glucose; it is then further converted to levulinic acid in the presence of acids (Li *et al*. 2006).

Several kinetics studies have been conducted using carbohydrate (cellulose) as a starting material (Otsuka *et al*. 1973; Feather *et al*. 1973; McKibbins *et al*. 1962; Moye. 1964; Schaufnagel and Rase. 1975; Rackemann and Doherty. 2012) although the mechanism was not clear and numerous intermediates were found:

(i) The carbohydrate is first hydrolyzed by acid catalyst to form glucose

(ii) 5-HMF is the intermediate formed from D-fructose by an overall first order reaction and

(iii) 5-HMF is finally hydrated and cleaved into levulinic acid and formic acid in equal ratios

The production of LA from cellulose is shown in fig. 2.4, where cellulose is acid hydrolyzed to form glucose, the glucose is dehydrated to form 5-HMF and 5-HMF is rehydrated to form LA and formic acid.

Rackemann and Doherty (2012) reported that the maximum theoretical yield of LA is 71.6 wt% from cellulose.

Timokhin *et al*. (1999) and Arato *et al*. (2005) produced LA from the carbohydrate in two different preparation methods:

(i) Acid hydrolysis of hexoses such as glucose, fructose, mannose, or galactose from polymeric carbohydrate, such as cellulose, hemicellulose, starch, or mono- and disaccharides via formation of D-fructose and 5-hydroxy methylfurfural (5-HMF)

(ii) Acidic treatment of pentoses such as xylose and arabinose from hemicellulose to produce furfural, followed by catalytic reduction of furfural alcohol and subsequent ring-opening in water).

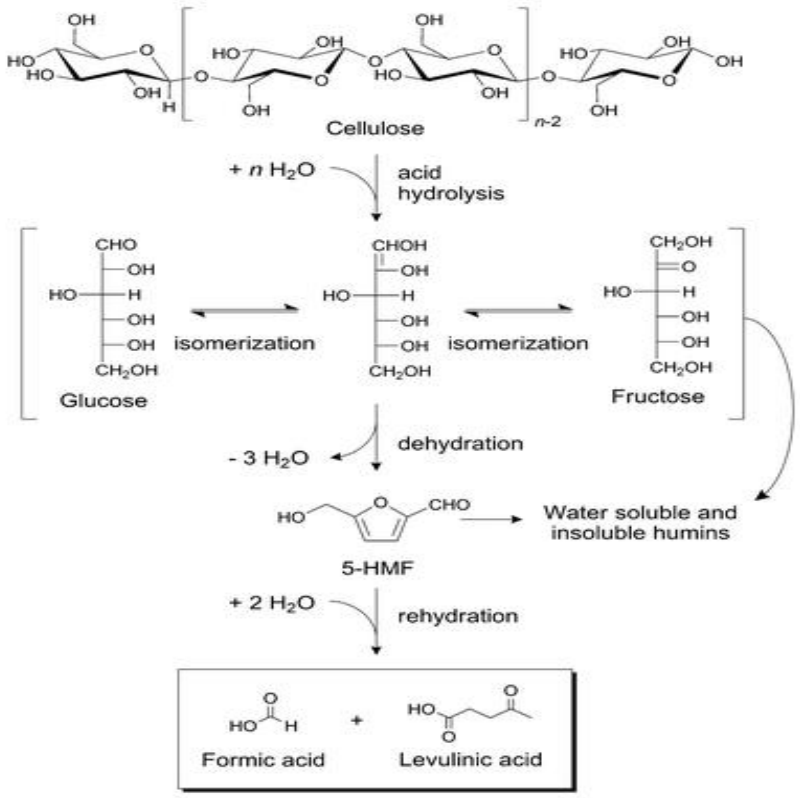


Figure 2.4 Proposed reaction pathways for the acid-catalyzed hydrolysis of cellulose to levulinic acid.

2.5 Production of LA from Sugars

The most abundant hexose sugar found in nature is glucose which is available in the polysaccharides in a form of starch, cellulose in bagasse and in disaccharide as sucrose, derived from glucose or fructose (Rackemann *et al*. 2011). Table 2.2 shows the LA yield produced from sugars

Table 2.2: LA yield from sugars (Kuster and Van der Baan 1977; Chang *et al*. 2006)

Sugars	**Acid (meq)**	**Temperature (°C)**	**Yield of LA based on theoretical yield (%)**	**Reference**
Fructose	HCl 3.6-7.2%	95	~81	Kuster and Van der Baan 1977
Glucose	H_2SO_4 5%	170	80.7	Chang *et al*. 2006

LA is commonly produced from glucose or fructose but it is easier to produce LA from fructose than from glucose. Glucose and xylose were acid catalysed by methanesulfonic acid (MSA) and sulphuric acid to produce LA, where methanesulfonic acid was found to be the suitable catalyst, the highest yield of LA obtained was 63.1 mol % and for sulphuric acid it was 65.4 mol % in same conditions: 0.50 M catalyst, 0.10 M glucose, 180 ^{o}C and for 15 minutes. The MSA is preferred for the production of LA because it is less corrosive and environmental friendly compared with sulphuric acid. It was also observed that the mixture of glucose and xylose produced a slightly higher LA yield compared to glucose solution, although the presence of xylose increases the amount of solid residue formed (Rackemann *et al*. 2014).

Rackemann and Doherty (2011) reviewed the production of levulinic acid and furanics from sugars and they found that organic acids such as LA and their esters are valuable platform chemicals that can be produced through acid-catalysed dehydration and hydrolysis of carbohydrates, the major component of sugar molasses and bagasse, but for the commercial production of LA and furfural from carbohydrates there are problems regarding the use of corrosive mineral acid catalyst which increase equipment and operating cost and can lead to waste disposal issues, they also tested different strong sulfonic acids as replacement for sulphuric acid, they were found to provide similar selectivity to sulphuric acid in the reaction of simple sugars to levulinic acid and furfural. Fig. 2.5 shows the production of LA from

hexose and pentose sugar. Hydroxymethylfurfural (HMF) is an intermediate which is hydrolysed to equal amounts of LA and formic acid, furfural alcohol is hydrolysed to produce LA (Rackemann and Doherty 2012).

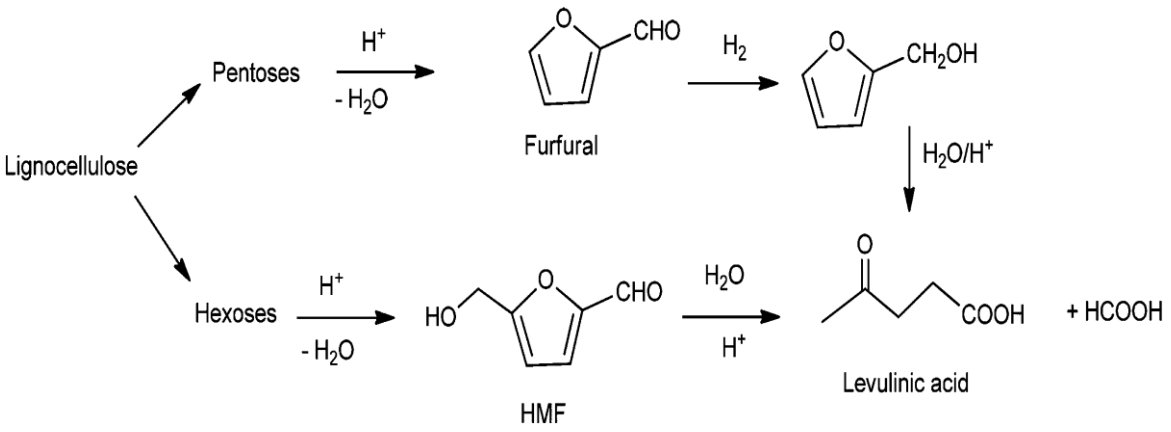


Figure 2.5 Production of LA from pentose and hexose sugars (Rackemann and Doherty 2012)

Girisuta *et al*. (2006) did a kinetic study on the conversion of glucose to LA; the experiments were carried out in temperature range of 140-200 ^{o}C, using sulphuric acid as the catalyst for the concentration range of 0.05-1.0 M and glucose concentration of 0.1 and 1.0 M. It was observed that the yield of LA increases in dilute glucose solution at high acid concentration.

2.6 History of Levulinic Acid

Levulinic acid was first described in 1875 by Freiherrn von Grote and Tollens. The authors obtained the acid by heating sugar candy in equal amounts with concentrated acid in water for several days. Formic acid water and large amount of humin were obtained for this reaction. The authors gave this substance the name levulinic acid because the levorotary fructose, called levulose, was the reactant for the acid generation.

Previously, Malaguti (1836) and Mulder (1840) had reacted sucrose with different acids, concentrated or diluted and they didn't discover LA. After 1875, many investigations were conducted using different routes and it was said that LA did not reach the commercial use in significant volumes (Dautzenberg *et al*. 2011), although it was recognized as an industrial chemical intermediate because of the functional groups present in the structure of LA such as oxygen which have unbonded electrons, which makes this compound to be highly reactive and it is capable of donating or accepting electrons.

Since 1956 LA was regarded as a platform chemical with high potential (Leonhard. 1956). However, the relatively cost-intensive production that proceeded through the dehydration of hexoses, formation of 5-hydroxymethyl-furfural and successive cleavage of one-mole formic acid, foreclosed the possibility that LA chemistry could compete with other chemical intermediates derived from fossil raw material.

During the 1970s, the attention was focused again on LA as a chemical raw material (Kitaro *et al*. 1975; Schaufnagel *et al*. 1975). An alternative and cost-efficient production directly from biomass was demonstrated by the biofine process (Fitzpatrick. 1990, 1997; Bozell *et al*. 2000). It seemed that the problems concerning expensive raw material, low yields, excessive equipment costs, and physical properties detrimental to easy recovery and handling could be overcome. Since then, LA productions regain attention (Ghorpade and Hanna 1999; Farone and Cuzens. 2000; Cha and Hanna 2002; Fang and Hanna 2002; Seri et al. 2002; Chang *et al*. 2007; Yan *et al*. 2008). The chemistry of LA (Timokhin *et al*. 1999) and its derivatives (Manzer. 2006) were studied.

The U.S. Department of Energy identified LA by screening approximately 300 substances as one of the 12 potential platform chemicals in the biorefinery concept (Werpy and Peterson 2004). The broad range of possible LA secondary products, many of high potential for industrial applications and as intermediates in organic chemistry, has been the fertile terrain of intense research efforts during the last decade. New synthetic routes that deliver chemical compounds of industrial relevance (Bozell *et al*. 2000; Hayes et al. 2006; Geilen *et al*. 2010), specifically for application as solvents (Manzer. 2006), monomers (Isoda and Azuma 1996; Manzer. 2004; Brandenburg *et al*. 2005; Manzer. 2006; Lange *et al*. 2007; Dautzenberg *et al*. 2011).

Rackemann and Doherty (2011) mentioned that one of the reasons behind the non-commercialization of LA is the high cost for material used for the production. The main method for production of high purity LA in use today involves the petrochemical conversion route from maleic anhydride (Moens. 2002) or hydrolysis of furfuryl alcohol (Timokhin *et al*. 1999; Yan *et al*. 2008). These conversation routes are more complex than the acid hydrolysis of biomass and result in the relatively high market price of LA of ~US$10/kg (Moens. 2002). Affordable feedstocks are required as techno economic evaluations show that the raw materials are the largest cost contributor to the production process (Wayman. 2007). This is currently been addressed with investigates on cellulosic feedstocks such as lignocellulosics

and municipal solid waste as suitable sources of sugars (Huber *et al*. 2006; Corma *et al*. 2007; Demirbas. 2001).

2.7 Commercial Production of LA

2.7.1 Biofine Processes

The process that is currently used for the production of LA from biomass is called biofine process. This process is one of the most used technologies for LA industrial production. Many feedstocks have been used as starting materials. This process involves the use of dilute sulphuric acid as a catalyst, and it takes place in two separate hydrolysis reactors. In the first step biomass and sulphuric acid solution are mixed and continuously supplied to a small diameter tubular reactor that operates at a temperature within the range of 210 to 220 °C and a pressure of 25 bar. The residence time in this first reactor is 12 seconds, in order to depolymerize the polysaccharides into their soluble monomers (e.g. hexoses, pentoses, HMF). The outflow mixture is then fed to the second continuously stirred tank reactor that operates at a lower temperature and pressure (190 to 200 °C, pressure 14 bar) (Hegner *et al*. 2010). LA with purity of up to 98 % is produced, while the H_2SO_4 catalyst is recovered and recycled. Around 50 % of the mass of the 6-carbon sugars are converted to LA, with 20 % forming formic acid and 30 % tars, while in the rest is converted to char (Hayes *et al*. 2006; Mukherjee *et al*. 2015). Fig. 2.6 shows the schematic representation of the two stage reaction of producing LA in a pilot scale.

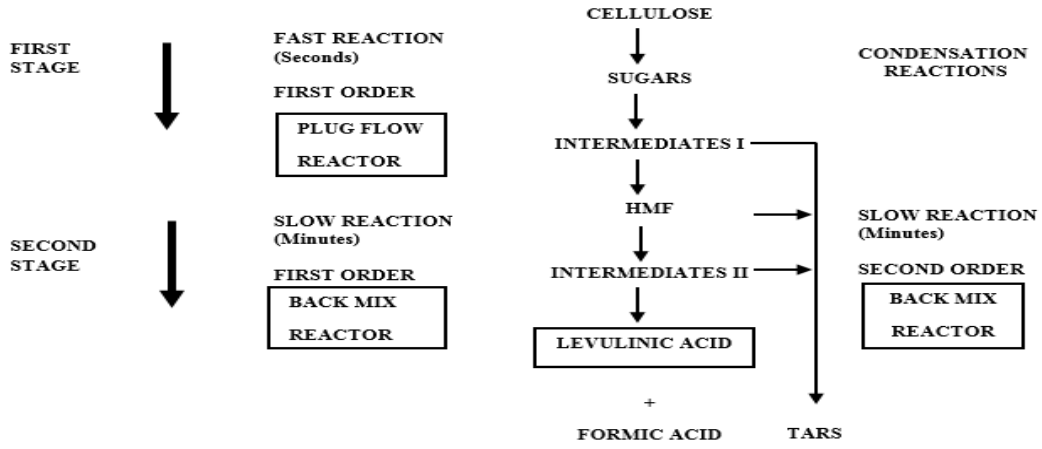


Figure 2.6 Schematic representation of the chemical conversion of cellulose to LA (major product), formic acid (by product), and tars (minor condensation products) in the two biofine reactors (Hayes *et al*. 2006).

2.8 Global Levulinic Acid Market, 2014 to 2020 Industry Survey

The global market for LA is expected to grow and reach 917.2 kilo tons by 2020, according to a study conducted by Grand View Research, Inc. (www.grandviewresearch.com, last accessed in March 2015). Increasing application for LA, particularly in pharmaceuticals, pesticides, cosmetics and solvents is expected to grow over the next coming years. High R&D spending, particularly to reduce costs to competitive levels is a critical dynamic for market participants, as substitution for synthetic products in potential applications such as fuel additives, polymers, bio-actives can only be achieved at low levulinic acid prices. As of 2013, LA was a small segment of the platform chemicals in application markets including Methyltetrahydrofuran (MTHF), DALA, ethyl levulinate and diphenolic acid, with potential addressable demand estimated to be over 25,000 kilo tons by 2020. Some industries have developed patented technologies for levulinic acid commercialization through renewable sources. These companies are trying to develop processes that will reduce production costs of LA. LA used to costs between USD 5 to 8 per kilo, with a target price of less than USD 1 per kilo, because of that the increase in the application scope of LA is expected to increase (www.grandviewresearch.com, last accessed in March 2015). Fig 1.4 shows the LA market volume share by applications.

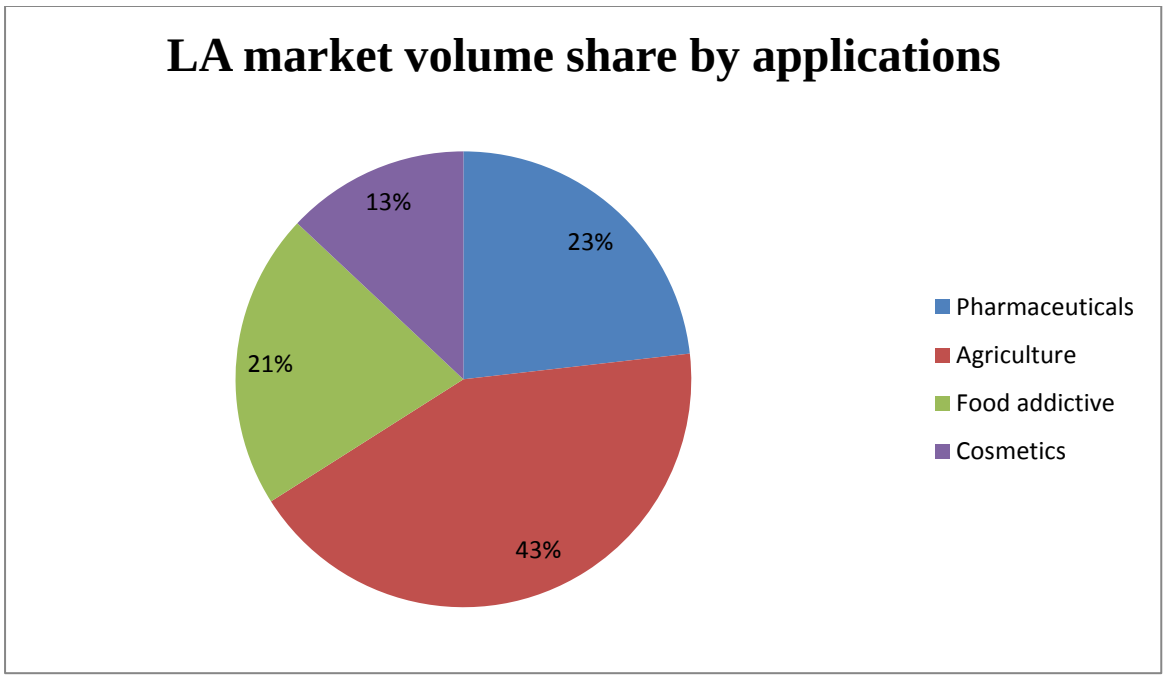


Figure 2.7 Pie chart for LA market volume share by application (www.grandviewresearch.com, last accessed in March 2015)

CHAPTER 3

EXPERIMENTAL METHODS

3.1 Introduction

The sugarcane bagasse samples that were used for this investigation were obtained from a Stanger paper mill except for part A where pure fructose was used as a starting material. The following experiments were conducted:

Part A: Production of levulinic acid from pure fructose

Part B: Enzymatic saccharification of acid and alkali pretreated depithed sugarcane bagasse

Part C: Production of levulinic acid from mill-run and depithed sugarcane bagasse

Part D: Production of levulinic acid from sugarcane bagasse using liquid hot water (LHW)

3.2 Part A: Production of Levulinic Acid from Pure Fructose

The following materials and procedure was used.

3.2.1 Materials

D-Fructose (≥ 99 %), sulphuric acid (72 %) and ethyl acetate they were all purchased from Sigma Aldrich.

3.2.2 Procedure

The reaction was carried out in a 250 ml flat bottom flask, 6.0 M (150 ml) of sulphuric acid was prepared from 72 % sulphuric acid, it was heated to 98 ^{o}C, when it reached the temperature and 0.33 M (30 ml) of fructose which was prepared from D-fructose was slowly

added, while stirring. The solution becomes yellow then black with a residue. It was heated for an hour and 30 min. The mixture was then cooled to room temperature, filtered by vacuum filtration. Black solids on the filter paper were kept for the analysis. The filtrate was then placed in a separating funnel and 250 ml of ethyl acetate was added, two layers were observed, an organic and an aqueous layer. The organic layer was extracted and placed in a fume hood to evaporate the solvent, the organic layer was first yellow but as the solvent evaporated, it became brown. The aqueous and organic layer was kept for analysis. Fig. 3.1 shows the chemical reaction for the production of LA from pure D-fructose.

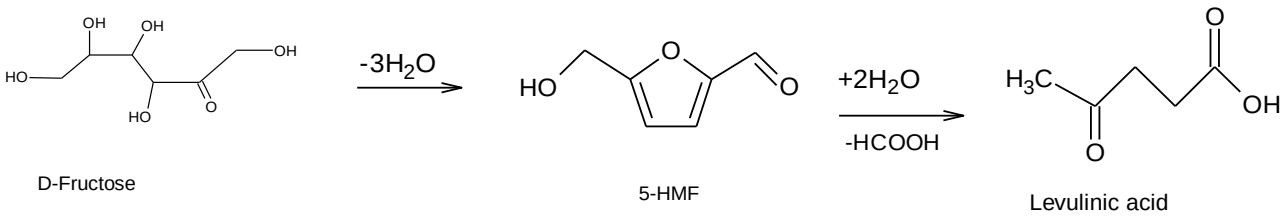


Figure 3.1 Chemical reaction for the production of levulinic acid from D-fructose

3.2.3 Characterisation Methods

The following qualitative and quantitative analyses were performed:

(i) Fourier Transforms InfraRed (FTIR) Spectroscopy

The FTIR spectra of the organic layer samples were recorded using a Shimadzu FTIR 8400) in the range of 500 – 4500 cm^{-1} with a resolution of 4 cm^{-1}.

(ii) High Performance Liquid Chromatography (HPLC)

The composition of the reaction mixture before separation of an organic and aqueous layers was determined using a HPLC system (PerkinElmer) consisting of a PerkinElmer binary LC pump, a Rezex ROA-Organic acid column, and a refractive index detector. The mobile phase consisted of aqueous sulphuric acid (2.5 mM) which was set at a flow rate of 0.5 ml/min. The column was operated at a temperature set at 55 ^{o}C. The pH of the reaction mixture must range from 4 to 7. The reaction mixture was very acidic therefore sodium hydroxide was added to the reaction mixture to increase the pH.

(iv) Proton Nuclear Magnetic Resonance (^{1}H NMR)

The product obtained after the organic layer was extracted and placed in a fume hood to evaporate the solvent i.e. the brown residue was dissolved in chloroform and it was analysed in the Bruker Avance III 600 MHz Nuclear Magnetic Resonance (NMR) spectrometer.

3.3 Part B: Enzymatic Saccharification of Acid /Alkali Pretreated Depithed Sugarcane Bagasse

The following materials and procedure were used.

3.3.1 Materials

Carboxymethyl cellulose, para nitro phenol β-D glucopyranoside and 3,5-Dinitrosalicylic acid (DNSA) was purchased from Sigma (St. Louis, USA), Media components were purchased from Himedia, USA. While rest other chemicals viz. sulphuric acid (98 %), sodium hydroxide, Tween 80, and citric acid, sodium di hydrogen ortho phosphate, of analytical grade were purchased locally in India. Cellulase was synthesized in-house using Trichoderma sp. RCKC65 at University of Delhi, South Campus, New Delhi, India. Sugarcane bagasse was obtained from a local mill (KwaZulu-Natal, South Africa).

3.3.2 Procedure

3.3.2.1 Preparation and Compositional Analysis of Sugarcane Bagasse

100 g of sugarcane bagasse was dried, ground and sieved to 40 mesh particle size. The cellulose, hemicellulose, lignin, moisture, and ash contents of raw and pre-treated biomass were determined according to the method described by TAPPI (α-Cellulose–TAPPI Method T203 om–83; Klason lignin–TAPPI Method T222 om–83; Pentosans–TAPPI Method T223 hm–84; Moisture–TAPPI Method T208 om–84 and Ash–TAPPI method211om–93).

The untreated and treated bagasse was characterized by:

(i) Fourier Transform InfraRed (FTIR) Spectroscopy

The FTIR spectra of the organic layer samples were recorded using a Shimadzu FTIR 8400) in the range of 500 – 4500 cm^{-1} with a resolution of 4 cm^{-1}.

(ii) ThermoGravimetric Analysis (TGA)/ Differential Scanning Calorimetry (DSC)

Simultaneous Differential Scanning Calorimeter and Thermo-Gravimetric Analyser (model SDT) manufactured by TA instruments, model SDT Q600, temperature range from (ambient) 20-1500 °C, Heating rate Up to 50 °C/min and the sample amount must range from 5-10 mg

(iii) Scanning Electron Microscopy (SEM)

Environmental scanning electron microscope manufactured by Carl Zeiss, model EVO 15 HD was used for the SEM analysis of raw, acid and acid/alkali treated bagasse, the sample was coated with gold to reflect electrons (sputter-coating)

3.3.2.2 Pretreatment

(i) Acid

To remove the hemicelluloses, the 40 mesh sugarcane bagasse (100 g) was pre-treated with 3 % (v/v) sulphuric acid (1.0 L) for 30 minutes in an autoclave at 121 ^{o}C. The solution was allowed to cool, then filtered, washed to a neutral pH and dried overnight at temperature of 50 ^{o}C in an oven. The filtrate was kept and analysed for reducing sugars.

(ii) Alkali

The oven dried substrate (65 g) from acid pre-treatment was further pretreated with 4 % (w/v) sodium hydroxide (550 ml) for 15 minutes in an autoclave at 121^{o}C to remove lignin. The solution was allowed to cool, filtered, washed to a neutral pH and oven dried overnight at a temperature of 50 ^{o}C. The cellulose bagasse produced was used for enzymatic saccharification.

3.3.2.3 Buffer Preparation for Enzymatic Hydrolysis

Two solutions were prepared:

(a) 0.1 M solution of citric acid ($C_6H_8O_7$) was prepared by weighing 3.9 g of citric acid (192, 13 g/mol) and adding 200 ml of distilled water.

(b) 0.2 M solution of sodium phosphate dibasic anhydrous was prepared by weighing 11.5g of Na_2HPO_4 (141.96 g/mol) and adding 400 ml of distilled water.

The two solution were mixed, 24.3 ml of solution (a) and 25.7 ml of solution (b). The pH of the mixture must be 5, for this mixture it was less than 5 therefore solution (b) was added until the pH of 5 was reached. This buffer was used for enzymatic saccharification.

3.3.2.4 Saccharification of Pretreated Sugarcane Bagasse

The acid and alkali pre-treated sugarcane bagasse was saccharified using crude enzyme extract produced by fungal RBT strain (8 FPU/mL). Enzymatic hydrolysis of pretreated material (1.0 g each) was carried out at 10 % (w/v) substrate consistency in 50 mM citrate phosphate buffer (pH 5.0). The substrate with buffer was pre-incubated at 50 ºC on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 150 rpm for 1 hr. Thereafter, to obtain the optimized enzyme doses for the saccharification of pretreated biomass, varied doses of exoglucanase (5-40 U/gds) were added to the pre-incubated slurry. The reaction continued up to 24 hrs. Samples were withdrawn at regular intervals of 8 hrs. centrifuged at 12000 rpm for 10 min in a centrifuge (Sigma, Germany) and the supernatant was analysed for total reducing sugars released and saccharification yield.

Optimization of:

(i) Enzyme load and time, (ii) surfactant dose and time and (iii) solid loading

(i) Optimization of Enzyme Load and Time

Four samples were prepared in duplicate with different enzyme load: 10, 20, 30 and 40 FPU/g with the same substrate (depithed cellulose) of 1.0 g and the samples were placed in the incubator at a speed of 150 rpm at 50 ^{o}C for 24 hrs. The samples were placed in the

incubator at 10 pm and the first samples (200 µL) were taken at 6 am and after every 4 hrs. until 10 pm. The samples were placed in a refrigerator after sampling before further analyses.

(ii) Optimization of Surfactant Dose and Time

After the optimization of enzyme load and time, four samples were prepared in duplicate with different tween 80 volumes: 0.30 %, 0.40 %, 0.50 % and 0.60 % w/v with the same substrate (depithed cellulose) of 1.0 g using the enzyme load of 30 FPU/g that gave the highest reducing sugars which was made from a reaction mixture of 7.5 ml of the enzyme and 2.5 ml of the buffer. Samples were placed in the incubator speed 150 rpm at 50 ^{o}C for 24 hrs. The samples were placed in the incubator at 10 pm and the first samples (200 µL) were taken at 6 am and after every 4 hrs. until 10 pm. The samples were placed in a refrigerator after sampling before further analyses.

(iii) Optimisation of solid loading

After optimization surfactants dose and time, four samples were prepared in duplicate with different substrate mass: 10 %, 12 %, 14% and 16 % w/v with the same substrate (depithed cellulose) using the enzyme load of 30 FPU enzyme (7.5 ml) and buffer (2.5 ml) and a surfactant dose of 0.4 % (tween 80) that gave the highest reducing sugars. Samples were placed in the incubator speed 150 rpm at 50 ^{o}C for 24 hrs. The samples were placed in the incubator at 10 pm and the first samples (200 µL) were taken at 6 am and after every 4 hrs. until 10 pm. The samples were placed in a refrigerator after sampling before further analyses.

3.3.2.5 Analysis of the Reducing Sugars

The refrigerated samples from enzymatic saccharification were centrifuge at 10000 rpm for 5 minutes. DNS method (Miller. 1959) was employed which detects the whole range of reducing sugars. The method consists of reading the absorption from the UV-Vis spectrometer at 540 nm in a solution composed of the sample containing reducing sugars and the DNS reagent. The intensity of the colour (absorbance) is used to calculate in mg the amount of sugars using a standard calibration curve. Yao *et al*. (2007); Nasirpour *et al*. (2014); Pandey and Negi. (2015); Das *et al*. (2015); Li *et al*. (2016) used DNS method to determine the reducing sugars.

3.4 Part C: Production of Levulinic Acid from Mill-run and Depithed Sugarcane Bagasse

The following materials and procedure were used.

3.4.1 Materials

Sulphuric acid (98 %), ethyl acetate, formic acid (98 %), methanesulfonic acid (99 %), HMF, levulinic acid (98 %) and acetic acid (99 %) were all purchased from Sigma Aldrich.

3.4.2 Procedure

The preparation for the depithed and mill-run sugarcane bagasse is described below.

The untreated substrate (mill-run/depith bagasse) was obtained from a local sugar mill in KwaZulu-Natal, South Africa. The bagasse was air dried, then oven dried for 24 hrs. at a temperature of 50 ^{o}C. It was later sieved to 40 mesh particle size.

3.4.2.1 Pretreatment

(i) Acid

The 40 mesh bagasse (100 g) and sulphuric acid was mixed in a ratio of 1:10. It was then autoclave for 30 minutes, at temperature of 121 °C and 15 psi. It was cooled to room temperature, and then filtered. The solid samples were washed with deionized water until the pH was neutral, it was then air dried for 4 hrs. and oven dried overnight at a temperature of 50 °C.

(ii) Alkali

The acid treated bagasse was weighed and mixed with 4 % of NaOH in a ratio of 1:100. It was autoclave for 15 minute, at 121°C and 15 psi; it was cooled to room temperature, and then filtered. The solid residue was washed with deionized water until the pH was neutral, air dried and oven dried overnight at a temperature of 50 °C.

(iii) Acid Hydrolysis

Cellulose produced from the acid and alkali pretreatment and 5 % sulphuric acid/MSA solutions were mixed in a ratio of 2:100 in a glass flask at temperature of 90 ^{o}C for 2 hrs.

(iv) Dehydration and Hydrolysis

Glucose solution obtained from the acid hydrolysis of cellulose and the catalyst (sulphuric acid and MSA) were mixed in ratio of 1:5. The catalyst was boiled to 98 ^{o}C then the glucose solution was slowly added while stirring for 2 hrs. and then cooled to room temperature, after cooling, the mixture was filtered.

(v) Extraction

250 ml ethyl acetate was added to the mixture to extract LA in a separating funnel; two layers were obtained; an organic and aqueous layer. Approximately 1.0 g of sodium sulphate was added to extract water in the organic layer, the organic layer was then placed in a fume cupboard for the solvent to evaporate and LA was the residue.

3.4.2.2 Characterisation Methods

The following qualitative, quantitative, thermal and microscopic analyses were performed:

(i) Fourier Transform InfraRed (FTIR) Spectroscopy

The FTIR spectra of the untreated bagasse and LA samples were recorded using a Shimadzu FTIR 8400 in the range of 500-4500 cm^{-1} with a resolution of 4 cm^{-1}.

(ii) High Performance Liquid Chromatography (HPLC)

The composition of the reaction mixture was determined using HPLC system (PerkinElmer) consisting of a PerkinElmer binary LC pump, a Rezex ROA-Organic acid column, and a refractive index detector. The mobile phase consisted of aqueous sulphuric acid (2.5 mM) which was set at a flow rate of 0.5 ml/min. The column was operated at a temperature of 55 ^{o} C, the pH of the reaction mixture must range from 4 to 7. The reaction mixture were very acid so sodium hydroxide was added to increase the pH.

(iii) Thermogravimetric Analysis (TGA)/ Differential Scanning Calorimetry (DSC)

Simultaneous Differential Scanning Calorimeter and Thermo-Gravimetric Analyser (model SDT) manufactured by TA instruments, model SDT Q600, temperature range from (ambient) 20-1500 °C, heating rate up to 50 °C/min and the sample amount must range from 5-10 mg.

(iv) Scanning Electron Microscopy (SEM)

Environmental scanning electron microscope manufactured by Carl Zeiss, model EVO 15 HD was used for the SEM analysis of raw, acid and acid/alkali treated bagasse, the sample was coated with gold to reflect electrons (sputter-coating).

3.5 Part D: Production of Levulinic Acid from Sugarcane Bagasse Using Liquid Hot Water (LHW)

The following materials and procedure were used.

3.5.1 Materials

Sulphuric acid (95-97 %), D(+)-glucose monohydrate, D(+)-fructose (99 %) were obtained from Merck, Darmstadt, Germany. Sodium hydroxide (99 %), D(+)-xylose (99 %) and methanesulfonic acid (99.5 %) were provided by Carl Roth, Karlsruhe, Germany.

3.5.2 Procedure

The composition analysis of bagasse (pellets) was conducted using Agilent HPLC

3.5.2.1 Sugarcane Bagasse Pretreatment

(i) Pelletization

The bagasse was milled with a pellet press type 14 175 from Amandus Kahl (Reinbek, Germany) with 175 x 24 x 6 mm metal matrix. Afterwards the bagasse was conditioned to a moisture content of 12 wt %. For pelletization the same press was used with a 175 x 36 x 6 mm metal matrix at a roller speed of 0.73 m/s. For both the roller shell and the metal matrix,

the smallest version available (3.7 x 30 mm) was used to enable maximum pressure per area during the pelletizing.

(ii) Liquid Hot Water (LHW) Pretreatment

A 1.0 kg of pelletized bagasse was used for the LHW hydrolysis at a temperature of 200 ^{o}C in a 3.0 L high pressure fixed bed reactor for 30 minute with a volume flow 250 ml/min of water. Hemicellulose dissolved in the water that was flowing throughout the reaction and a solid residue was collected from the reactor, which is known to have cellulose and lignin (cellulignin). The parameters were selected according to Reynolds *et al*. (2015), who optimized the process parameters with regards to maximum enzymatic sugar recovery.

(iii) Enzymatic Hydrolysis

The solid residue (cellulignin) from the LHW was then enzymatically treated in 10 L glass reactor, at a temperature of 50 °C for 72 hrs. A ratio of 1:10 for bagasse (cellulignin) and water respectively was used for the enzymatic hydrolysis. The enzyme mixture used for enzymatic degradation of cellulignin was Cellic® CTec2 received from Novozymes A/S. According to manufacturer information Novozymes A/S (Hg.) (2010), CTec2 is an industrial standard cellulase blend for the degradation of lignocelluloses to fermentable sugars containing aggressive cellulases, a high level of β-glucosidases as well as some hemicellulases. Optimal reaction conditions at ambient conditions are 45-50 °C and pH 5. A CTec2 activity of 161 FPU/ml has been determined experimentally. The determination method has been adopted from the NREL (Adney *et al*. 1996) instruction "Measurement of Cellulase Activities". Cellulase activity is usually measured in "filter paper units" (FPU) per milliliter of original undiluted enzyme solution. The Cellic Ctech 2 (23.3 ml) with a concentration of 15 FPU was used and sodium hydroxide was added to reach a pH of 5. The product produced after 72 hrs. were centrifuged at approximately 2500 times for 45 minutes for separation. The liquid solution (glucose solution) was kept in a refrigerator for analysis and the solid residue (lignin) was oven dried at a temperature of 50 ^{o}C overnight.

(iv) Bagasse Moisture Content

The solid bagasse moisture content has been determined gravimetrically according to a laboratory procedure published by NREL (Sluiter. 2008) the wet biomass samples were mixed thoroughly and weighed into pre-dried aluminium pans. The samples were dried in an oven at a temperature of 105 °C for a minimum of four hours. The samples were cooled in a desiccator, weighed and further dried to constant mass (± 0.1 % change).

(v) pH Determination

The pH of LHW hydrolysate and sugar solutions from enzymatic hydrolysis of pretreated biomass was determined using a WTW pH 340. pH measurement might represent a cheap alternative to the expensive and time-consuming analysis of sugars and degradation products executed. The pH for the mixture (cellulignin, water and enzyme) was adjusted to 4.8 using 2.0 M sodium hydroxide solution before enzymatic hydrolysis reaction started.

(vi) Hydrolysate Density

Similar to the pH determination, the hydrolysate density can also serve as a simple evaluation factor for the treatment severity in LHW and enzymatic treatment. An Anton Paar DMA 4500M density meter was used to determine the density of LHW hydrolysate and sugar solution from enzymatic hydrolysis.

3.5.2.2 Sugar Analysis

For the determination of reducing sugars concentrations in LHW and enzymatic hydrolysates, three different analytical methods were applied. For the photometric analyses, a Thermo Scientific Evolution 300 UV-photometer equipped with a xenon lamp has been used.

(i) Photometric Analysis of Total Reducing Sugar Using DNS Reagent

As described in the NREL (Adney *et al*. 1996) laboratory procedure for the determination of reducing sugar concentration DNS reagent can be used for the photometric measurement of reducing sugar concentration under alkaline conditions. The DNS reagent reacts with the functional aldehyde group (R-CHO) in reducing sugars like glucose and oxidizes it to a carboxyl group (R-C(O)OH). In parallel, 3,5-dinitrosalicylic acid is reduced to 3-amino-5-

nitrosalicylic acid, which strongly absorbs light at 540 nm (Miller. 1959). The formation of 3-amino-5-nitrosalicylic acid is related to the amount of reducing sugars in the hydrolysate samples.

First, the hydrolysate samples were centrifuged at approximately 2500 times for 10 minutes. The supernatant was diluted in order to match the valid absorbance range of the applied UV-photometer. Afterwards, 0.167 ml of the diluted sample 0.333 ml, 0.05 M citrate buffer and 1.0 ml DNS reagent were pipetted into 2.0 ml centrifuge tubes and mixed thoroughly. The mixture was boiled in a water bath for 5 minutes and cooled in iced water immediately in order to stop the reaction. The color of the samples should have changed from bright yellow to orange or red. 0.1 ml of the boiled sample was diluted with 1.25 ml of deionized water in a polystyrene cuvette and UV-absorbance was measured at 540 nm. A calibration curve for xylose and glucose in a range of 0 – 10 g/l was prepared prior to the sample analyses.

(ii) Glucose Analysis Using a D-glucose Test-kit

For an accurate analysis of D-glucose without being dependant on HPLC-analysis, enzymatic methods can be used. The applied glucose test-kit was purchased from r-biopharm with chemicals from Roche. Sugar determination is based on the enzyme-catalyzed 11phosphorylation of d-glucose to D-glucose-6-phosphate in the presence of hexokinase and adenosine-5'-triphosphate (ATP) which simultaneously reacts to adenosine-5'-diphosphate (ADP). A second enzyme, glucose-6-phosphate dehydrogenase, catalyzes the oxidation of D-glucose-6-phosphate to D-gluconate-6-phosphate with parallel reduction of nicotinamide-adenine dinucleotide phosphate (NADP) to reduced NADPH. The amount of NADPH formed during this reaction is stoichiometric to the consumed amount of D-glucose. The D-glucose concentration is measured indirectly by UV light absorbance of NADPH at 340 nm.

(iii) Oligomer Analysis

LHW hydrolysates were analyzed for sugar monomer and oligomer content using an Agilent HPLC with refraction index detection. The samples were centrifuged at 16 000 times and diluted if necessary. Free glucose, xylose and arabinose monomers in the hydrolysates as well as HMF and furfural are measured directly. For oligomer analysis, the samples are hydrolyzed with 4 % sulphuric acid for polysaccharide degradation. Sugar-monomers, HMF and furfural concentrations were all determined.

(iv) Agilent High Performance Liquid Chromatography (HPLC)

The Agilent High Performance Liquid Chromatography with a refractive index detector, VA 300/7.8 Nucleogel sugar Na (Machery-Nagel) column was used. The detection limit was 100 mg/L free sugar monomers, HMF and furfural were measured directly from centrifuged and diluted samples. Further aliquot from the LHW extract was hydrolysed with 4 % sulphuric acid at a temperature of 120 ^{o}C for 1 hr. afterwards, the samples were neutralized with $CaCO_{3,}$ centrifuged and analysed for oligomer content via the same chromatographic system. In both cases distilled water was used as the eluent.

The solid biomass sample composition was analyzed by the Thunen Institute of wood Research in Hamburg, Germany. Lignin and sugar contents were determined using a two-step sulphuric acid hydrolysis (72 and 4 %, respectively). The remaining solid (Klason-lignin means acid insoluble lignin) was dried and weighed, while the acidic solution was analysed by High Performance Anion Exchange Chromatography (HPAEC) and photometric detection.

3.5.2.3 Acid Hydrolysis of Glucose Solution to Produce LA

The glucose solution produced from the enzymatic hydrolysis of cellulignin was used as the main substrate for the acid hydrolysis to produce LA, other substrates were also used such as: pure glucose, pure glucose/xylose and pure fructose (see table 3.1). LA synthesis was conducted in 40 ml stainless steel screening reactors with PTFE inlets. The temperature adjustment over heating jackets was controlled by internal temperature measurement. Mixing was achieved by magnetic stirrers. The reaction volume of each experiment was 30 ml. For the preliminary experiments with the model solutions, sugars were dissolved in distilled water to reach the equivalent concentration as in the solution obtained from the biorefinery process. Either the model solutions as well as the real substrate were preheated in an oven at 80 °C for 30 min in the PTFE inlet. Then the acid was injected over a cannula and the PTFE inlet was inserted into the preheated reactor (30 min, 140 °C). The pressure in the reactors was adjusted with nitrogen to 50 bar. The reactors that were used are shown in fig. 3.2

Figure 3.2 Batchies set up, 40 ml batch reactors with 30 ml reaction volume and pressure of 50 bar

Table 3.1: Acid hydrolysis details for glucose solution obtained from sugarcane bagasse using both MSA and sulphuric acid

NR.	Substrate	Glucose concentration [g/L]	Xylose concentration [g/L]	Fructose Concentration [g/L]	Total Sugar Concentration [g/l]	Catalyst	Duration [min]	Temperature [°C]
1	Fructose	0.0	0.0	42.7	42.7	0,5 M H_2SO_4	45	180
2	Fructose	0.0	0.0	42.7	42.7	0,5 M H_2SO_4	45	180
3	Glucose	42.7	0.0	0.0	42.7	0,5 M H_2SO_4	45	180
4	Glucose	42.7	0.0	0.0	42.7	0,5 M H_2SO_4	45	180
5	Glucose + xylose	42.7	9.03	0.0	52.2	0,5 M H_2SO_4	45	180
6	Glucose + xylose	42.7	9.03	0.0	52.2	0,5 M H_2SO_4	45	180
7	Glucose solution	42.7	9.03	0.0	52.2	0,5 M H_2SO_4	45	180
8	Glucose solution	42.7	9.03	0.0	52.2	0,5 M H_2SO_4	45	180
9	Fructose	0.0	0.0	42.7	42.7	0,5 M MSA	45	180
10	Fructose	0.0	0.0	42.7	42.7	0,5 M MSA	45	180
11	Glucose	42.7	0.0	0.0	42.7	0,5 M MSA	45	180
12	Glucose	42.7	0.0	0.0	42.7	0,5 M MSA	45	180
13	Glucose + xylose	42.7	9.03	0.0	52.2	0,5 M MSA	45	180
14	Glucose + xylose	42.7	9.03	0.0	52.2	0,5 M MSA	45	180
15	Glucose solution	42.7	9.03	0.0	52.2	0,5 M MSA	45	180
16	Glucose solution	42.7	9.03	0.0	52.2	0,5 M MSA	45	180

LA was also produced from cellulignin and pure cellulose using MSA (see table 3.2).

Table 3.2: Acid hydrolysis on the cellulignin from LHW pretreatment and pure cellulose using MSA for the production of LA

Substrate	**Substrate conc (1.7 %)**	**Temperature [°C]**	**Catalyst**	**Duration [min]**
Cellulose (pure)	1.7	150	1M	120
Cellulose (pure)	1.7	150	1M	120
Cellulignin (LHW)	1.7	150	1M	120
Cellulignin (LHW)	1.7	150	1M	120

3.5.2.4 Design of Experiments (DoE)

Stat-Ease Design-Expert 8.0.7.1 was used for the experimental design and analysis. Set-up and analysis of the designs were conducted according to the standard procedure implemented in the software. DoE provides a set of powerful tools for the effective planning and evaluation of experimental designs by minimizing the number of required experiments.

Central Composite Design (CCD) for LA Production

For the optimization of LA production, a central composite design was used. Parameters that were used for optimization settings are given in table 3.3, after inserting the minimum and maximum of the three parameters given in table 3.3, a set of experiments were given from the software and table 3.4. shows those experiments that needed to be done to measure the amount of LA produced. The glucose solution from bagasse (pellets) was used as a starting material for this experiments and only MSA was used as a catalyst, using a 40 ml batch reactor with a 30 ml reaction volume and 50 bar pressure.

Table 3.3: Parameters used for optimization LA production

Parameter	Minimum	Maximum
Temperature	160 °C	200 °C
Reaction time	30 min	90 mi
Acid	0.25 M	1.0 M

Table 3.4: Parameter optimization for the production of LA obtained from fig. 3.3

Std	Run	Temperature [^{o}C]	Time [min]	Acid [M]	Acid [g]
13	1	180	60	0.131	0.38
16	2	180	60	0.625	1.80
3	3	160	90	0.250	0.72
9	4	153.7	60	0.625	1.80
7	5	160	90	1.000	2.88
6	6	200	30	1.000	2.88
19	7	180	60	0.625	1.80
12	8	180	99.5	0.625	1.80
17	9	180	60	0.625	1.80
2	10	200	30	0.250	0.72
15	11	180	60	0.625	1.80
10	12	206.3	60	0.625	1.80
11	13	180	20.5	0.625	1.80
5	14	160	30	1.000	2.88
18	15	180	60	0.625	1.80
4	16	200	90	0.250	0.72
1	17	160	30	0.250	0.72
14	18	180	60	1.119	3.23
8	19	200	90	1.000	2.88
20	20	180	60	0.625	1.80

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Part A: Production of Levulinic Acid from Pure Fructose

(i) Confirmation of the production of LA

The FTIR spectra for the LA standard, LA synthesized from fructose and LA from literature, respectively are given in figs. 4.1, 4.2 and 4.3. The important vibrations of LA are all seen in these FTIR spectra, vibration such as O-H (3300-2500 cm^{-1}) stretch for carboxylic group, C=O (1715 cm^{-1}) stretch for ketone group and for carboxylic group, and C-H (3000-2850 cm^{-1}) stretch for alkane groups. Tables 4.1 and 4.2 lists the general and experimental characteristic of FTIR absorption bands and when comparing figs. 4.1, 4.2 and 4.3, they are almost the same and when looking at tables 4.1 and 4.2 it is observed that they all have the important vibration of LA functional groups, confirming that LA was produced.

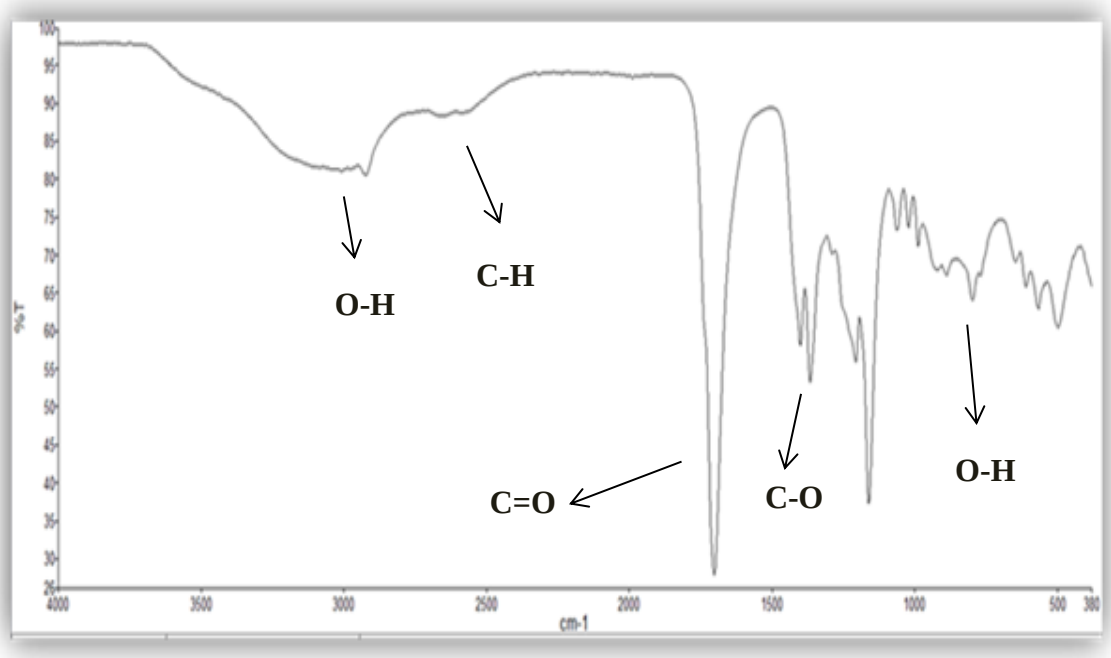


Figure 4.1 FTIR spectrum of LA standard

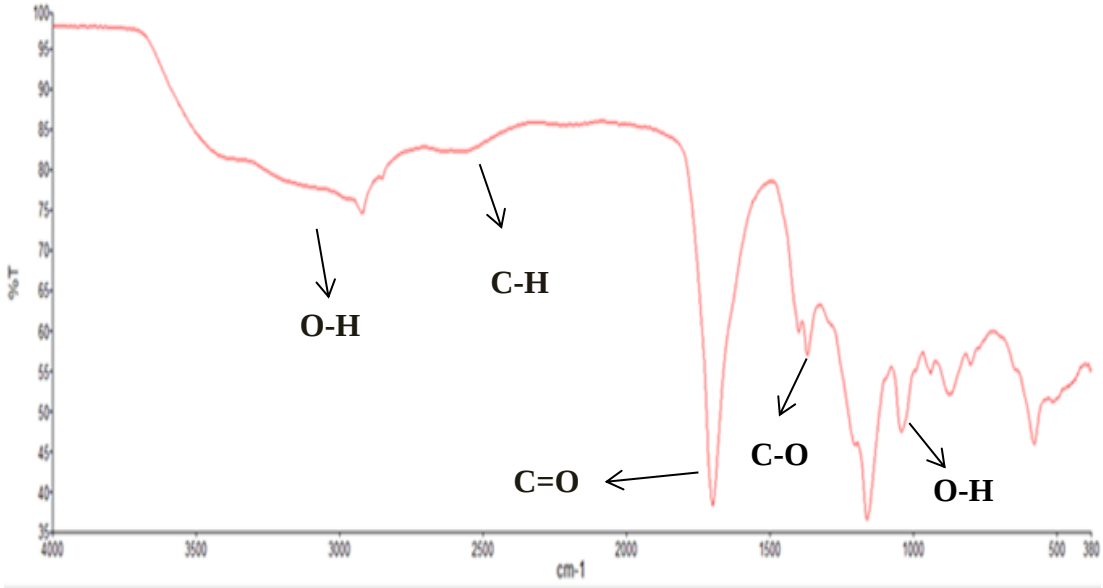


Figure 4.2 FTIR spectrum of LA synthesized from fructose

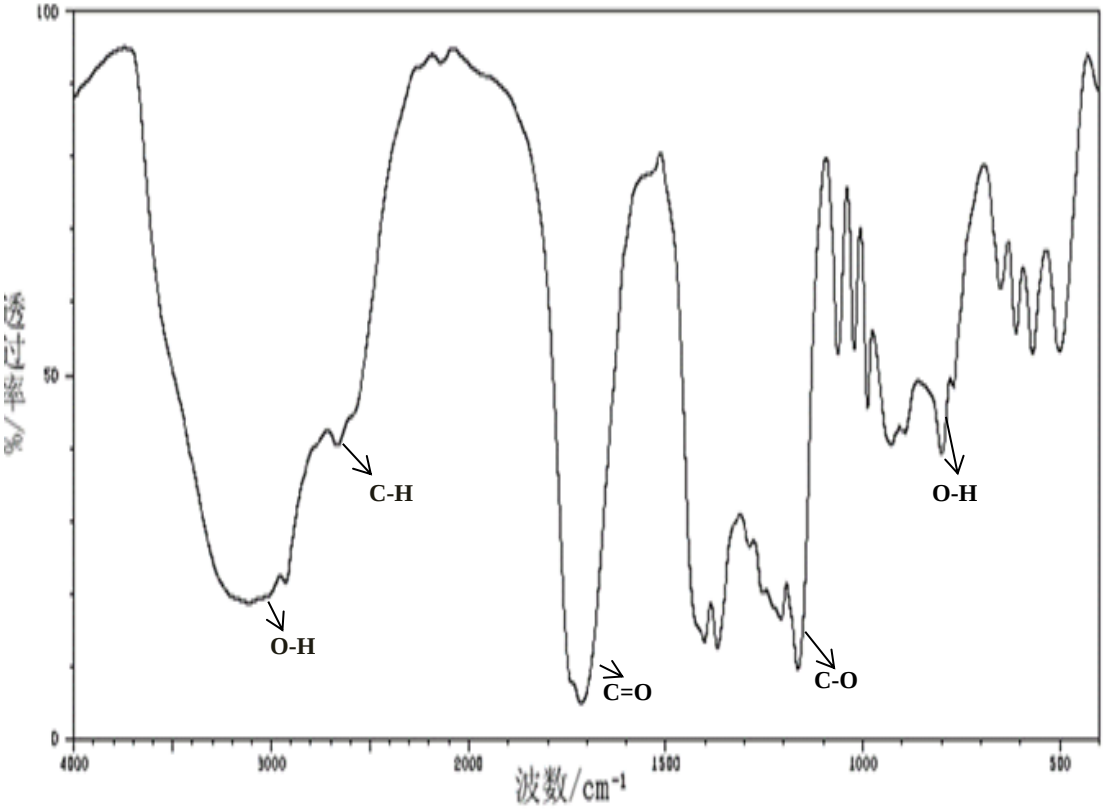


Figure 4.3 FTIR spectrum of LA literature (www.hanhonggroup.com, last accessed in July 2014)

Table 4.1: Characteristic FTIR absorptions bands for the vibration of interest

Frequency , cm^{-1}	**Bond**	**Functional group**
3300- 2500 (m)	O-H stretch	Carboxylic acid
3000 – 2850 (m)	C-H stretch	Alkanes
1760 – 1690 (s)	C=O stretch	Carboxylic acid
1715 (s)	C=O stretch	Ketone
1370 – 1350 (m)	C-H rock	Alkane
1320- 1000 (s)	C-O stretch	Carboxylic acid
950 – 910 (m)	O-H bend	Carboxylic acid

Where (m) – medium peak, (s) - sharp peak

Table 4.2: Experimental FTIR absorption bands for fig. 4.1, 4.2 and 4.3

LA std	**LA synthesized**	**LA literature**	**Bond**	**Functional group**
Frequency cm^{-1}				
3196.6	2919.7	~ 3100	O-H stretch	Carboxylic acid
2920.2	2849	~ 2900	C-H stretch	Alkanes
1701	1702.2	1715	C=O stretch	Ketone
1370.4	1367.9	1369	C-H rock	Alkane
1205	1158.6	1208	C-O stretch	Carboxylic acid
931.4	941.2	930	O-H bend	Carboxylic acid

(ii) Identification of components in the mixture

The reaction mixture produced by reacting fructose and sulphuric acid was analysed by the HPLC to identify the components in the mixture. The HPLC chromatogram of the standards and reaction mixture are given in figs. 4.4, 4.5, 4.6 and 4.7. The chromatogram of the reaction mixture is given in fig. 4.7; from the chromatogram it was observed that the mixture contained a small amount of fructose, formic acid, a very small amount of acetic acid and LA. Each of these compounds (LA, formic acid, acetic acid and fructose) was determined by the use of their standards given in figs. 4.4, 4.5 and 4.6. The standards were prepared and analysed in HPLC and fig 4.4, 4.5 and 4.6 were obtained with retention time for the compounds in the standards and when the reaction mixture was analysed the retention time were matched with the standard retention time to identify, the components in the reaction

mixture. Retention time is the time taken by the compound to travel through the column to the detector.

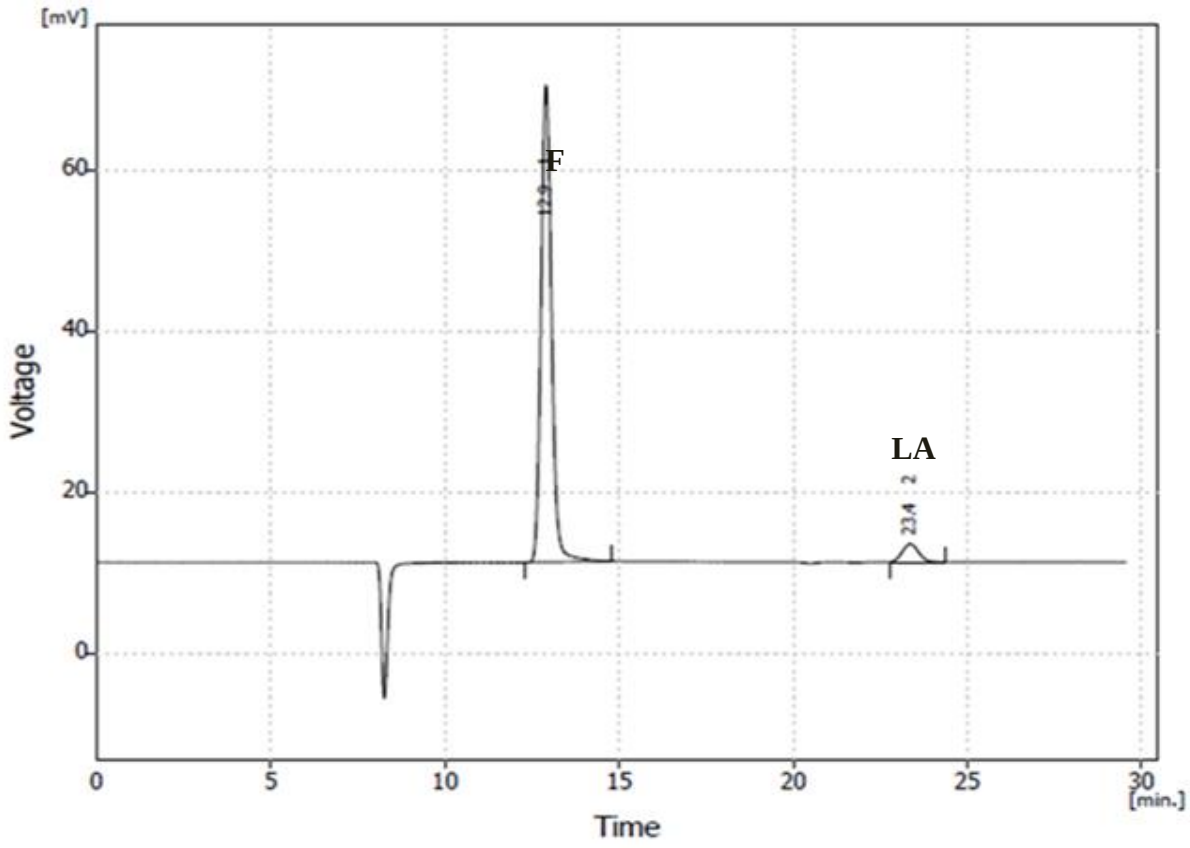


Figure 4.4 HPLC chromatogram for mixture standards of fructose (1000 ppm) and LA (100 ppm)

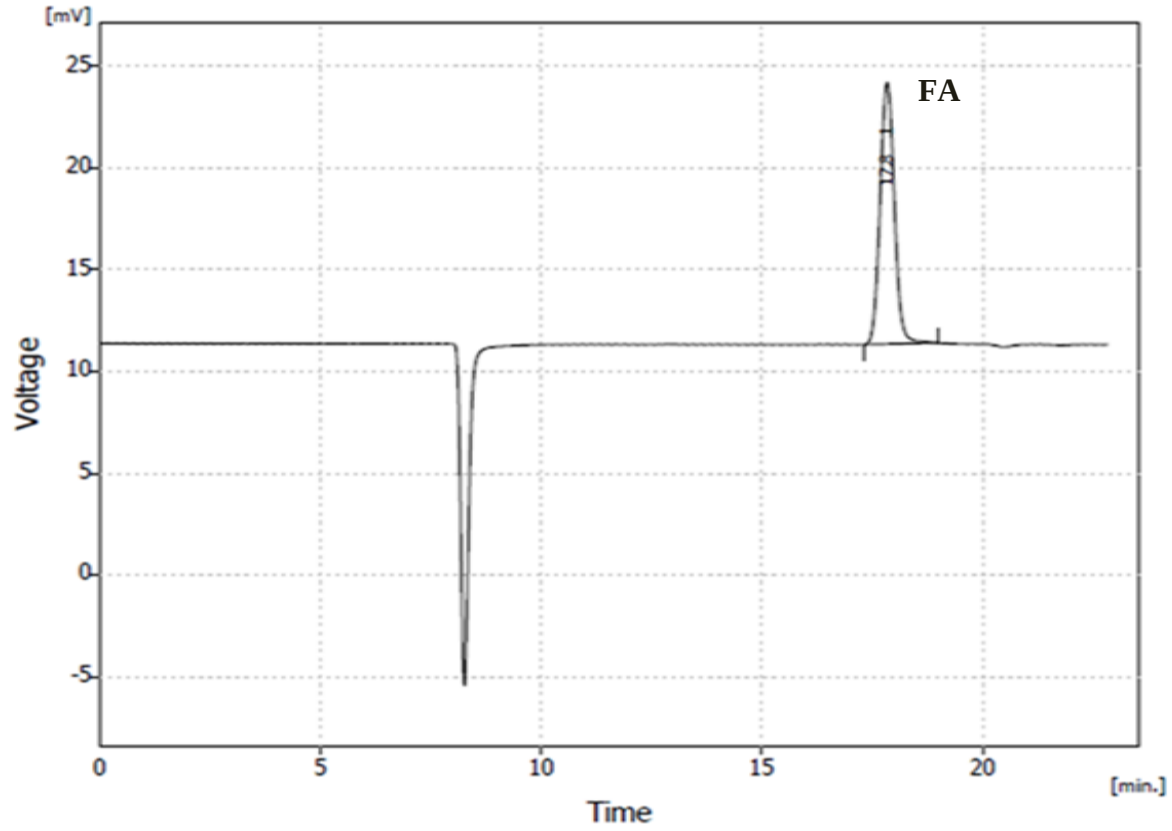


Figure 4.5 HPLC chromatogram for formic acid (100 ppm)

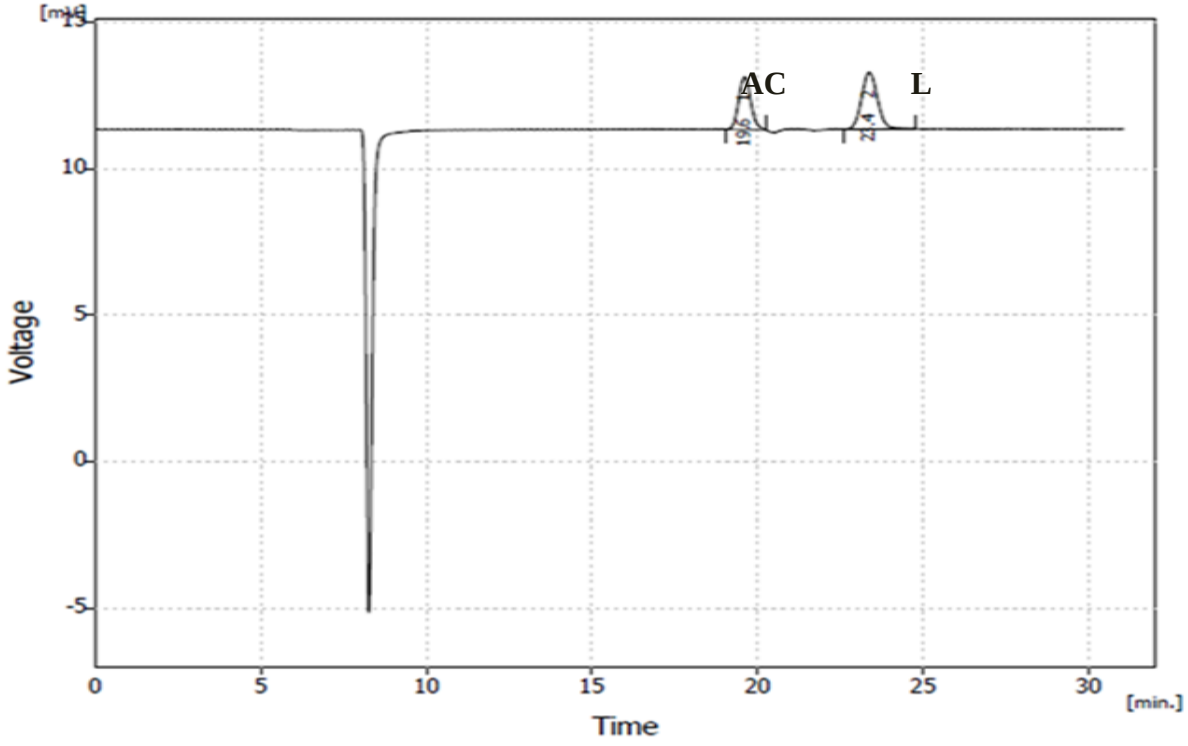


Figure 4.6 HPLC chromatogram for the mixture standards of acetic acid and LA (100 ppm)

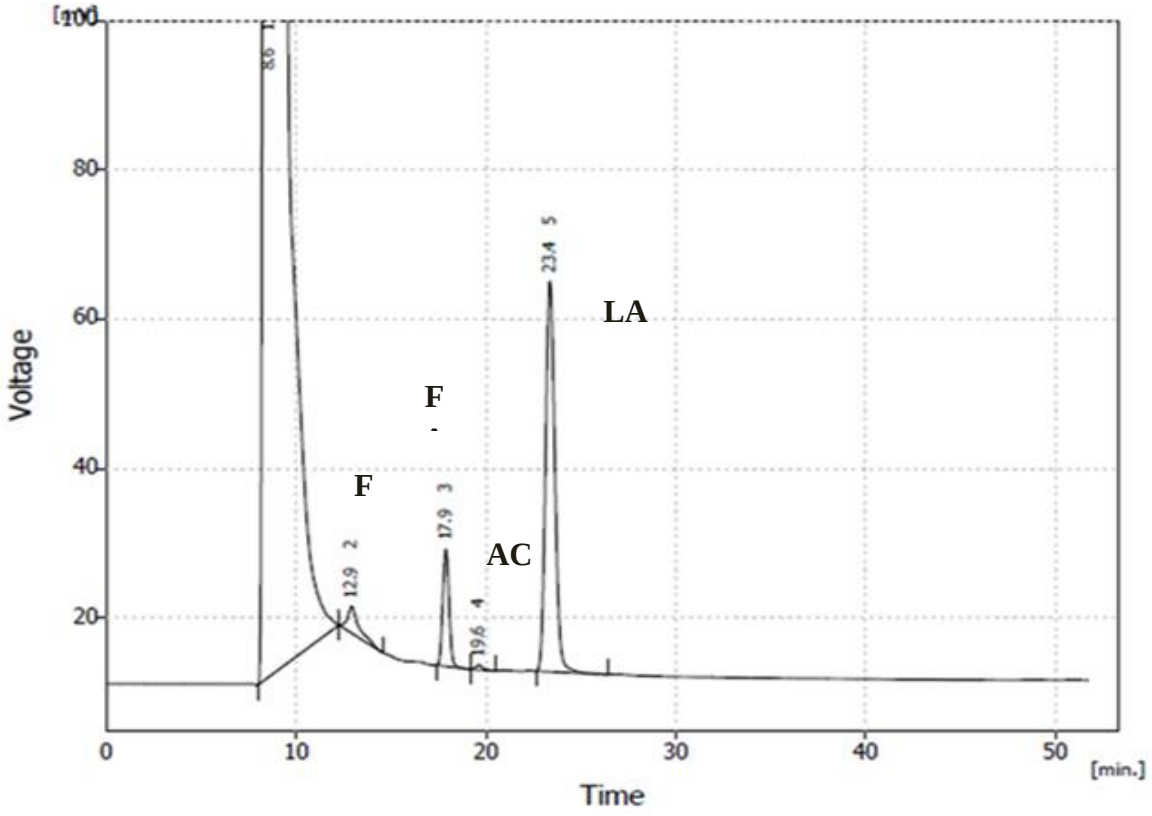


Figure 4.7 HPLC chromatogram for fructose reaction mixture, where the first peak with a retention time of 8.61 min is the solvent peak, AC – acetic acid, LA – levulinic acid, FA- formic acid and F- fructose

(iii) Identification of the chemical structure of the product

The ^{1}H NMR analysis was done on the synthesized and purchased LA to identify the chemical structure of the product. The purchased LA was used as the standard. The ^{1}H NMR scan for LA standard is given in fig. 4.8 and the ^{1}H NMR scan for the synthesized LA is given in fig. 4.9. In fig. 4.8 the peak at 2.205 ppm is assigned to methyl (CH_3) protons, the peak at 2.2773 ppm is assigned to CH_2 protons, the peak at 2.613 ppm is assigned to CH_2 and the peak at 10.4 ppm is assigned to carboxylic acid (COOH) proton. When comparing figs. 4.8 and 4.9, the difference observed is that the proton (a) in fig. 4.10 has the chemical shift of 10.4 ppm where as it is 7.2399 ppm in fig. 4.9 and when comparing figs. 4.9 and 4.10 the only difference observed in these spectra is the 1.2348 ppm peak which may have been caused by the impurities. Table 4.3 shows that proton b, c, and d from fig. 4.11 in the spectra should range from 0.9 and 1.3 but because of the neighbouring carbon bearing oxygen, the

chemical shifts are greater than that because oxygen is more electronegative thus it causes the proton to be deshielded. Fig. 4.11 shows the protons in the structure of LA and table 4.4 shows the exact chemical shift of all the protons in the NMR spectra of synthesized, std and literature LA.

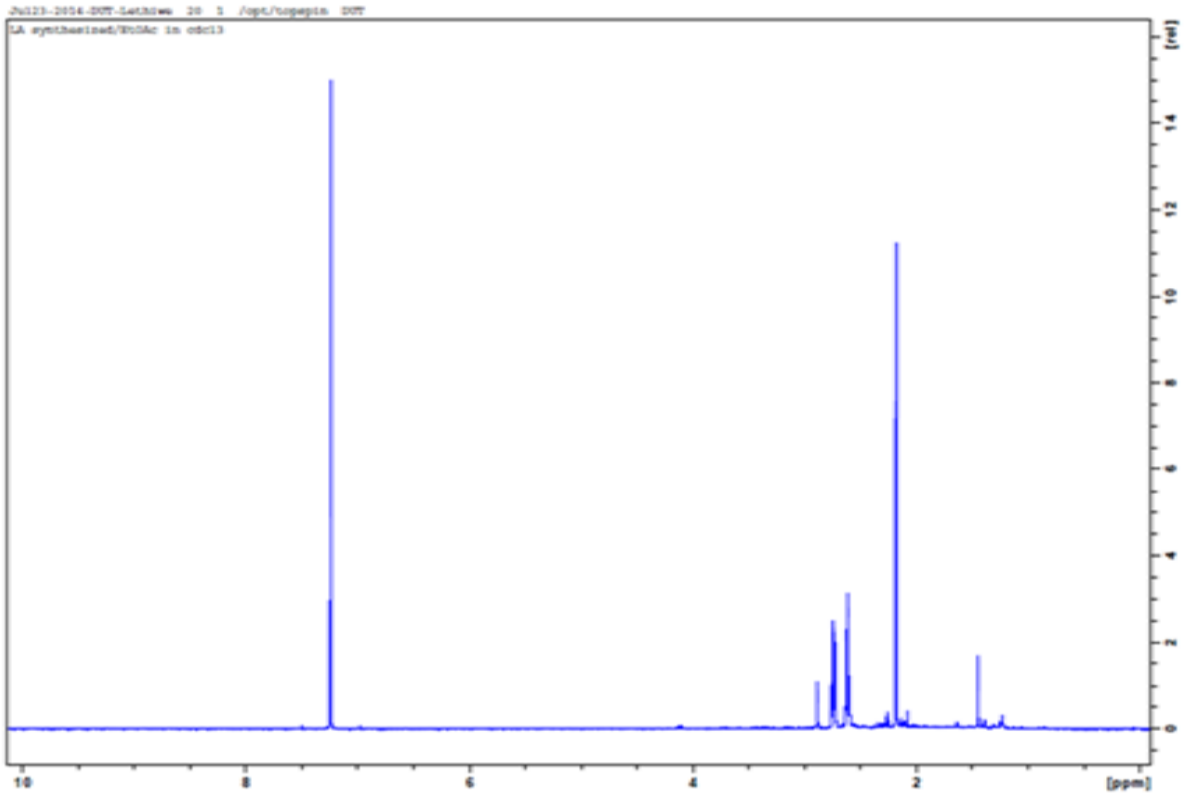


Figure 4.8 ^{1}H NMR for the LA standard

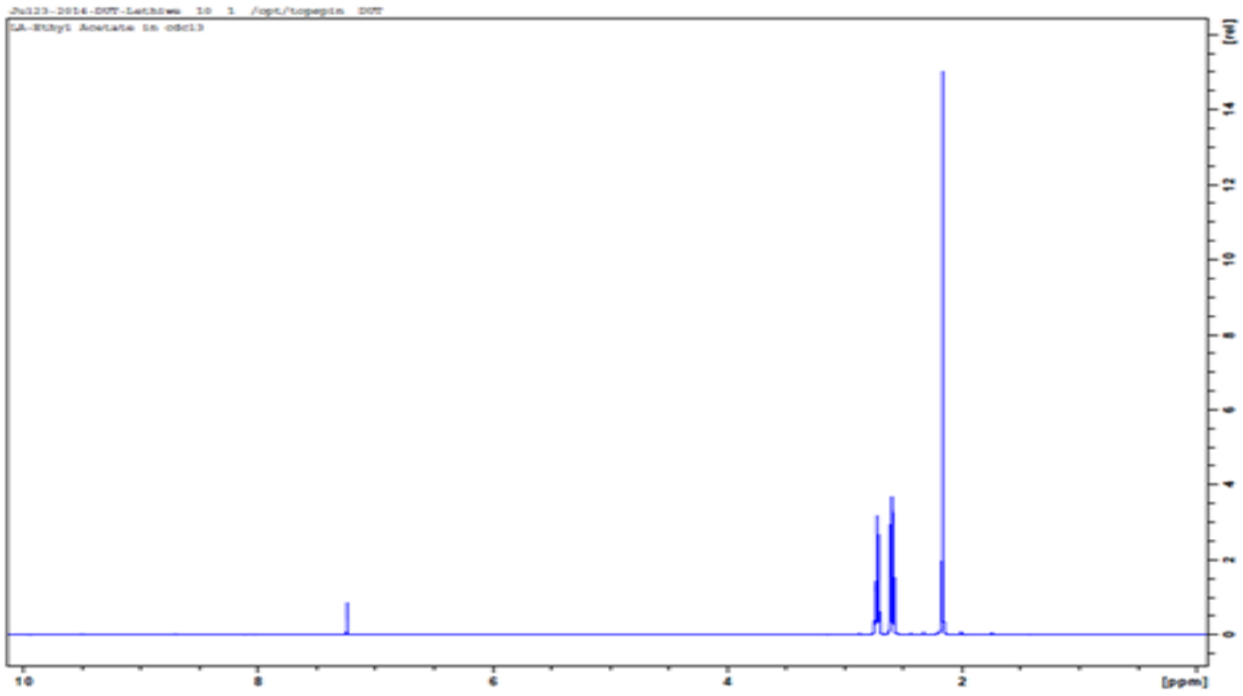


Figure 4.9 ^{1}H NMR for the synthesized LA

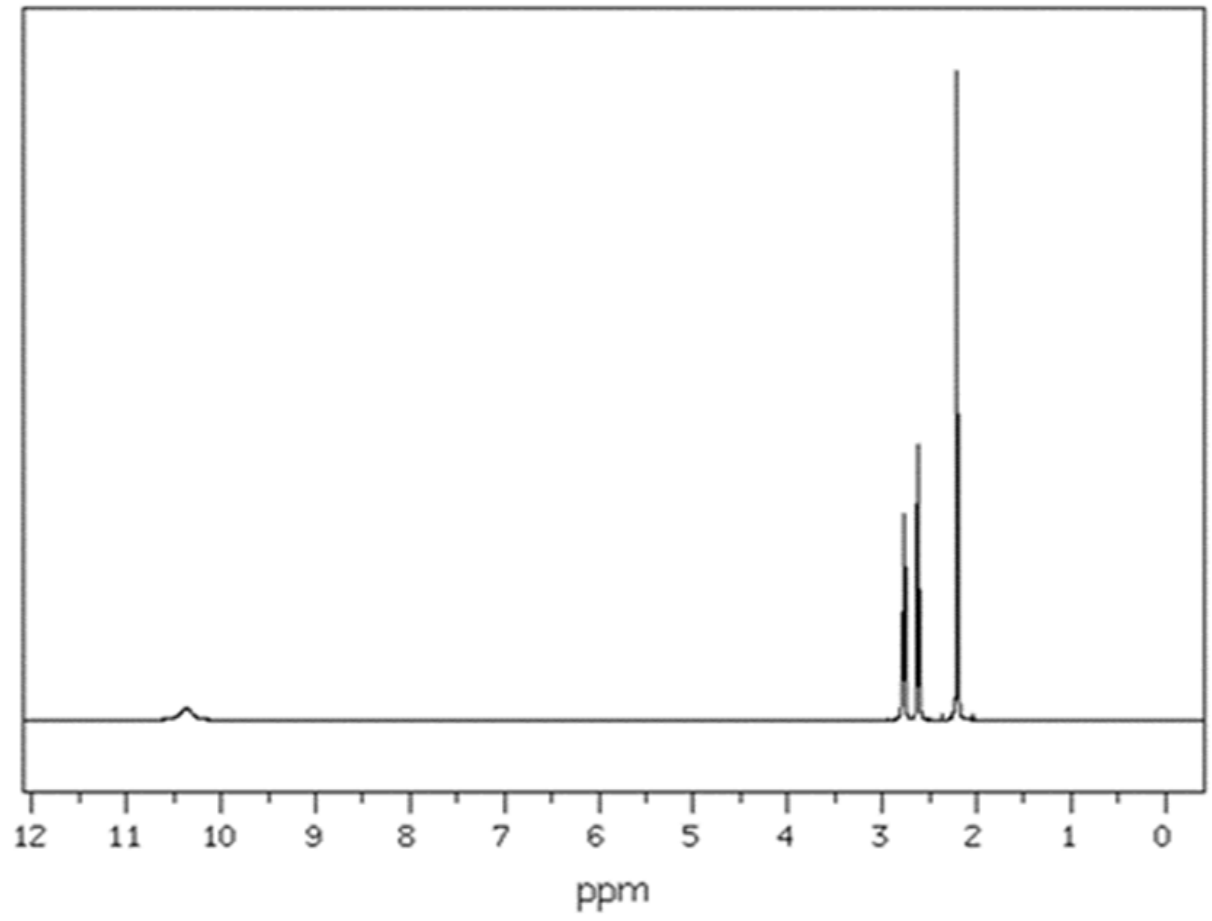


Figure 4.10 ^{1}H NMR for the literature LA (www.hanhonggroup.com, last accessed in July 2014)

Table 4.3: Characteristic ^{1}H NMR chemical shifts for the protons of interest

Type of proton	Type of compound	Chemical shifts (ppm)
1. RCH_3	I° aliphatic	0.9
2. R_2CH_2	2° aliphatic	1.3
3. RCOOH	Carboxylic	10 -13.2

Table 4.4: Chemical shifts for the ^{1}H NMR spectra for figures 4.8, 4.9 and 4.10

Name	Proton	Chemical shifts (ppm)
LA literature	(a)	10.4
	(b)	2.613
	(c)	2.773
	(d)	2.205
LA STD	(a)	7.2399
	(b)	2.6086
	(c)	2.7360
	(d)	2.1643
LA Synthesised	(a)	7.2402
	(b)	2.6388
	(c)	2.8848
	(d)	2.1807

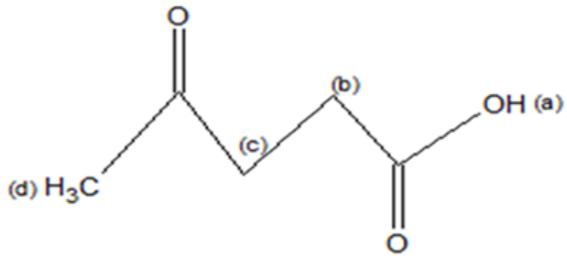


Figure 4.11 LA structure proton labelled

4.2 Part B: Enzymatic Saccharification of Acid /Alkali Pretreated Depithed Sugarcane Bagasse

4.2.1 Compositional Analysis of Sugarcane Bagasse

The composition analysis of the untreated and treated depith sugarcane bagasse is given in table 4.5. The compositional analysis of untreated depith bagasse sample revealed that the biomass contains cellulose (55 %); hemicellulose (28.2 %), and lignin (7.2 %). The pre-treatment of substrate with 3 % H_2SO_4 decreased the proportion of hemicellulose by 50 %. The pre-treatment of substrate with 4 % NaOH increased the proportion of cellulose by 62 % and reduced the lignin content by 55 % in the sugarcane bagasse (see table 4.5).

Table 4.5: Compositional analyses of untreated and pretreated depith sugarcane bagasse

Bagasse Sample	**Components (wt %)**			
	Cellulose	Hemicellulose	Lignin	Ash
Untreated	55.0	28.2	7.2	6.1
Acid pre-treated	74.1	13.1	3.6	5.2
Acid/Alkali Pre-treated	89.1	1.7	3.24	5.7

4.2.2 Characterization of Untreated and Pretreated Sugarcane Bagasse

(i) Determination of functional groups/molecular conformation

The FTIR spectroscopy was performed to detect changes in functional groups and/ molecular conformation that was caused by the pre-treatment process. Fig. 4.12 illustrates the FTIR spectra of the raw and regenerated biomass after acid and alkali pre-treatment. Table 4.6 shows the main functional groups of the biomass components namely cellulose, hemicellulose and lignin. It is noticeable that these components are mostly consisting of alkenes, esters, aromatics, ketone and alcohol with different oxygen containing functional groups for example OH (3500 – 3200 cm^{-1}), C=O (1770 – 1710 cm^{-1}), C-O-C (1280 cm^{-1}) and C-O-H (1055 cm^{-1}) etc. (Demibras. 2000). The depithed sugarcane bagasse, acid/alkali pre-treated showed the lowest OH absorption and raw bagasse shows the highest OH

absorption. In comparison with raw and acid pretreated bagasse, a big difference is noticeable in the fingerprint region (1830 - 730 cm^{-1}) for acid alkali pretreated bagasse.

Table 4.6: Main functional groups of bagasse components

Wavenumber (cm^{-1})	**Peak Assignment**	**Compound**
3600 – 3000	O-H stretching	Acid, methanol
2860 – 2970	C-H stretching	Alkyl, aliphatic and aromatic
1700 – 1730; 1510 - 1560	C=O stretching	Ketone and carbonyl
1632	C=C stretching	Benzene stretching ring
1613; 1450	C=C stretching	Aromatic skeletal mode
1470 – 1430	OCH_3	Methoxyl-O-CH_3 acid
1440 – 1400	OH bending	
1402	C-H bending	
1232	C-O-C stretching	Aryl-alkyl ether linkage
1215	C-O stretching	Phenol
1170; 1082	C-O-C stretching vibration	Pyranose ring skeletal
1108	O-H in plane deformation	C-OH
1060	stretching-O stretching vibration and C-O deformation	C-OH (ethanol)

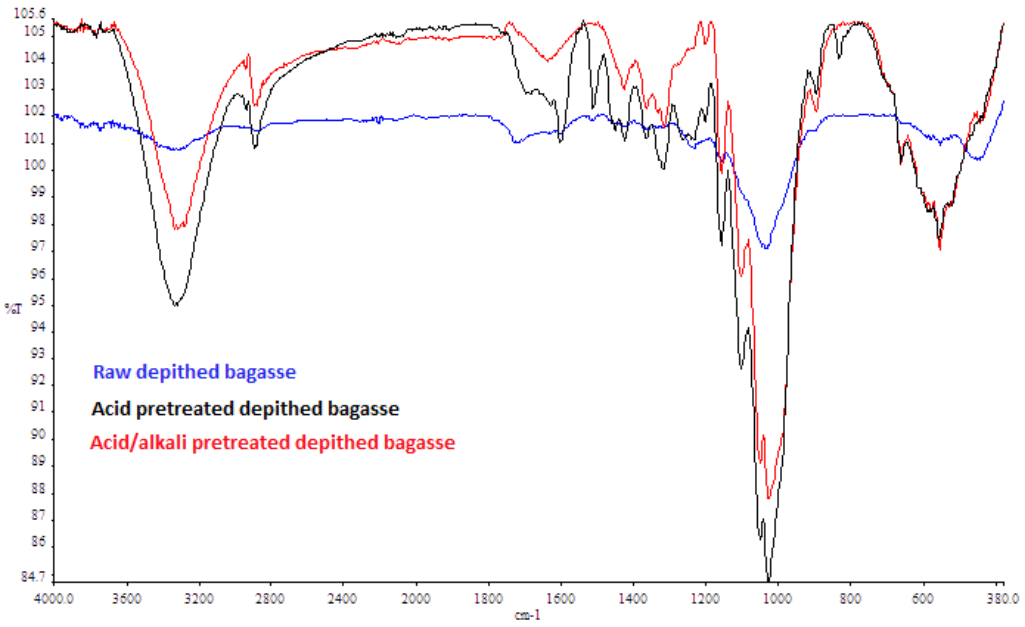


Figure 4.12 FTIR spectra of depithed sugarcane bagasse (untreated, acid pretreated and acid/alkali pretreated)

(ii) Assessment of morphology of raw and reqenerated SB

Scanning Electron Microscope (SEM) was used to study the morphology of raw and regenerated sugarcane bagasse after acid/alkali pretreatment. Fig. 4.13 shows the SEM images at 800 x magnification. The images show that the surfaces of the raw fibers (fig. 4.13a) appear smooth and the surfaces of the pre-treated fibers appear rougher with more porosity (fig. 4.13 b and c). The changes may be attributed to the disruption of the structure and reduction in crystallinity. One of the main objectives of pretreatment is to increase the surface area and pore size for enzymatic hydrolysis (Alvira *et al*. 2010); therefore this objective was met as a significant increase in porosity is observed after pretreatment.

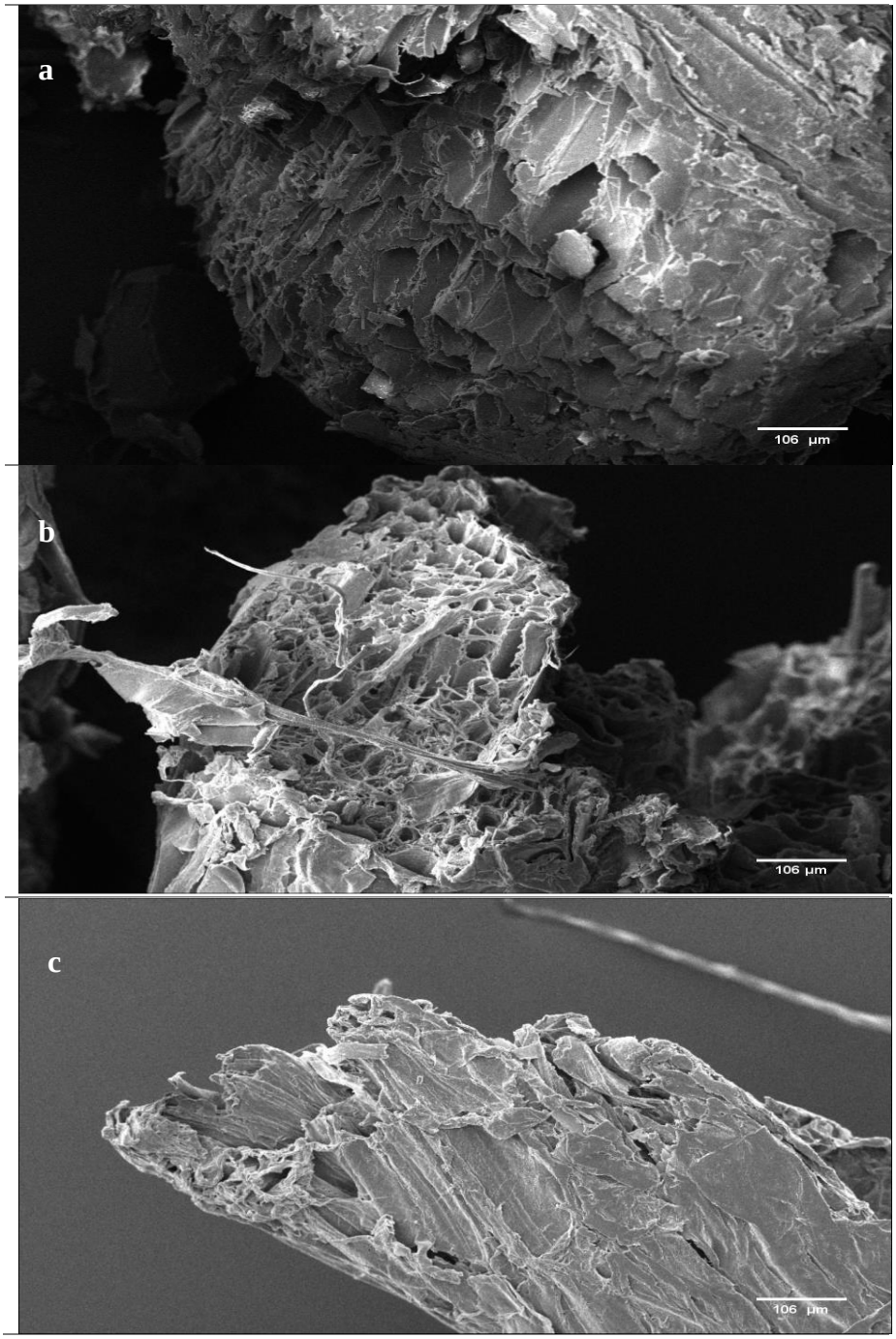


Figure 4.13 SEM images of depithed bagasse (a) untreated, (b) acid pretreated and (c) acid/ alkali pretreated

(iii) Thermal Analysis

DSC and TGA were used to study the thermal behaviour of the untreated and pretreated sugarcane bagasse in the temperature range of ambient temperature to 800 ^{o}C at a heating rate of 10 ^{o}C/ min under a nitrogen flow. Figs. 4.14 and 4.15 shows the DSC and TGA curves for depith (untreated, acid and acid/alkali pretreated sugarcane bagasse). The DSC curves in fig. 4.14 shows an exothermic peak at approximately 100 ^{o}C which is due to the removal of moisture when the sample was heated.

The TGA curves (fig. 4.15) exhibited three degradation steps. The initial weight loss is observed between 30 ^{o}C and 100 ^{o}C in all scans, this may be attributed to the evaporation of loosely bound moisture on the surfaces of the samples. The second step is observed between 250 ^{o}C and 350 ^{o}C and the third step between 350 ^{o}C and 500 ^{o}C. The exothermic peaks that appear at 350 ^{o}C and 400 ^{o}C are attributed to charring (Yang *et al*. 1997). Table 4.7 shows the weight loss and degradation temperatures of sugarcane bagasse fibers.

Table 4.7: Thermal properties of sugarcane bagasse fibers

Bagasse Sample		**Weight loss (%)**			**Degradation temperature (^{o}C)**		
		100 ^{o}C	350 ^{o}C	400 ^{o}C	100 ^{o}C	350 ^{o}C	400 ^{o}C
Depithed SB	Untreated	5.7	60.7	31.9	192.1	351	495
	Acid Pretreated	6.8	59.9	28.1	200.3	362.7	539.3
	Acid and Alkali Pretreated	8.7	62.8	28.1	190.9	356.2	480.2

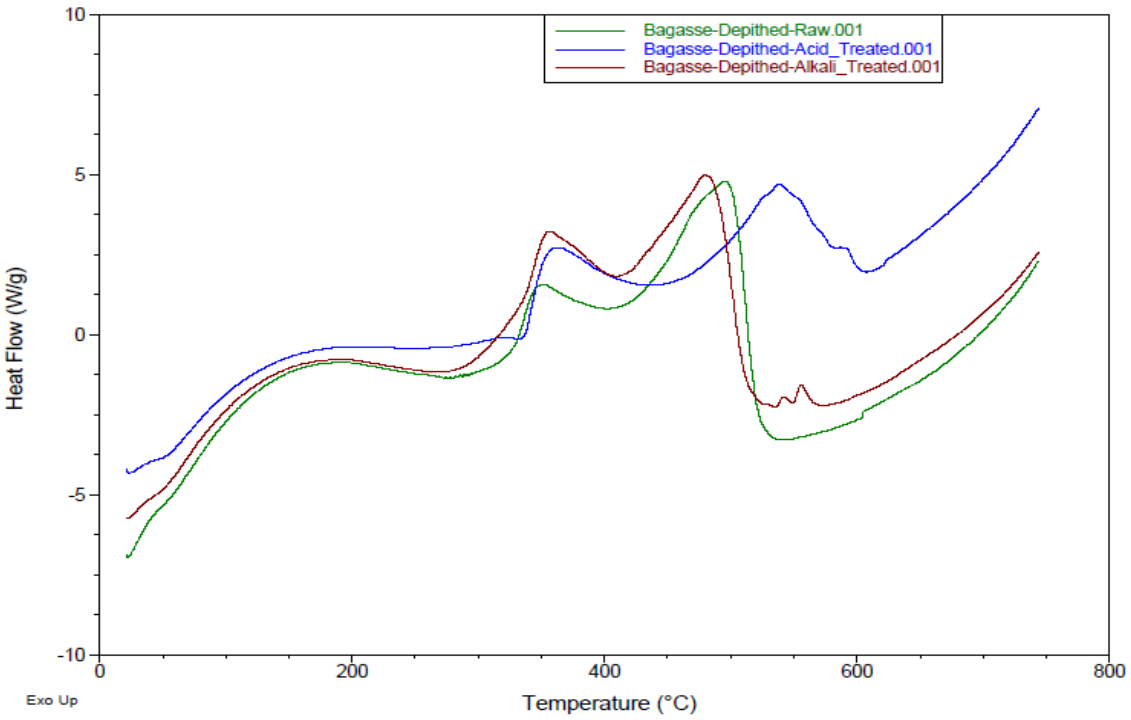


Figure 4.14 DSC thermograms for untreated and pretreated depithed bagasse

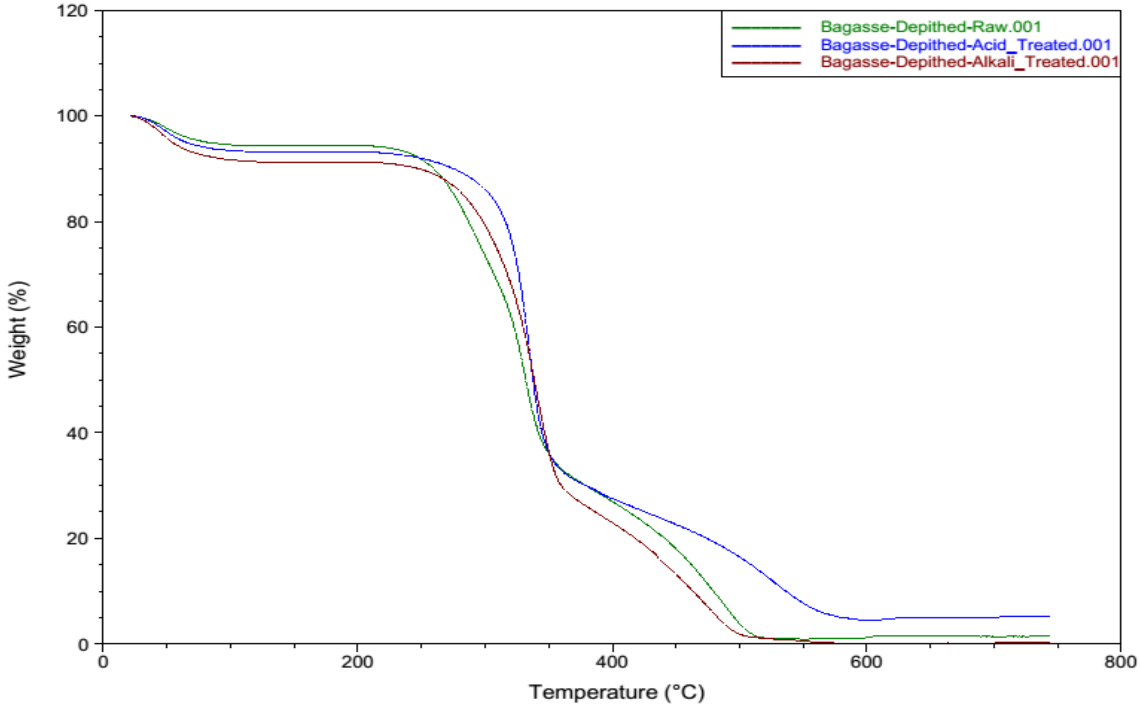


Figure 4.15 TGA thermograms for untreated and pretreated depithed sugarcane bagasse

4.2.3 Saccharification of Pretreated Sugarcane Bagasse

(i) Optimization of Enzyme Load and Time for Saccharification of Pretreated Sugarcane Bagasse

Fig. 4.16 illustrates the action of cellulase enzyme on alkali pretreated depithed sugarcane bagasse samples. The depithed sugarcane bagasse samples when hydrolysed with 30 FPU/g, released maximum amount of reducing sugars (536 mg/gds) after 20 hrs. 10 FPU/g gave the lowest reducing sugars.

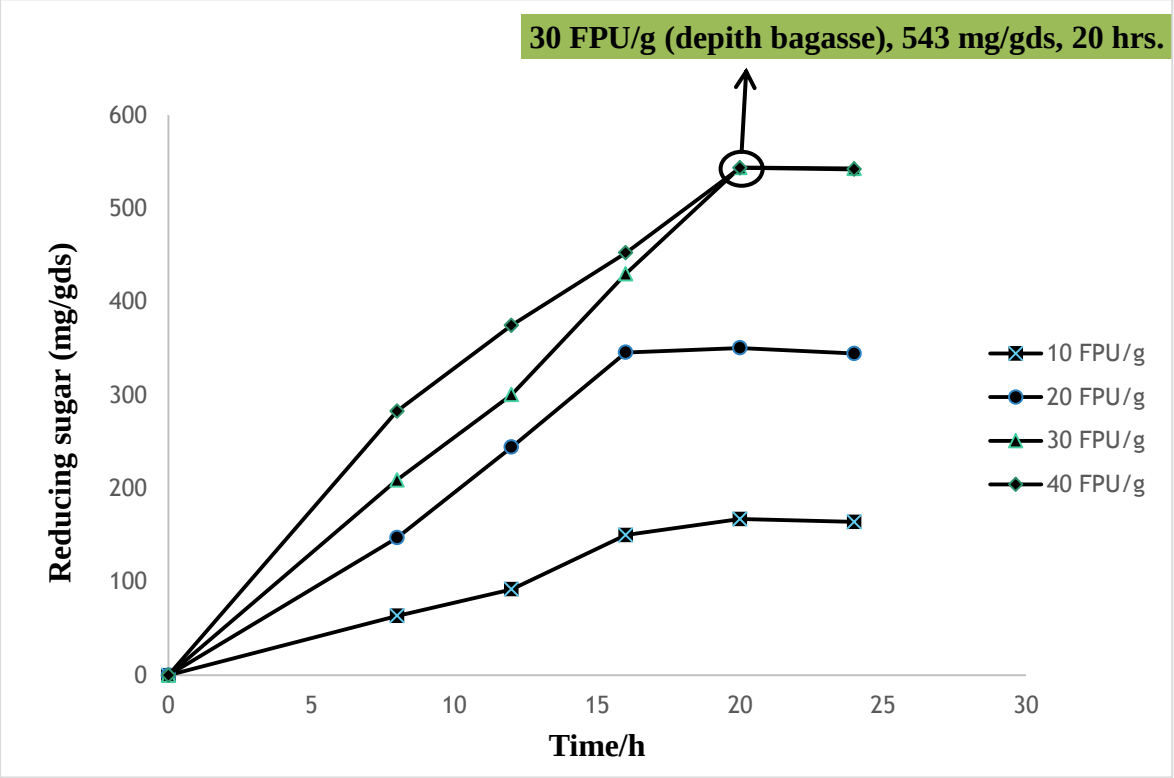


Figure 4.16 Effect of enzyme load on the hydrolysis of depithed sugarcane bagasse

(ii) Optimization of Surfactant Dose and Time for the Saccharification of Pretreated Sugarcane Bagasse

Fig. 4.17 shows the effect of different surfactant addition in saccharification of pretreated sugarcane bagasse. Among various dosages of surfactant (Tween 80) tested, Tween 80 at a dose of 0.4% (v/v) caused maximum increase in the saccharification efficiency with a release of 608 and 605 mg sugars/gds from depithed sugarcane bagasse samples (Fig. 4.17) after 20 hrs. of incubation. The addition of a surfactant to the enzymatic hydrolysis process increased the reducing sugars yield by 12 %. For this study Tween 80 was selected because it is known

not to denature the cellulolytic enzyme that brings about the saccharification (Wilke, 1978; Nystrom and Allen, 1976). The role of a surfactant is to lower the surface tension between two liquids or between a solid and a liquid (in the case of this study between pre-treated bagasse and cellulase enzyme) thereby increasing the spreading and wetting properties (www.britannica.com, last accessed in March 2015). Adsorption and orientation of the surfactant molecules at the solid-liquid interface should render the substrate readily wettable by the enzyme solution, bringing the substrate quickly into intimate contact with the enzymes and allowing the enzymes to reach the inaccessible substrate sites; moreover, interactions between the sorbed surfactant molecules, the substrate, and the adsorbed enzymes might result in a decrease of the force with which the enzyme is held on the substrate (Castanon and Wilke, 1981).

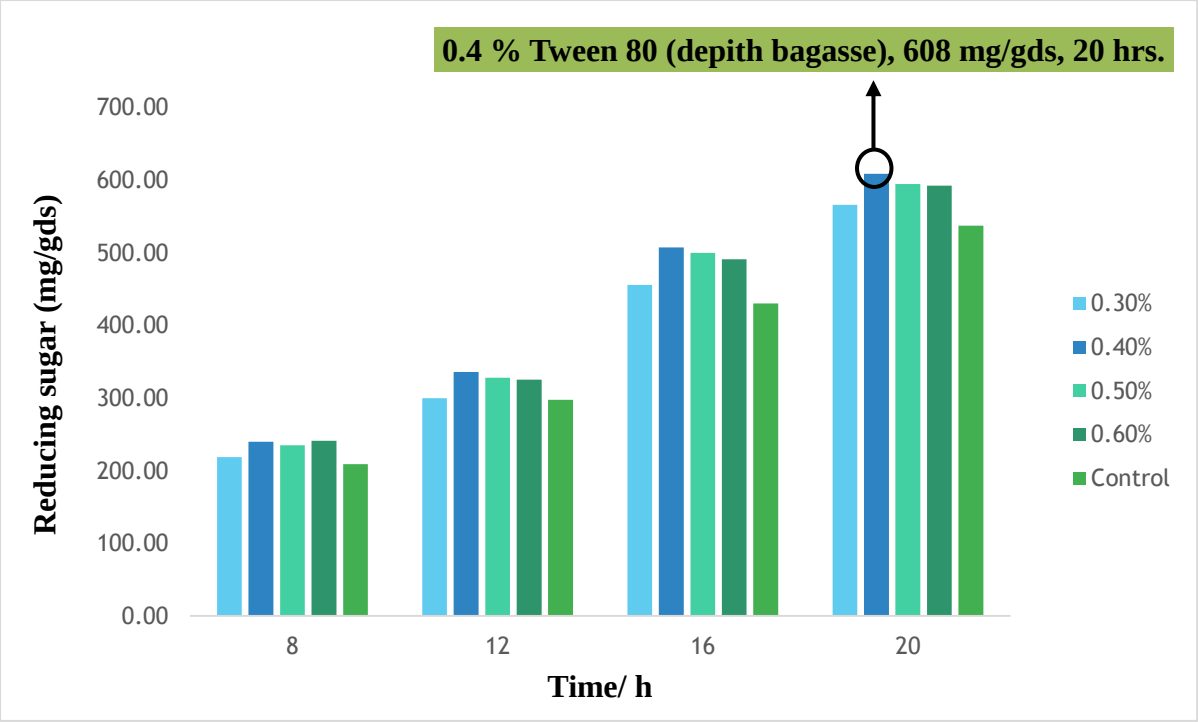


Figure 4.17 Effect of surfactant dose on the hydrolysis of depithed sugarcane bagasse.

(iii) Optimization of solid loading

The fig. 4.18 illustrates the effect of different substrate dose on the saccharification of pretreated sugarcane bagasse. The optimum conditions for enzyme load (30 FPU/g) and surfactant dose (0.4 %) were used to optimize the substrate consistency. The maximum reducing sugars was obtained at a 10 % solid loading with a sugar release of 609 mg/gds (see

fig. 4.18). 16 % solid loading gave the lowest reducing sugars. This shows that as the solid loading increases the reducing sugars decreases.

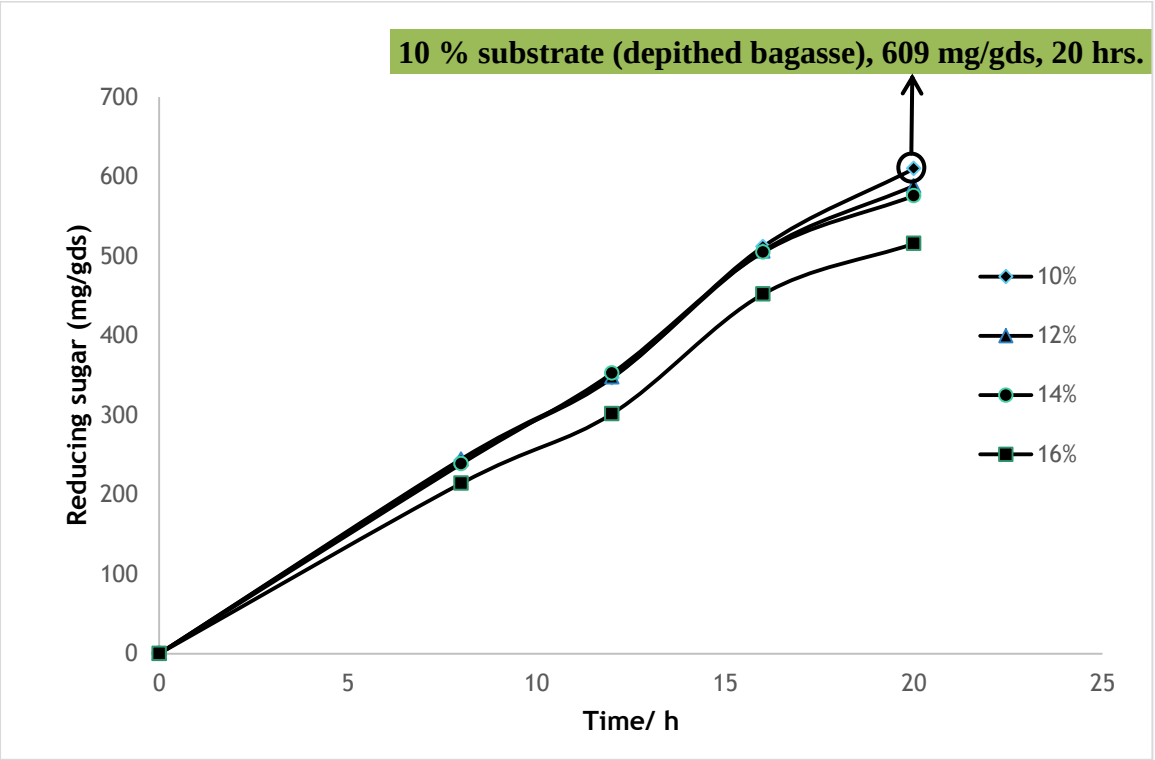


Figure 4.18 Effect of solid loading on the hydrolysis of depithed sugarcane bagasse

4.2 Part C: Production of Levulinic Acid from Mill-run and Depithed Sugarcane Bagasse

4.2.1 Characterizations of Sugarcane Bagasse and Produced LA

(i) Fourier Transform InfraRed (FTIR) Spectroscopy

(a) Acid and alkali pretreatment

The FTIR analysis was done to determine the changes from untreated SB; acid treated SB and after acid/alkali SB treated. Fig. 4.19 shows the FTIR spectrum of untreated mill-run SB, acid pretreated and acid/alkali treated bagasse and fig. 4.20 shows the FTIR spectrum of untreated depith SB, acid pretreated and acid/alkali treated bagasse. The band at 898 cm^{-1} is characteristic of the glycosidic bond β-(1 → 4) cellulose, C-H deformation in cellulose (Chandel *et al*. 2014; Pandey and Pitman 2003; Oh *et al*. 2005). The region between 1.200 and 1.100 cm^{-1} is a large contribution of hemicellulose and cellulose, which exhibits a maximum value around 1.035 cm^{-1} due to C-O stretching and 1.164 cm^{-1} for the asymmetrical stretching of C-O-C (Chandel *et al*. 2014; Colom *et al*. 2003; Pandey 2005; Pandey 1999). The region around 1.247 cm^{-1} was due to the stretching of C-O, which is characteristic of hemicellulose and lignin (Chandel *et al*. 2014; Pandey and Pitman 2003). A band around 1.458 cm^{-1} is reported to be a deformation of lignin CH_2 and CH_3, and 1.604 cm^{-1} is reported to be stretching of the C=C and C=O lignin aromatic ring. The band around 1.515 cm^{-1} is because of the C=C stretching of the aromatic ring in lignin (Chandel *et al*. 2014; Colom *et al*. 2003; Pandey 2005).

A band around 1.733 cm^{-1} is characteristic of C=O stretching of unconjugated hemicellulose. The peak around 2.850 cm^{-1} is reported due to the symmetric stretch of CH and CH_2, while the peak at 2.918 cm^{-1} is due to asymmetrical stretching of CH_2 and CH. Both denote the characteristics of cellulose (Chandel *et al*. 2014; Ivanova 1989). The region between 3.800 and 3.000 cm^{-1} covers the related crystalline structure of cellulose. This region represents the sum of the vibration of valence bands of the hydrogen bond of the OH group and the bands of intra-molecular and intermolecular hydrogen bonds (Chandel *et al*. 2014; Hinterstoisser and Salmen 1999).

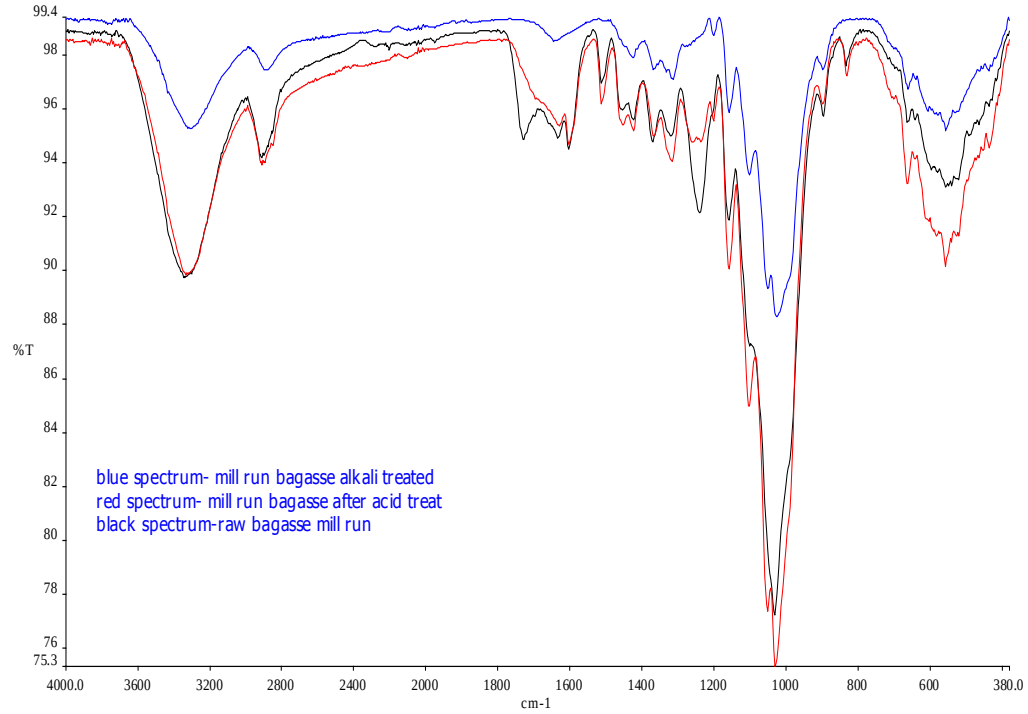


Figure 4.19 FTIR scan for mill-run untreated SB, acid treated and alkali treated bagasse

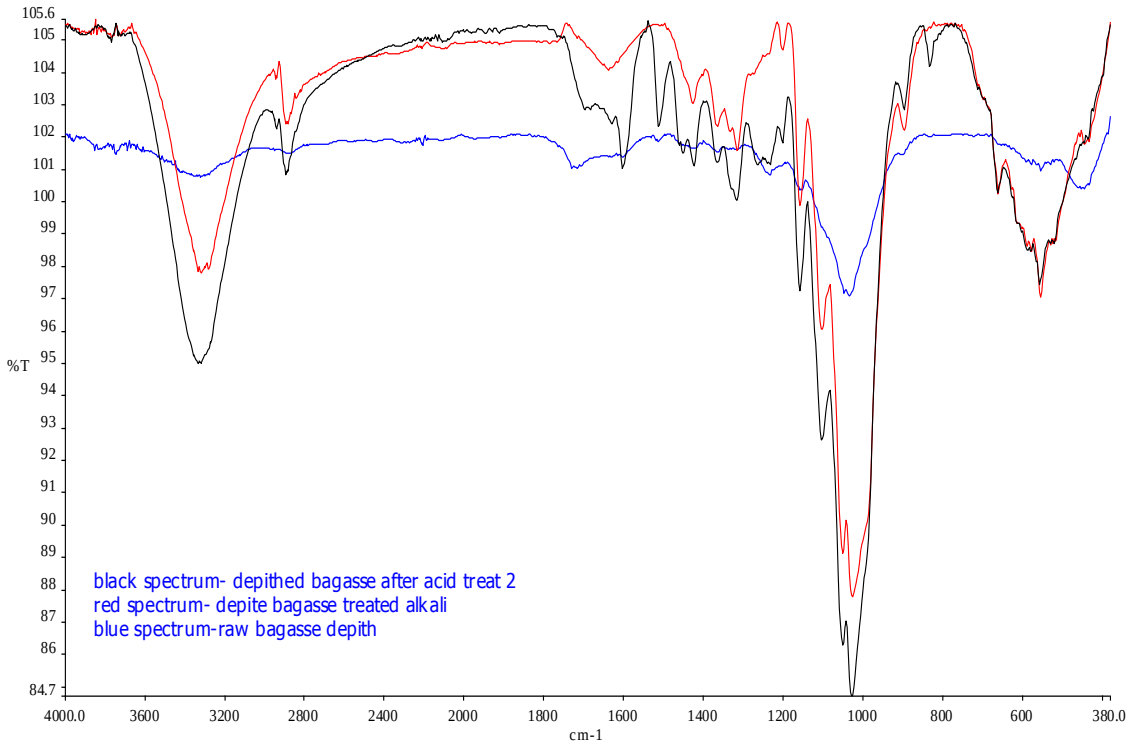


Figure 4.20 FTIR scan for untreated, acid treated and alkali treated depithed bagasse

(b) Levulinic acid from mill-run and depithed SB using both sulphuric acid and methanesulfonic acid as catalysts

The FTIR spectrums from figs. 4.21 – 4.24, show that there is a presence of the LA. The peak observed around 3300-2500 cm^{-1} is for the O-H stretch for the carboxylic acid group, 3000-2850 cm^{-1} for the C-H stretch for alkane group, 1760-1690 cm^{-1} for the C=O cm^{-1} stretch for carboxylic acid, 1715 cm^{-1} for C=O stretch for ketone group, 1370-1350 cm^{-1} C-H rock for alkane, 1320-1000 cm^{-1} C-O stretch for carboxylic acid group and 950-910 cm^{-1} for the O-H bend for carboxylic acid group. When looking at the structure of LA it can be seen that there is a carboxylic group, ketone group and alkanes and these functional group they are observed in the FTIR spectra for the LA produced from both depithed and mill-run SB using both MSA and H_2SO_4 for the hydrolysis. When comparing produced LA and literature spectra, they are almost the same. There is no difference observed for depith/mill run and also for the MSA/H_2SO_4 from the FTIR spectra of LA they are all similar.

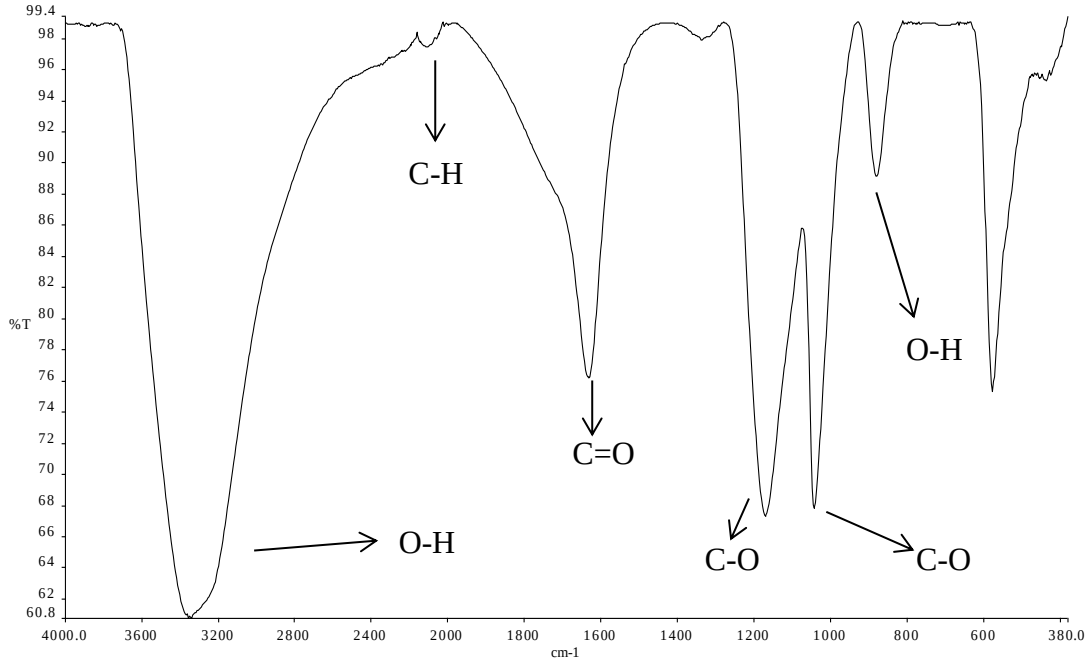


Figure 4.21 FTIR scan for LA produced using sulphuric acid from depithed sugarcane bagasse

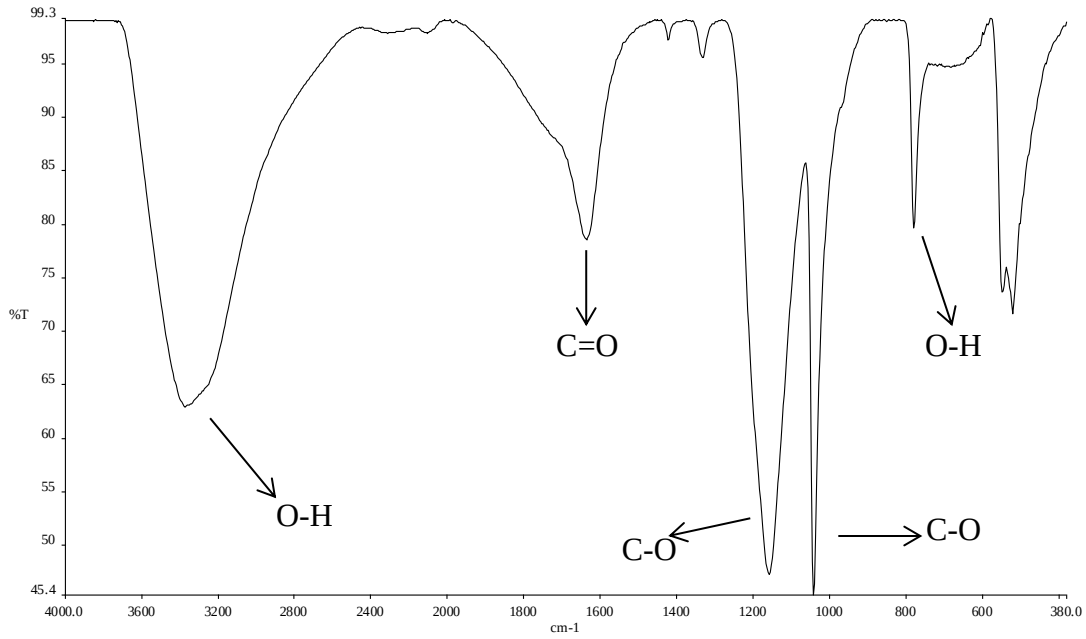


Figure 4.22 FTIR scan for LA from depithed sugarcane bagasse using MSA

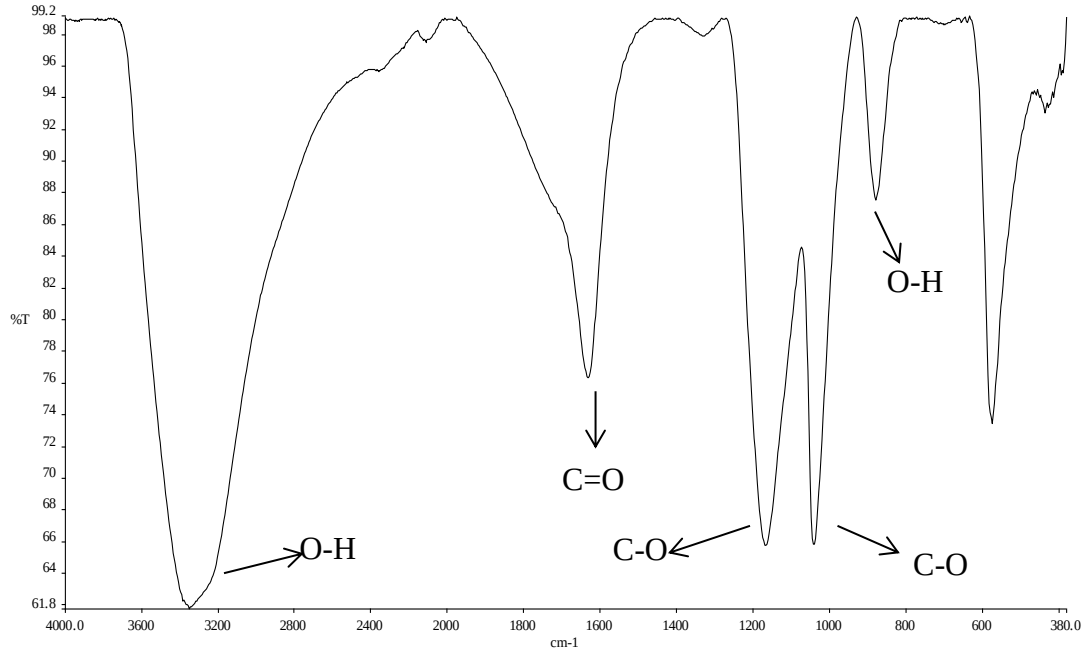


Figure 4.23 FTIR scan for LA produced from mill-run sugarcane bagasse using sulphuric Acid

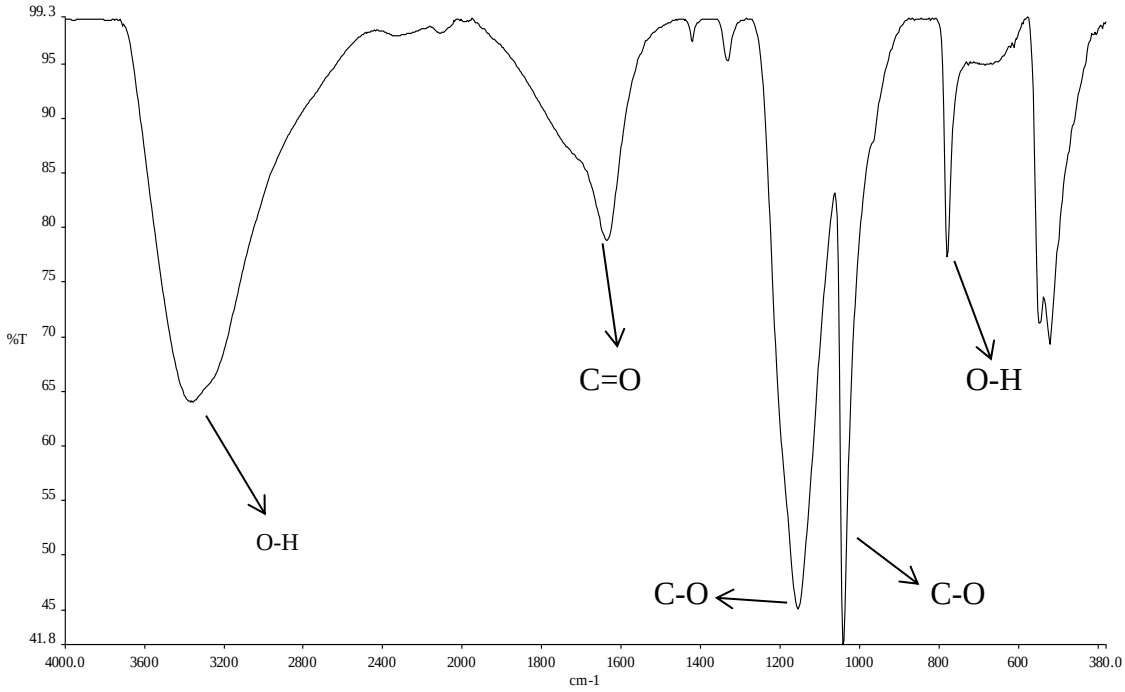
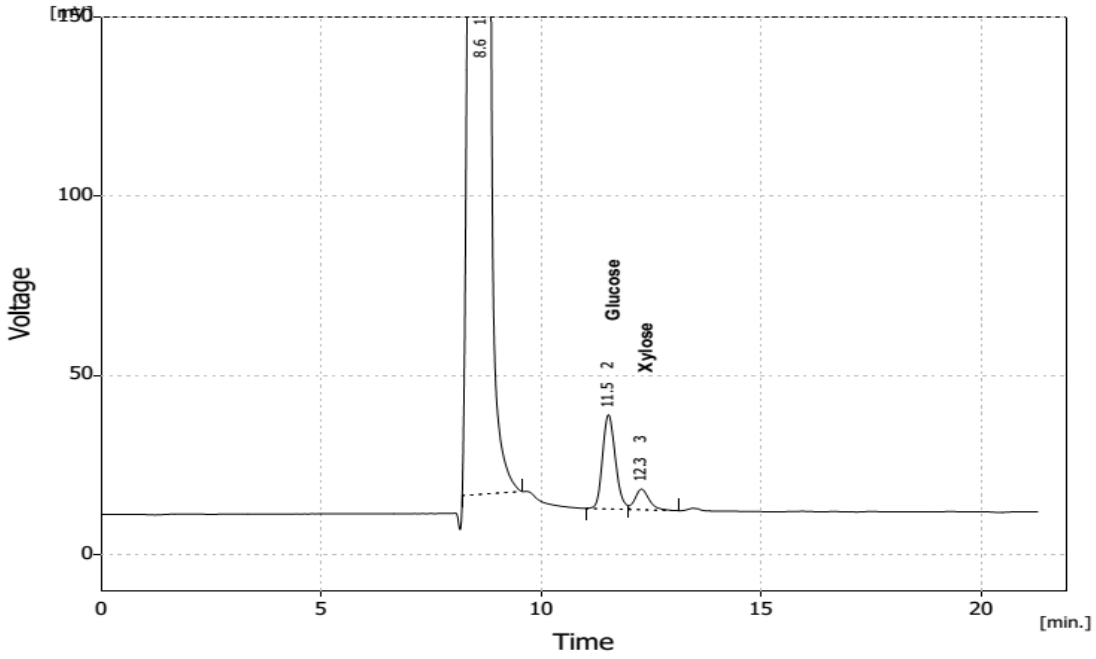


Figure 4.24 FTIR scan for LA produced from mill-run sugarcane bagasse using MSA

(ii) Analysis of the hydrolysate produced

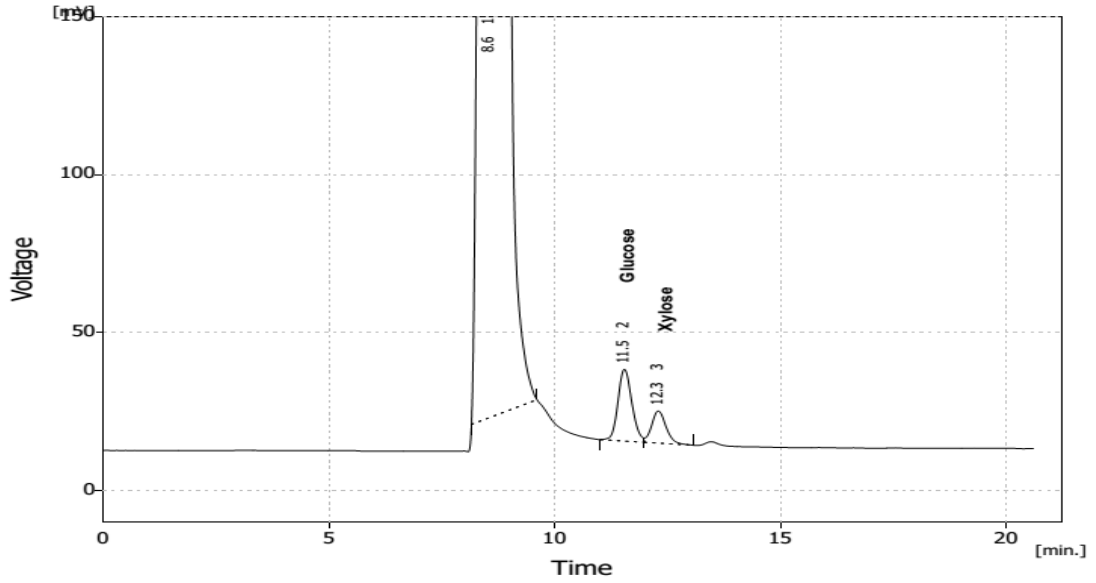
The HPLC was done for the qualitative and quantitative analysis of the hydrolysate produced from the acid hydrolysis of the cellulose extracted from depith and mill-run bagasse. Figs. 4.25 - 4.28 are the HPLC chromatogram for depith and mill-run samples that were hydrolysed by both sulphuric acid and methanesulfonic acid, there are three peaks observed, the first peak is for the solvent and the other two peaks are for glucose and xylose.



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ sample A 26-May-2015 11_00 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	25185.298	1233.055	97.4	97.5	0.32	0.000
2	11.5	533.462	26.287	2.1	2.1	0.32	0.000
3	12.3	128.448	5.783	0.5	0.5	0.34	0.000
	Total	25847.208	1265.125	100.0	100.0		0.000

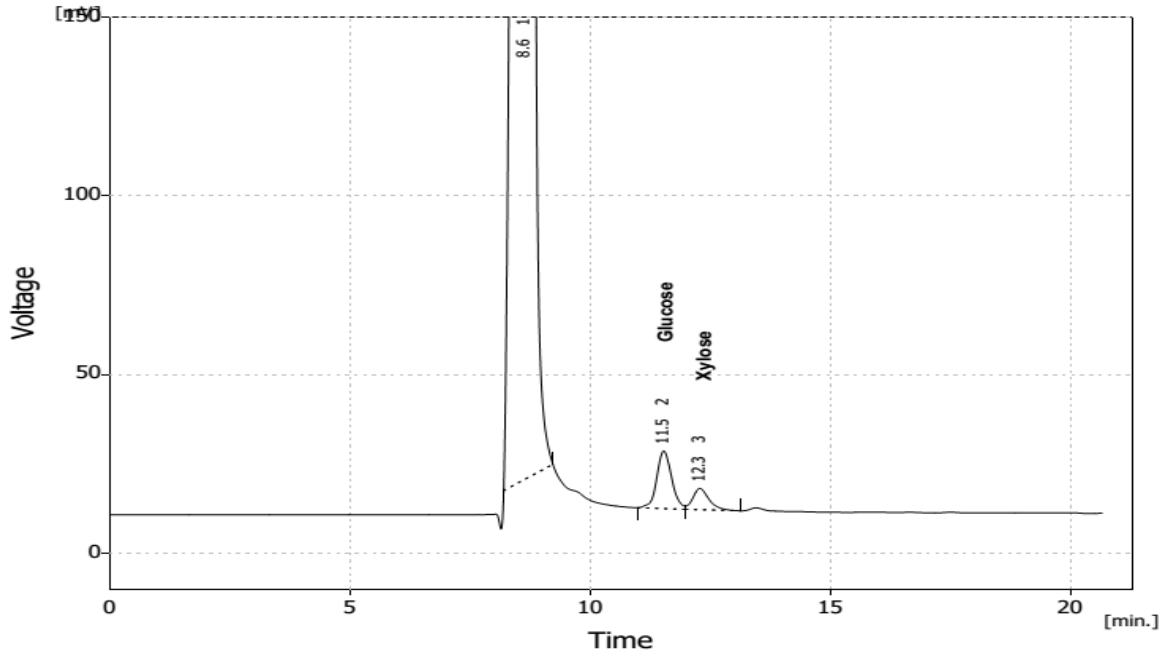
Figure 4.25 HPLC chromatogram for depith sugarcane bagasse hydrolysed with MSA



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ sample C 26-May-2015 11_47 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	42691.443	1226.852	98.4	97.4	0.56	0.000
2	11.5	487.501	22.720	1.1	1.8	0.33	0.000
3	12.3	224.849	10.166	0.5	0.8	0.34	0.000
	Total	43403.793	1259.738	100.0	100.0		0.000

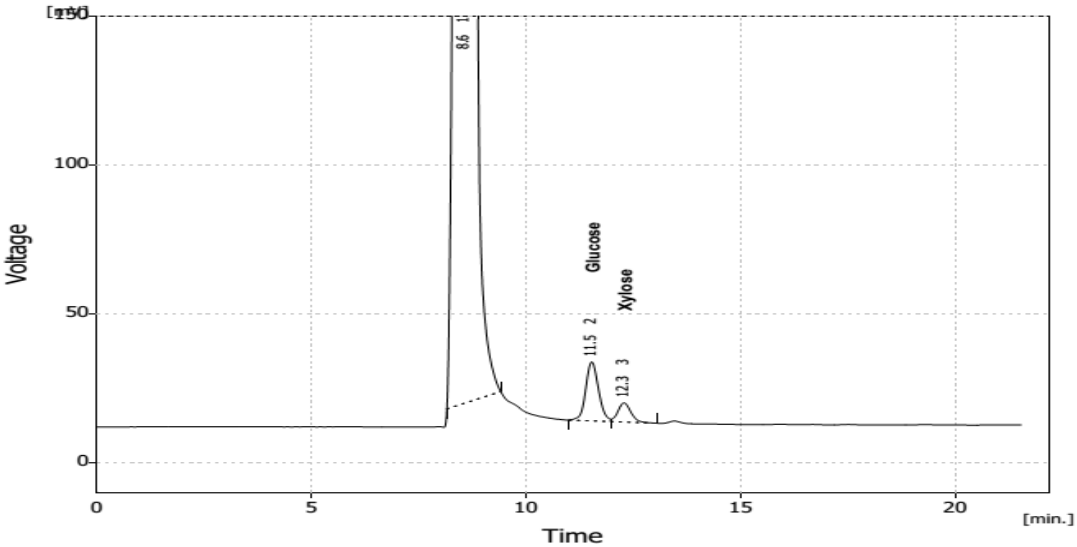
Figure 4.26 HPLC chromatogram for depith sugarcane bagasse hydrolysed with sulphuric acid



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ sample D 26-May-2015 12_19 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	26285.052	1229.236	98.2	98.2	0.34	0.000
2	11.5	346.792	16.146	1.3	1.3	0.33	0.000
3	12.3	138.861	5.944	0.5	0.5	0.35	0.000
	Total	26770.705	1251.326	100.0	100.0		0.000

Figure 4.27 HPLC chromatogram for mill-run sugarcane bagasse hydrolysed with MSA



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ sample B 26-May-2015 11_24 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	30200.032	1229.975	98.2	97.9	0.39	0.000
2	11.5	410.629	19.811	1.3	1.6	0.32	0.000
3	12.3	136.562	6.447	0.4	0.5	0.33	0.000
	Total	30747.224	1256.233	100.0	100.0		0.000

Figure 4.28 HPLC chromatogram for the mill-run sugarcane bagasse hydrolysed with sulphuric acid

Different standards and four samples were analysed by HPLC the calibration curve was drawn from those result (see fig. 4.29), using the calibration curve the concentration of glucose and xylose in the samples were determined. The graph in fig. 4.30 shows the concentration of glucose and xylose in the samples. These concentration of glucose are very small to be used for the production of LA because the glucose from these samples have to be acid hydrolysed to produce LA. The reaction starts from glucose, it then isomarises to fructose then HMF is formed which is further hydrolysed to LA and formic acid, because of that LA concentration that was formed from these samples were very small in that it was not possible to be quantified by the HPLC

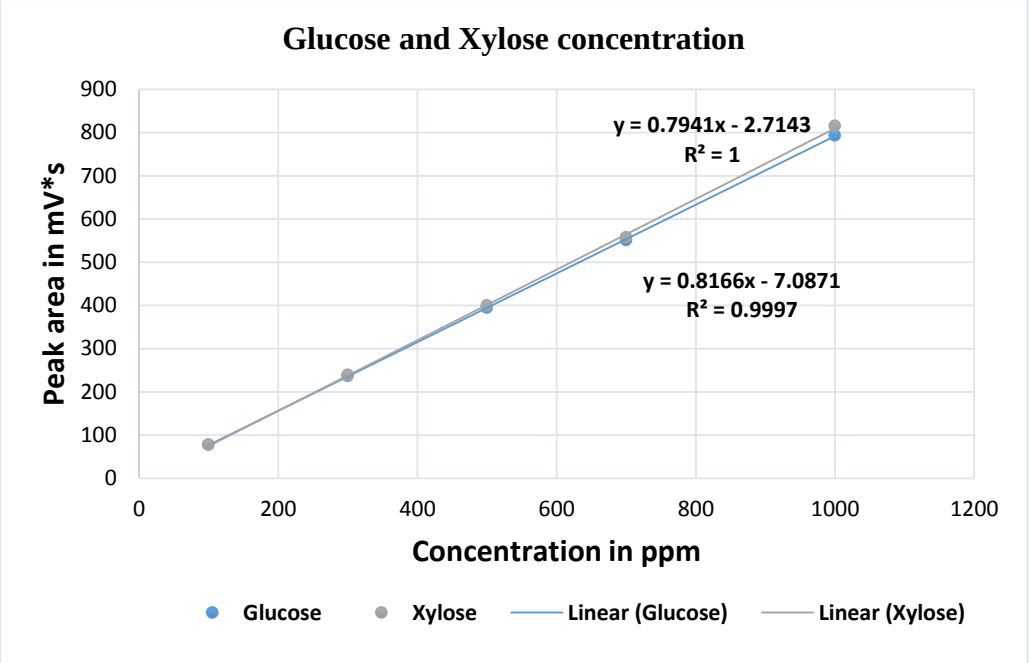


Figure 4.29 Calibration curve for the glucose and xylose standard

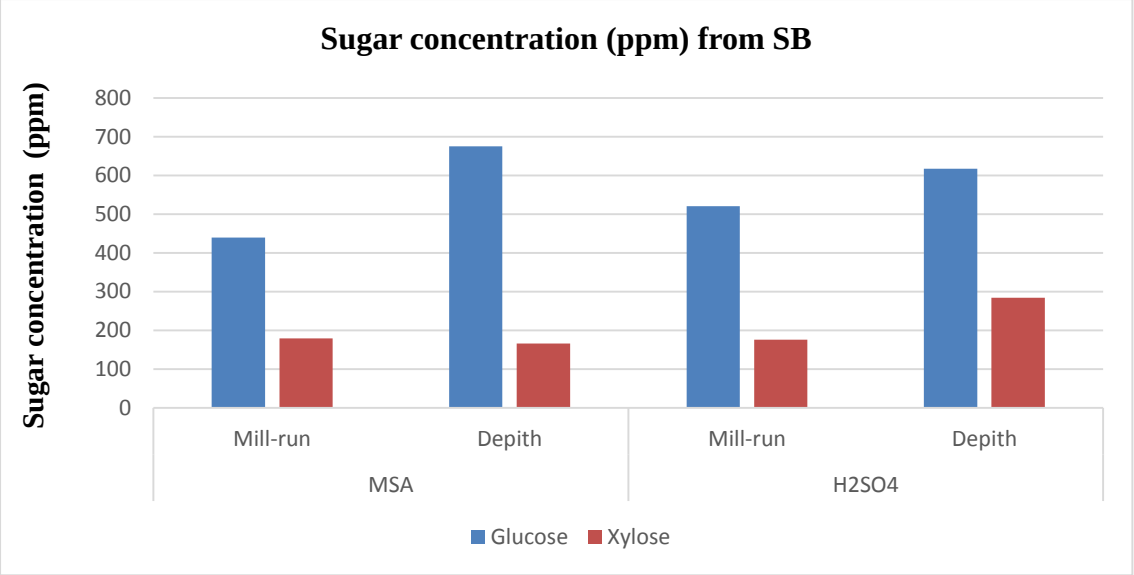
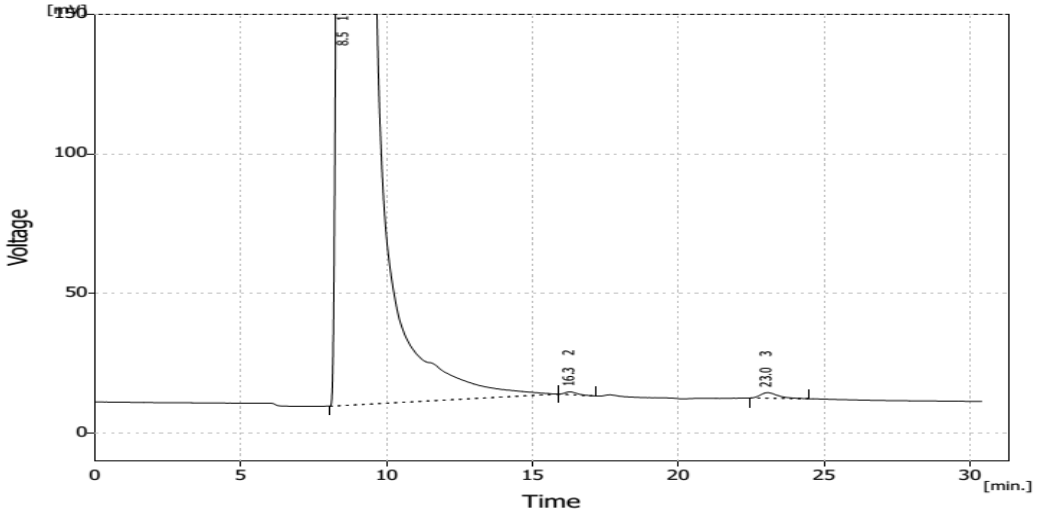


Figure 4.30 Glucose and xylose concentration from acid hydrolysed cellulose

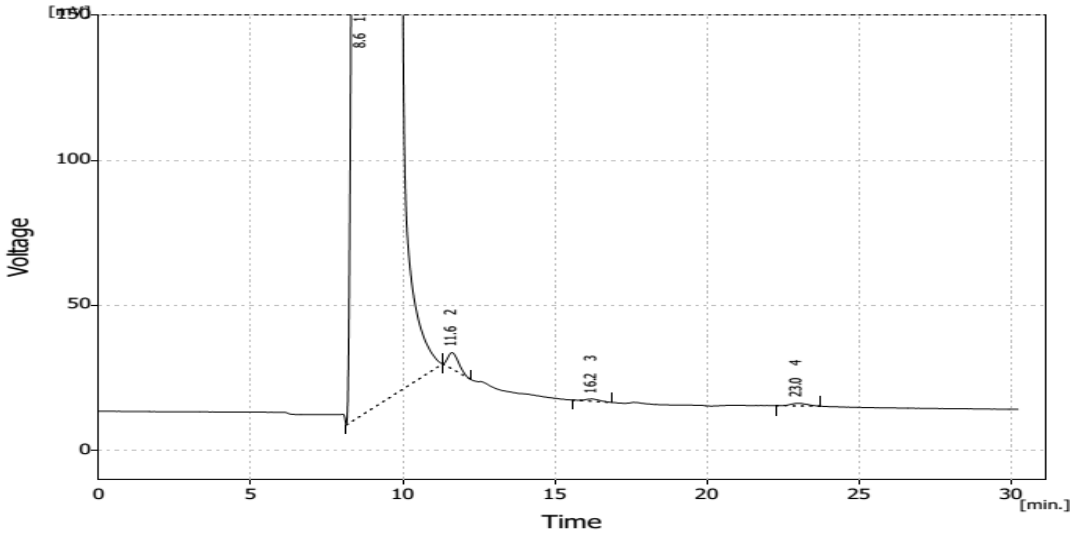
The HPLC chromatogram for the glucose solution that were hydrolysed by 6.0 M acid at 150 ^{o}C for 1 hr. are given in figs. 4.31 - 4.34, they all show that there is LA and formic acid this was determined by the analyzing the standard of LA and formic acid, but the concentration for the LA, formic acid and glucose was too small to be quantified.



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ depith h2so4 09-Jun-2015 13_39 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.5	87206.030	1240.009	99.9	99.8	1.07	0.000
2	16.3	29.668	1.015	0.0	0.1	0.43	0.000
3	23.0	84.281	2.003	0.1	0.2	0.63	0.000
	Total	87319.979	1243.026	100.0	100.0		0.000

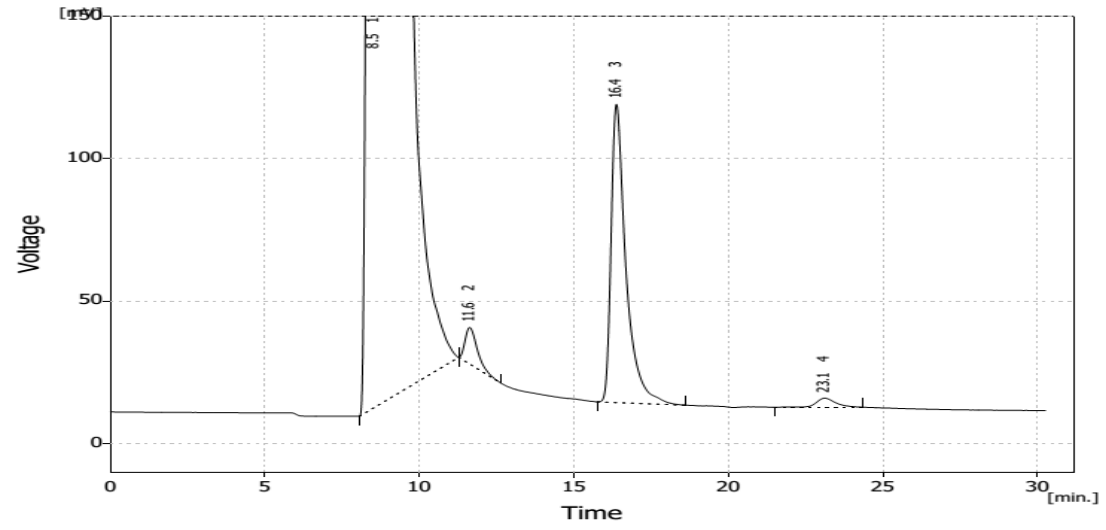
Figure 4.31 HPLC chromatogram for depith sugarcane bagasse hydrolysed by sulphuric acid



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ depith msa 09-Jun-2015 13_06 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	106708.774	1238.019	99.8	99.4	1.41	0.000
2	11.6	140.629	5.651	0.1	0.5	0.39	0.000
3	16.2	27.710	0.826	0.0	0.1	0.53	0.000
4	23.0	33.657	0.897	0.0	0.1	0.60	0.000
	Total	106910.771	1245.393	100.0	100.0		0.000

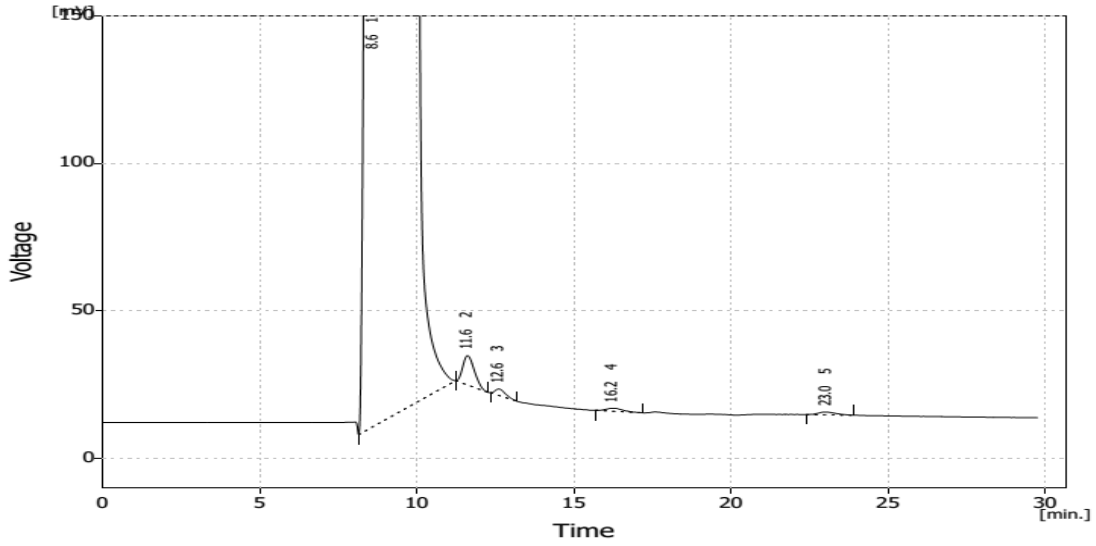
Figure 4.32 HPLC chromatogram for depith sugarcane bagasse hydrolysed by MSA



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ mill-rum h2so4 09-Jun-2015 14_11 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.5	88979.486	1237.287	95.7	91.1	1.11	0.000
2	11.6	359.195	12.825	0.4	0.9	0.40	0.000
3	16.4	3506.225	104.671	3.8	7.7	0.47	0.000
4	23.1	142.989	3.214	0.2	0.2	0.64	0.000
	Total	92987.895	1357.997	100.0	100.0		0.000

Figure 4.33 HPLC chromatogram for mill-run sugarcane bagasse hydrolysed by sulphuric acid



Result Table (Uncal - C:\Clarity\Prof Deenadayalu's students\LETHIWE\ millrun msa 09-Jun-2015 12_33 - Detector 1)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Amount
1	8.6	110026.718	1239.048	99.7	98.9	1.46	0.000
2	11.6	258.145	9.979	0.2	0.8	0.40	0.000
3	12.6	51.481	2.137	0.0	0.2	0.34	0.000
4	16.2	38.041	1.005	0.0	0.1	0.57	0.000
5	23.0	38.202	0.964	0.0	0.1	0.60	0.000
	Total	110412.588	1253.132	100.0	100.0		0.000

Figure 4.34 HPLC chromatogram for mill-run sugarcane bagasse hydrolysed by MSA

4.2.2 Differential Scanning Calorimetry/ ThermoGravimetric Analysis (DSC/TGA)

The DSC curve in fig. 4.35, shows an exothermic peak near 110 ^{O}C, which is due to the release of moisture as the sample was heated. Two exothermal peak appear at 310 ^{O}C and 385 ^{O}C, respectively, and they are attributed to charring, and the other endothermic peak at 365 ^{O}C is related to the cellulose fraction. This behaviour is related to the full decomposition of cellulose that might be attributed to quick devolatilization reactions, leading to very little solid residue (Pereira *et al*. 2011).

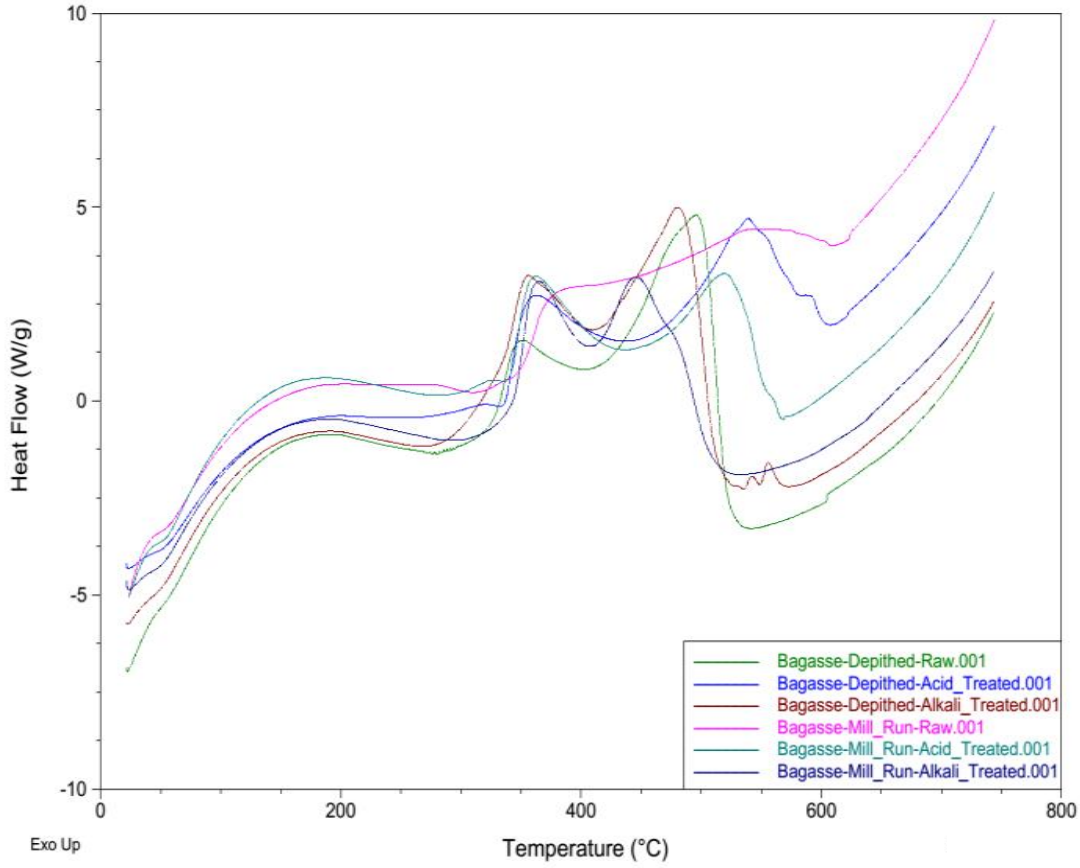


Figure 4.35 DSC thermograms for depithed sugarcane bagasse

Fig. 4.36 illustrates the thermal degradation curves of holocellulose and lignin in the biomass with three distinct stages of weight losses. Initial weight losses were observed between 30 ^{o}C and 100 °C, which can be regarded as the evaporation of residual water in the biomass samples. The second stage at a temperature range of 150 ^{o}C - 350 °C is the decomposition of holocellulose. Meanwhile, the degradation of lignin occurred at 350 ^{o}C - 520 °C. The residue is defined as the ash content. The temperature ranges are aligned with a previous TGA study of biomass composition (Ya'aini *et al*. 2012; Yin *et al*. 2011). Figs. 4.37 - 4.42 illustrate the DSC/TGA thermograms for untreated and treated mill-run and depith sugarcane bagasse.

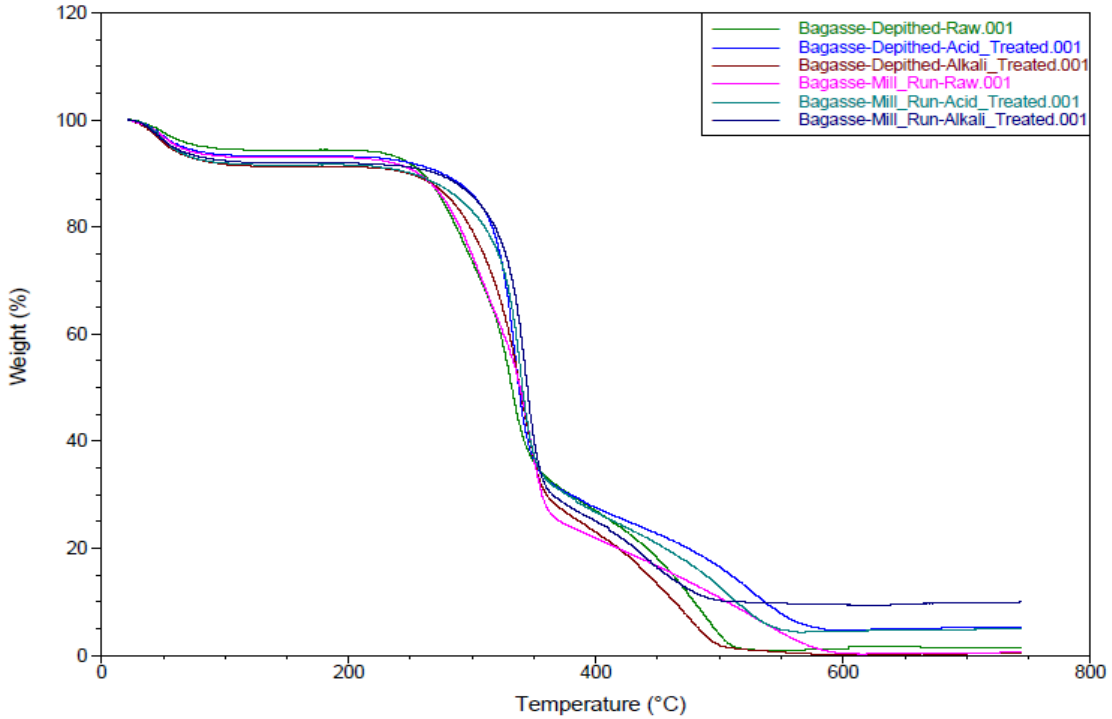


Figure 4.36 TGA thermograms for depithed and mill-run sugarcane bagasse

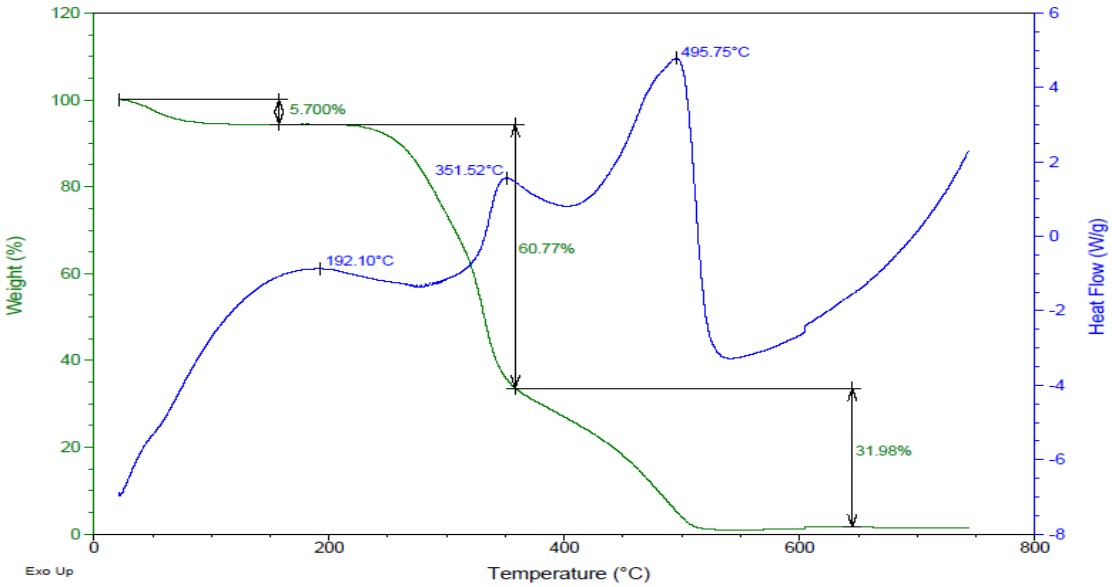


Figure 4.37 DSC/TGA thermograms for untreated depithed sugarcane bagasse

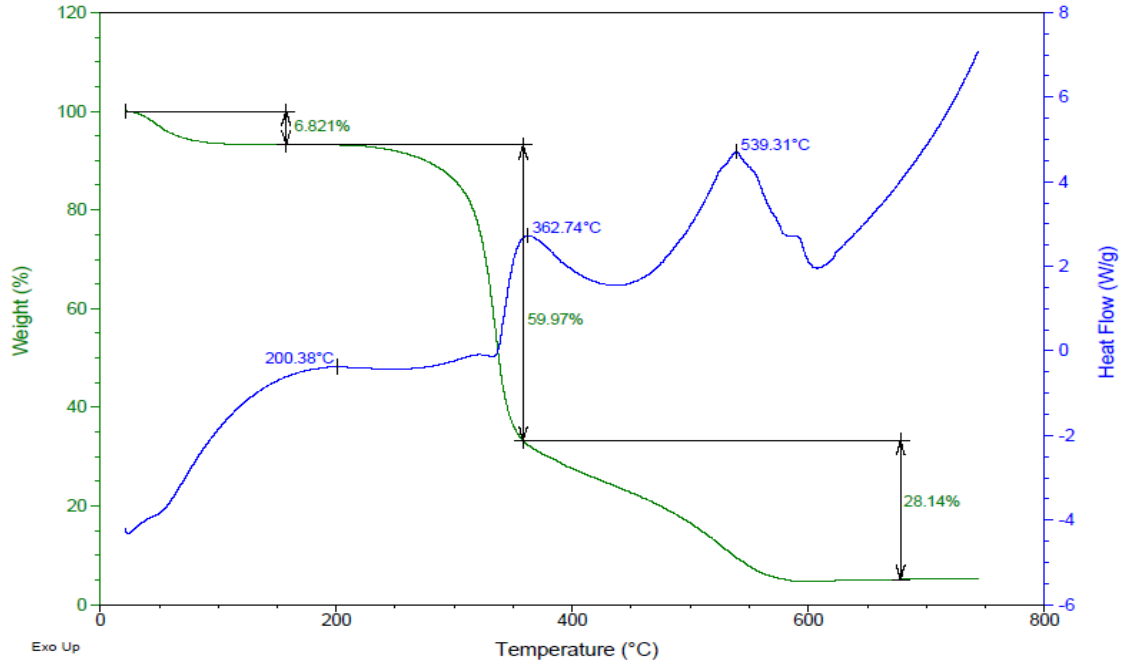


Figure 4.38 DSC/TGA thermograms for acid treated depithed sugarcane bagasse

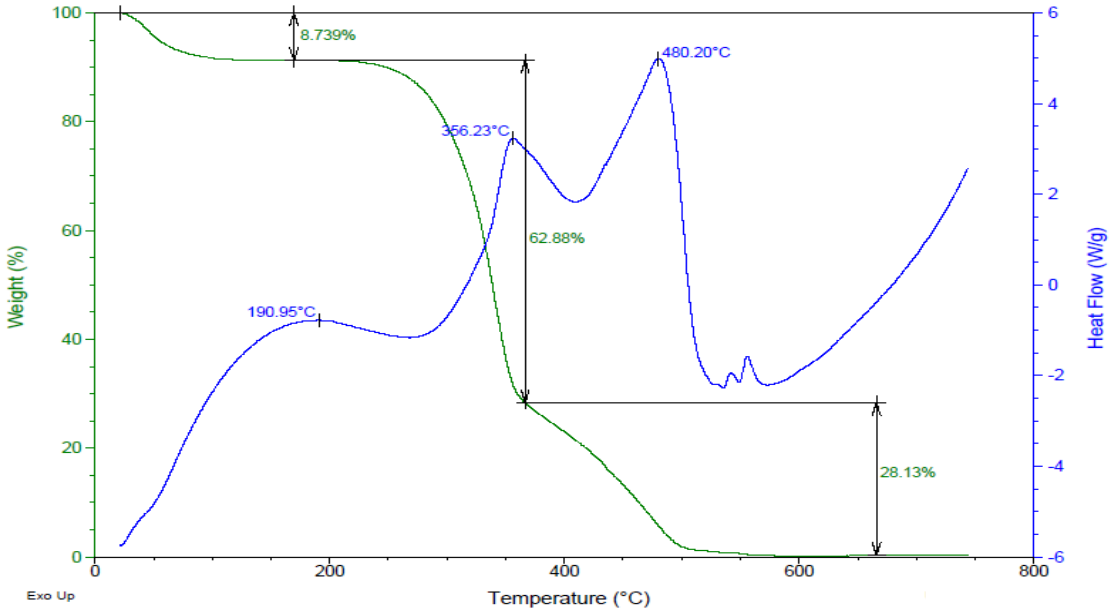


Figure 4.39 DSC/TGA thermograms for alkali treated depithed bagasse

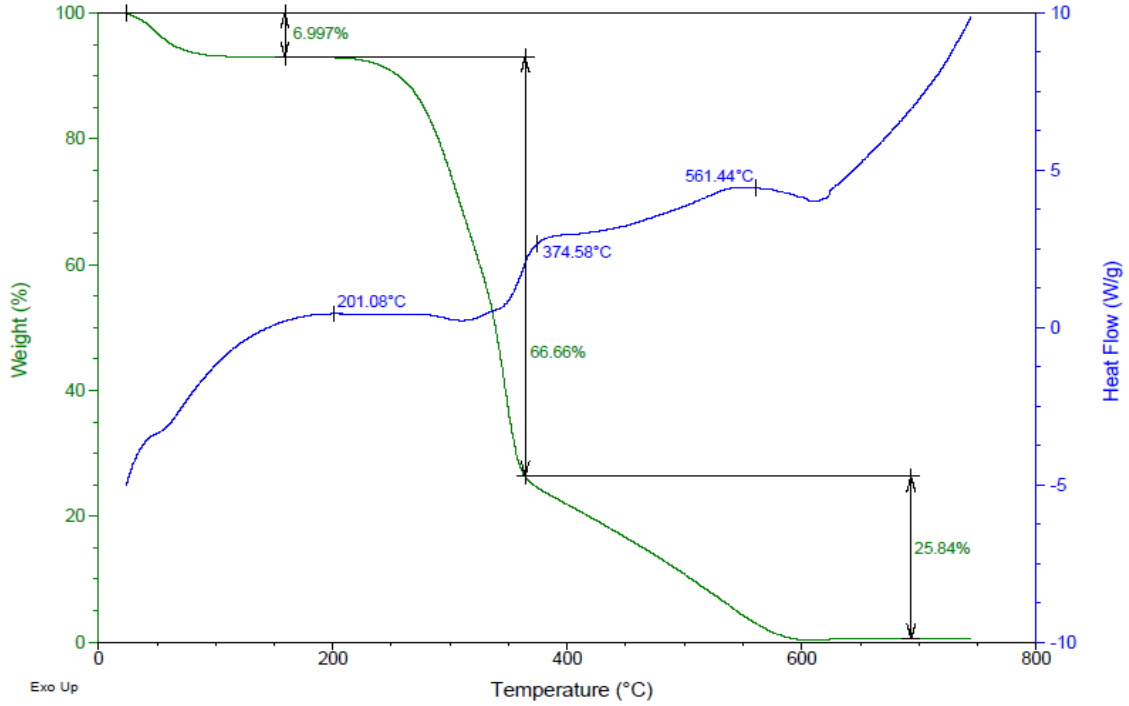


Figure 4.40 DSC/TGA thermograms for untreated mill-run sugarcane bagasse

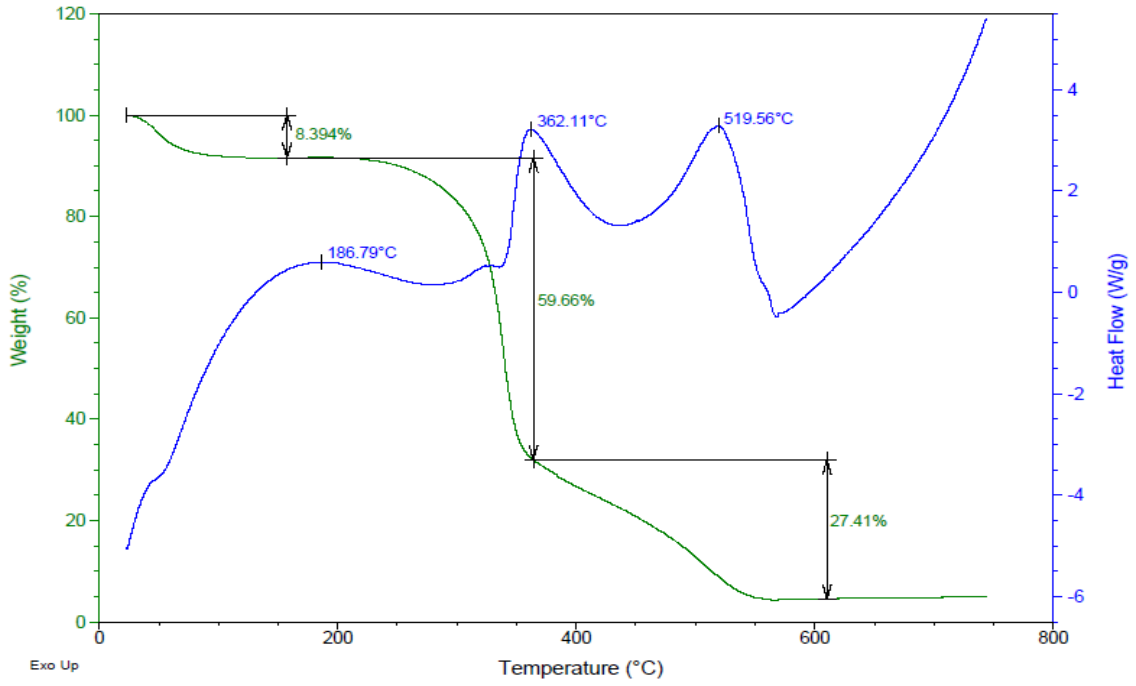


Figure 4.41 DSC/TGA thermograms for acid treated mill-run sugarcane bagasse

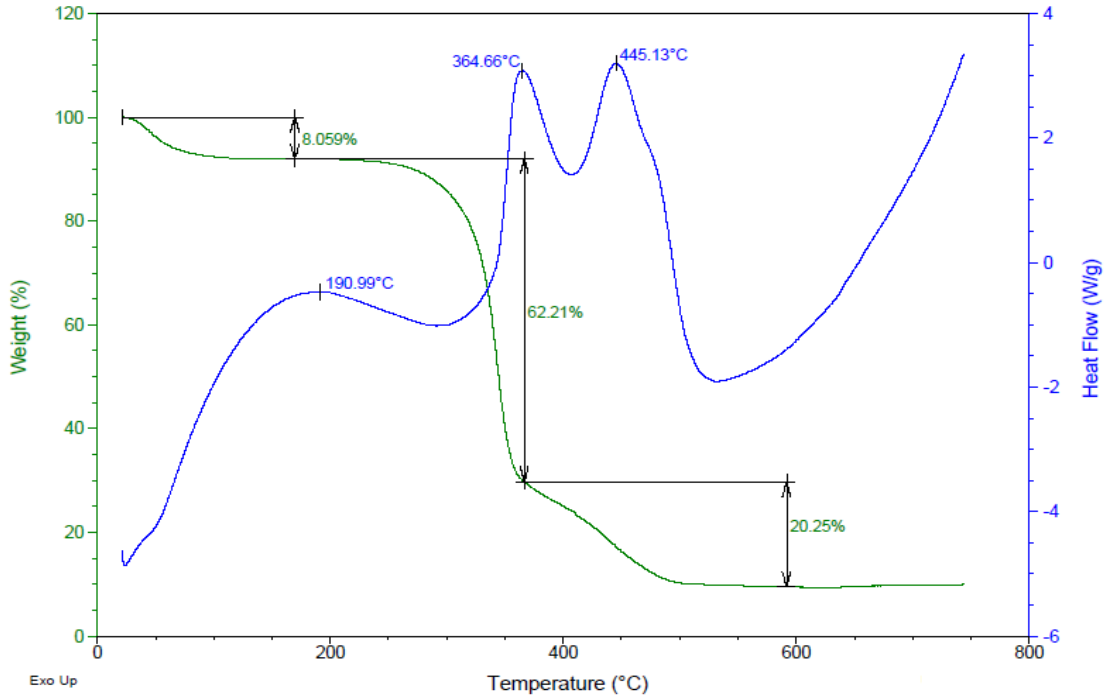


Figure 4.42 DSC/TGA thermograms for acid and alkali treated mill-run sugarcane bagasse

4.2.3 Scanning Electron Microscopy (SEM) Analysis

The ultra-structure of the untreated sugarcane bagasse by SEM was compared with the acid treated sugarcane bagasse (cellulignin) and alkali treated cellulignin. Figs. 4.43, 4.46 shows the anatomy of depithed and mill-run sugarcane bagasse which is compact, rough, and has thick-walled fiber cells interlinked with pith. Fibers are constituted by parallel stripes and are superficially covered with extractives. The most apparent effect of acid pretreatment is the separation of fibers from pith and loosening of the fibrous network. The acid pretreatment removes hemicellulose from bagasse disrupting the cell wall with a loose matrix figs. 4.44 and 4.47.

After the alkali pretreatment of cellulignin, lignin was removed substantially and the surface of the substrate was very smooth with the appearance of parallel sheaths. Appearance of pores and lignin droplets can also be seen in alkali pre-treated sugarcane bagasse figs. 4.45, 4.48. Rezende *et al*. (2011) also observed similar morphological changes in the bagasse surface after acid and alkali pretreatment, although our conditions are different. They used different percentage of NaOH and 1 % of H_2SO_4 whereas for this project 3 % H_2SO_4 and 4 % NaOH was used but they observed the removal of the pith from fibers during dilute sulfuric

acid pre-treatment followed by extensive dismantling of vascular bundles and detachment of fibers in NaOH pretreated cellulignin just like it can be observed from figs. 4.43 - 4.48 and also Chandel *et al*. (2014) observed the similar effect as it was observed for this project.

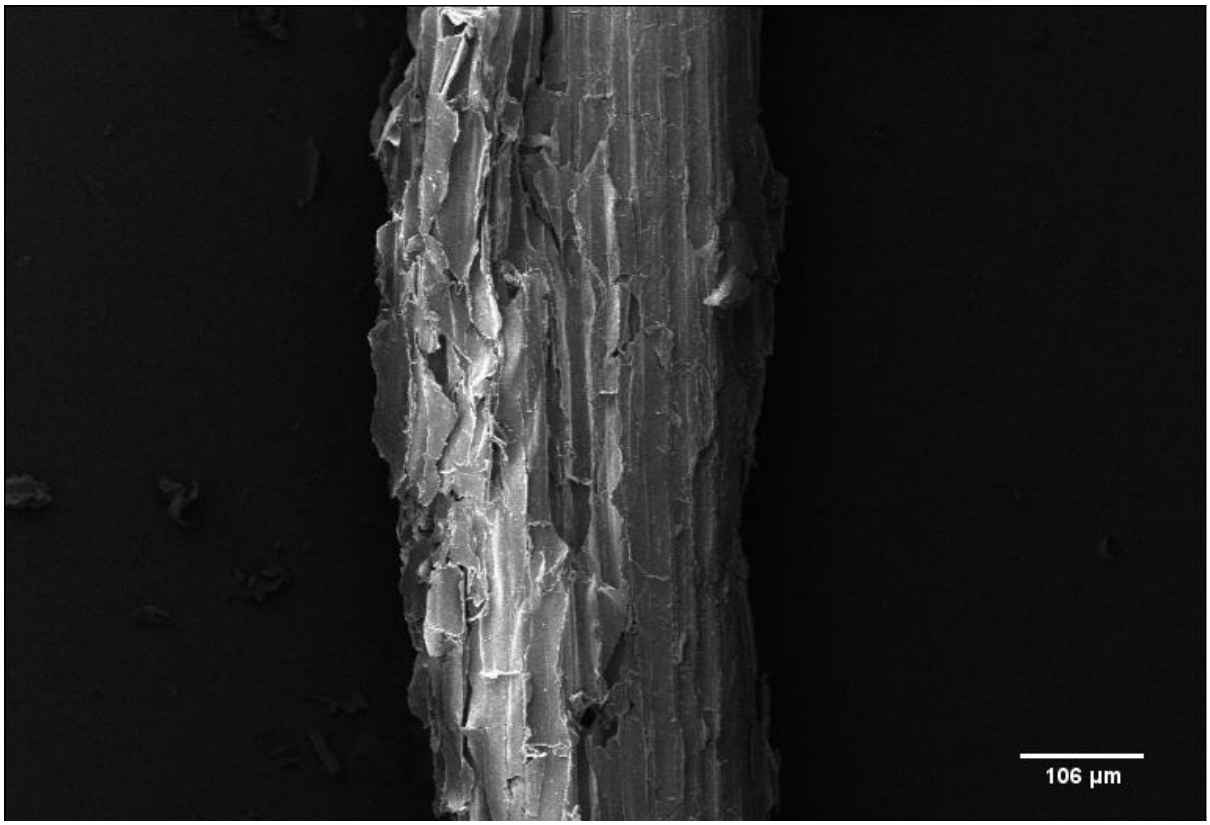


Figure 4.43 SEM image of untreated depithed sugarcane bagasse

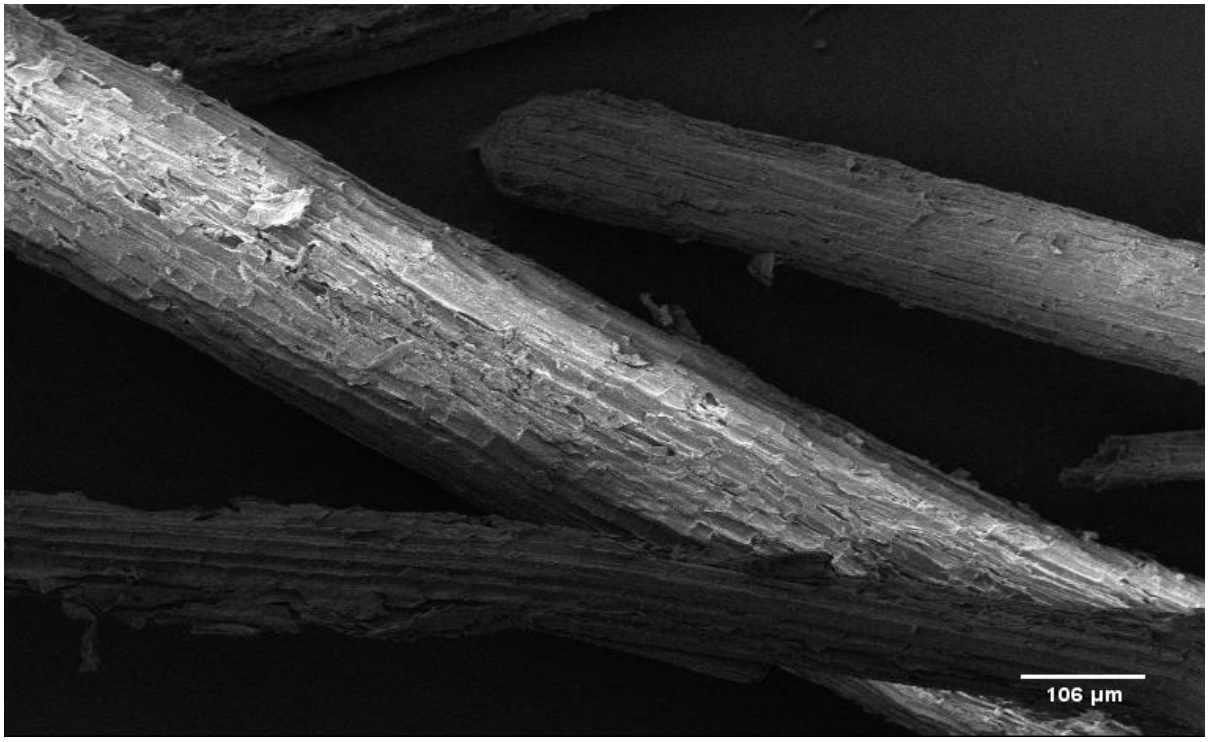


Figure 4.44 SEM image of acid treated depithed sugarcane bagasse

Figure 4.45 SEM image of acid/alkali treated depithed sugarcane bagasse

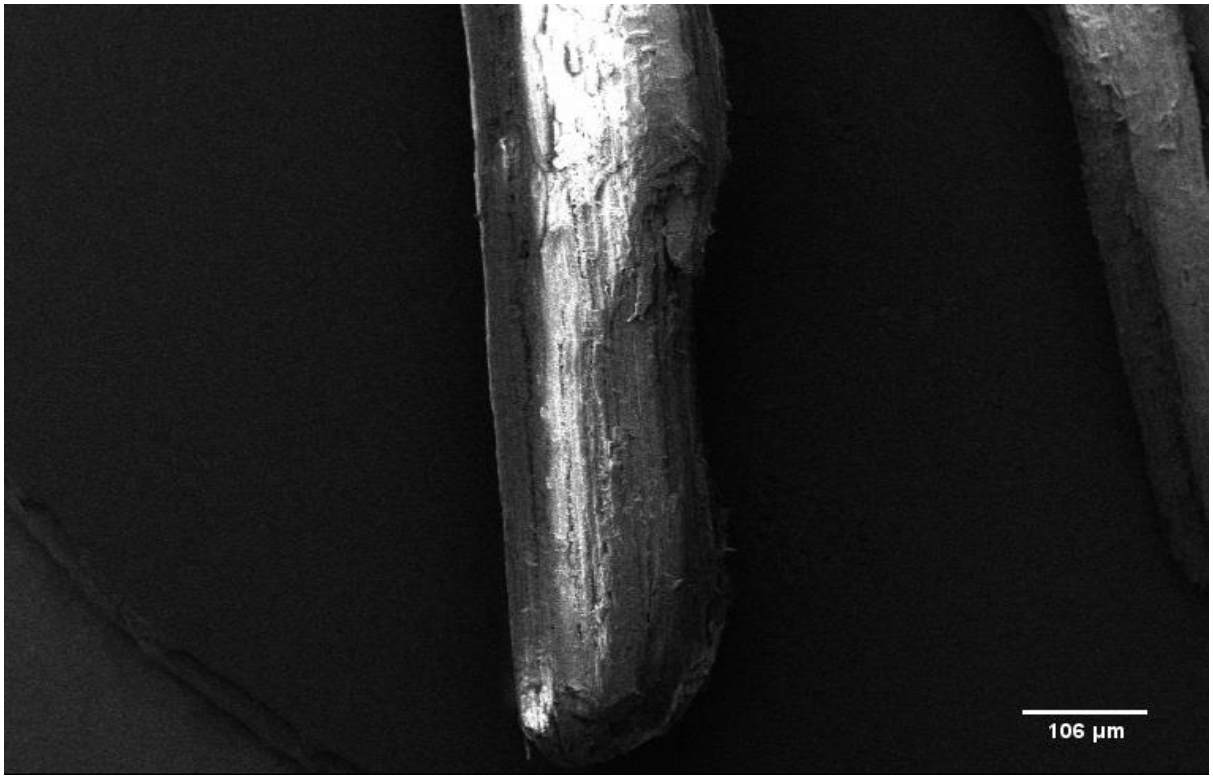


Figure 4.46 SEM image of untreated mill-run sugarcane bagasse

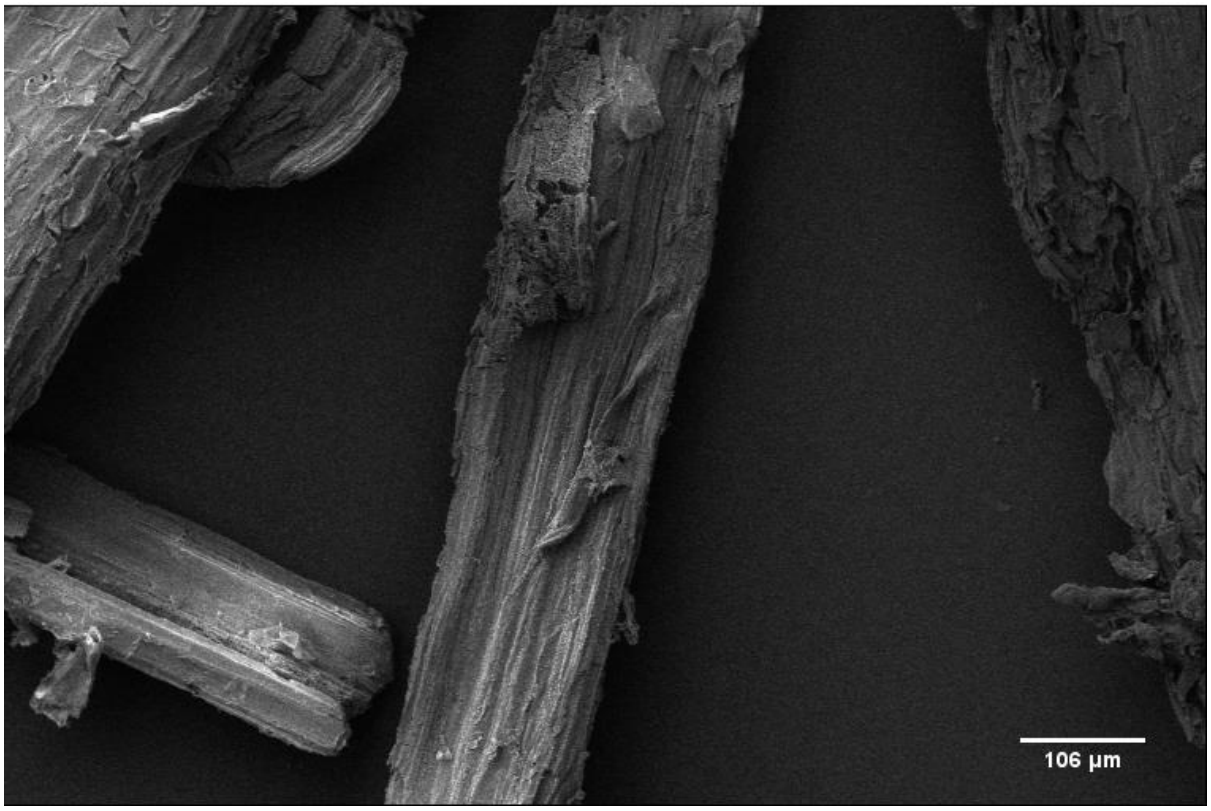


Figure 4.47 SEM image of acid treated mill-run sugarcane bagasse

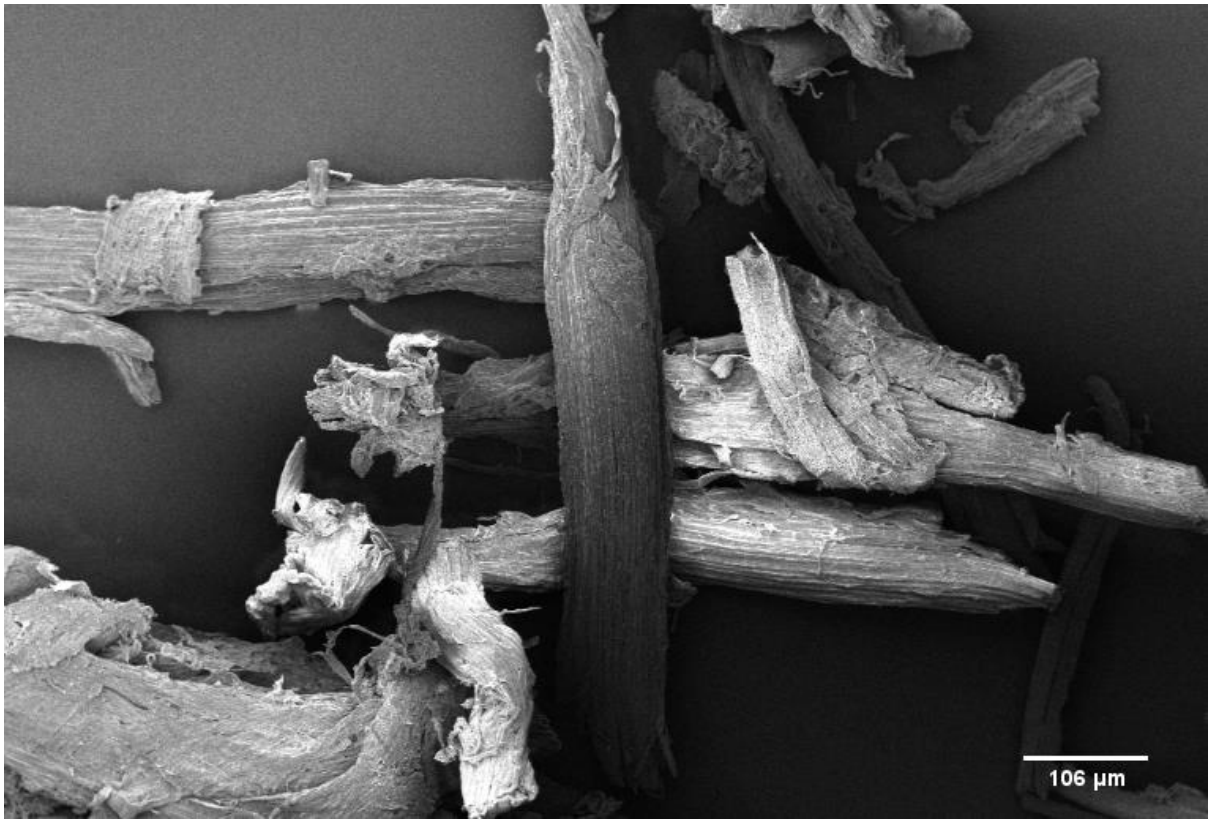


Figure 4.48 SEM image of acid/alkali treated mill-run sugarcane bagasse

4.3 Part D: Production of Levulinic Acid from Sugarcane Bagasse Using Liquid Hot Water (LHW)

4.3.1 Sugarcane Bagasse Pretreatment

(i) Compositional Analysis of Sugarcane Bagasse

The composition analysis of untreated sugarcane bagasse pellets is given in fig. 4.49. The SB pellets were analysed using HPLC (see table 4.8) and fig. 4.49 shows the main components of SB and there were also sugars that were found but in a very small amount. The ash content of the pellets was found to be 0.03 (see table 4.9).

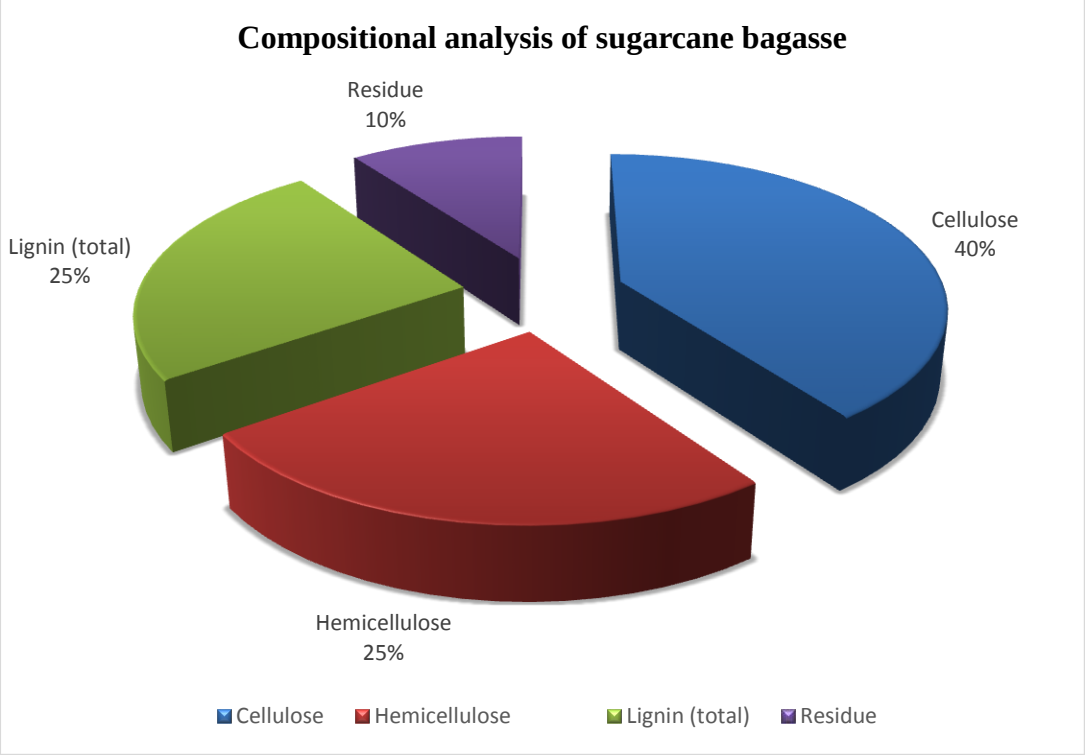


Figure 4.49 Compositional analysis of the SB pellets for the main components of SB

Table 4.8: Compositional analysis for SB pellets

	% abs
Hydrolysis-residue	22.4
Σ Sugar	65.4
Xylose	21.4
Glucose	40.0
Mannose	0.2
Galactose	0.8
Arabinos	2.9
Rhamnose	0.1
Hemicellulose	25.4
Cellulose	40.0
Acid soluble lignin	2.2
Klason-lignin	22.4
Lignin total	24.6
Rest	10.0

Table 4.9: Ash content and dry matter content for the SB pellets

	Value
Dry matter content	0.937
Ash content	0.03

(ii) LHW Pretreatment for Sugarcane Bagasse Pellets

After the LHW pretreatment a solid residue and liquid fraction were obtained, the solid residue which is called cellulignin was analysed with HPLC and the results are given in fig. 4.50 and table 4.10. It was observed that the solid residue have the components in table 4.10, when comparing the composition analysis of the untreated SB pellets and cellulignin it is observed that hemicellulose decreased in cellulignin and that was expected because LHW is used to remove hemicellulose in sugarcane bagasse.

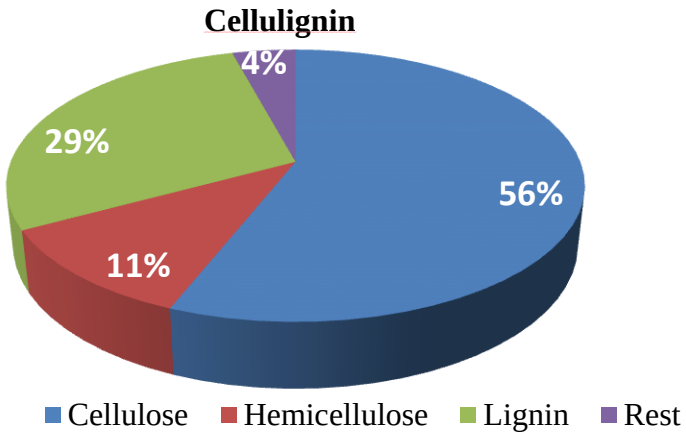


Figure 4.50 Pie chart for composition analysis of the solid residue from LHW

Table 4.10: Compositional analysis for cellulignin obtained from LHW

	% abs
Hydrolysis-residue	26.5
Σ Sugar	67.1
Xylose	10.0
Glucose	56.0
Mannose	0.2
Galactose	0.4
Arabinose	0.5
Rhamnose	0.0
Acid soluble lignin	2.2
Hemicellulose	25.4
Cellulose	40.0
Acid soluble lignin	2.2
Klason-lignin	22.4
Lignin total	24.6
Rest	10.0

The liquid fraction was analysed with HPLC and composition analysis is given in table 4.11. Table 4.11 show that the liquid fraction from LHW contains sugars, acid and other organic compounds.

Table 4.11: Hydrolysate composition from LHW

	Monomer (mg/L)	**Oligomers (mg\L)**
Cellobiose	<50	460
Glucose	115	1200
Xylose	1400	21300
Arabinose	1100	2300
Formic acid	<50	<100
Acetic acid	1100	3700
Levulinic acid	<50	<100
HMF	<50	<100
Furfural	370	570

Table 4.12 show that after LHW the cellulose increases where as hemicellulose and lignin decreases and that is expected since LHW pretreatment is for the removal of hemicellulose in the sugarcane bagasse and the hydrolysate contains 174.103 g of hemicellulose it show that the hemicellulose is dissolve in water during the LHW pretreatment and an amount of 9.009 g of cellulose is also removed to the hydrolysate.

Table 4.12: Sugarcane bagasse mass before, after LHW and hydrolysate mass

Sugarcane bagasse components	**Bagasse mass before LHW [g]**	**Bagasse mass after LHW [g]**	**Recovery [%]**	**Hydrolysate [g]**
Cellulose	232.228	353.503	156	9.009
Hemicellulose	367.396	26.765	55	174.103
Lignin	225.892	169.473	0.0	0.0

The moisture content of the sugarcane bagase is given in table 4.13, the moisture content for the cellulignin (solid residue produced from LHW) was found to be 70.2 % average.

Table 4.13: Moisture content of the sugarcane bagasse

Moisture Content						
Bagasse wet [g]	Bagasse dry [g]	Moisture [g]	Moisture content [%]	Mean value	Standard deviation	Student t-test
12.76	3.79	8.97	70.31			
14.74	4.44	10.30	69.91	70.15	0.21	0.16
12.76	3.80	8.97	70.24			

The pH of the hydrolysate (liquid fraction produced from LHW) was found to be 3.83 and the density of the hydrolysate was found to be 1.012 $g \cdot cm^{-3}$ at 20 ^{o}C.

(iii) Enzymatic hydrolysis

The cellulignin obtained from the LHW was hydrolysed with enzymes and the solution obtained was centrifuged and there was a solid residue and a liquid fraction.

The liquid fraction is assumed to be glucose solution but also it was tested with a DNS test, glucose assay kit, xylose assay kit and HPLC. From the DNS (fig. 4.51) it was found that there are 52.2 g/L of sugars in the liquid fraction and to identify which sugars were present the glucose assay kit gave 42.4 g/L of glucose in the liquid fraction, the xylose assay kit gave 9.03 g/L of xylose in the liquid fraction and the HPLC confirms the results obtained using the assay kit tests. Table 4.14 gives the compounds in the liquid fraction together with their concentration: glucose is 42800 mg/L and xylose is 9600 mg/L.

The solid residue was assumed to be lignin but it was tested and the results in fig. 4.52 shows that the lignin and other component was 51 % in the solid residue.

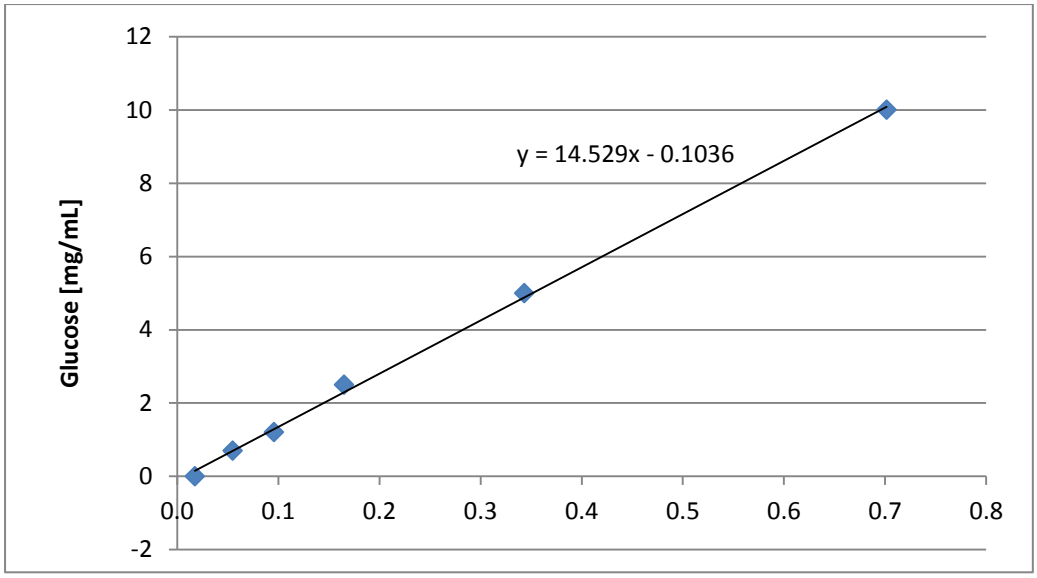


Figure 4.51 Graph for the DNS results for the reducing sugars

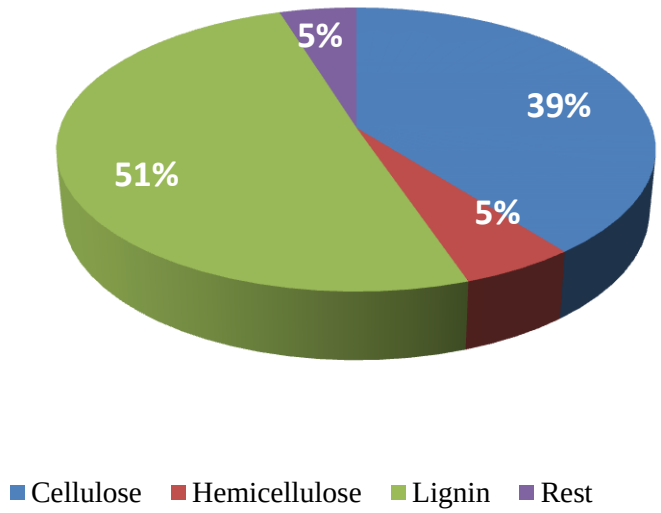


Figure 4.52 Pie chart for composition analysis of the solid residue after enzymatic hydrolysis

Table 4.14: Compositional analysis of the liquid fraction obtained after enzymatic hydrolysis

	Monomers (mg/L)	Oligomers (mg\L)
Cellobiose	1500	730
Glucose	42800	44000
Xylose	9600	11700
Arabinose	500	600
Formic acid	500	0.0
Acetic acid	2600	0.0
Levulinic acid	<50	0.0
HMF	60	160
Furfural	400	550

4.3.2 Acid hydrolysis

To produce LA which is the aim of this project acid hydrolysis was done from the glucose solution obtained after enzymatic hydrolysis of the cellulignin, other substrate were added such as pure fructose, pure xylose, pure glucose and a mixture of pure glucose and xylose. Also two different acid were used for the production of LA, this was done to observe if MSA could get the same or better yield of LA and table 4.15 and 4.16 shows that there is no much difference in the yield of LA when using sulphuric acid or MSA and it shows other compound that are produced such as formic acid ,acetic acid which are end product of this hydrolysis and furfural which is formed because the presence of xylose, it can be observed that the furfural is only formed in those reaction that have xylose as a substrate. Fig. 4.53 and 4.55 shows the calculated conversion rate and the yield of LA using both sulphuric acid and MSA and the conversion rate it shows that the glucose most of the time was all converted to forming products and the LA yield for fructose is very high compared to other substrate used but this is due to that glucose have to isomerise to fructose first before it forms LA whereas fructose just react with the acid to form LA. There is a small difference observe in comparing figs. 4.53 and 4.54 that means MSA can be used as the catalyst for the production of LA since it is more environment friendly, less corrosion effect compared to sulphuric acid.

Table 4.15: Glucose solution and other substrate hydrolysed with H_2SO_4 to produce LA

No.	Sample	Cellobiose	Glucose	Xylose	Arabinose	Formic Acid	Acetic Acid	Levulinic acid	HMF	Furfural
		mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
1	Fructose	< 50	< 50	< 50	< 50	8100	350	17700	< 50	< 50
2	Fructose	< 50	< 50	65	< 50	8100	330	17700	< 50	< 50
3	Glucose	< 50	< 50	< 50	< 50	6850	270	14500	< 50	< 50
4	Glucose	< 50	180	< 50	< 50	6900	260	14400	< 50	< 50
5	Glucose and xylose	< 50	220	80	< 50	7550	290	14700	< 50	260
6	Glucose and xylose	< 50	150	70	< 50	7650	320	15300	< 50	210
7	Glucose Solution	< 50	300	190	60	8400	2800	15600	< 50	290
8	Glucose Solution	< 50	180	170	70	8700	2850	16200	< 50	290

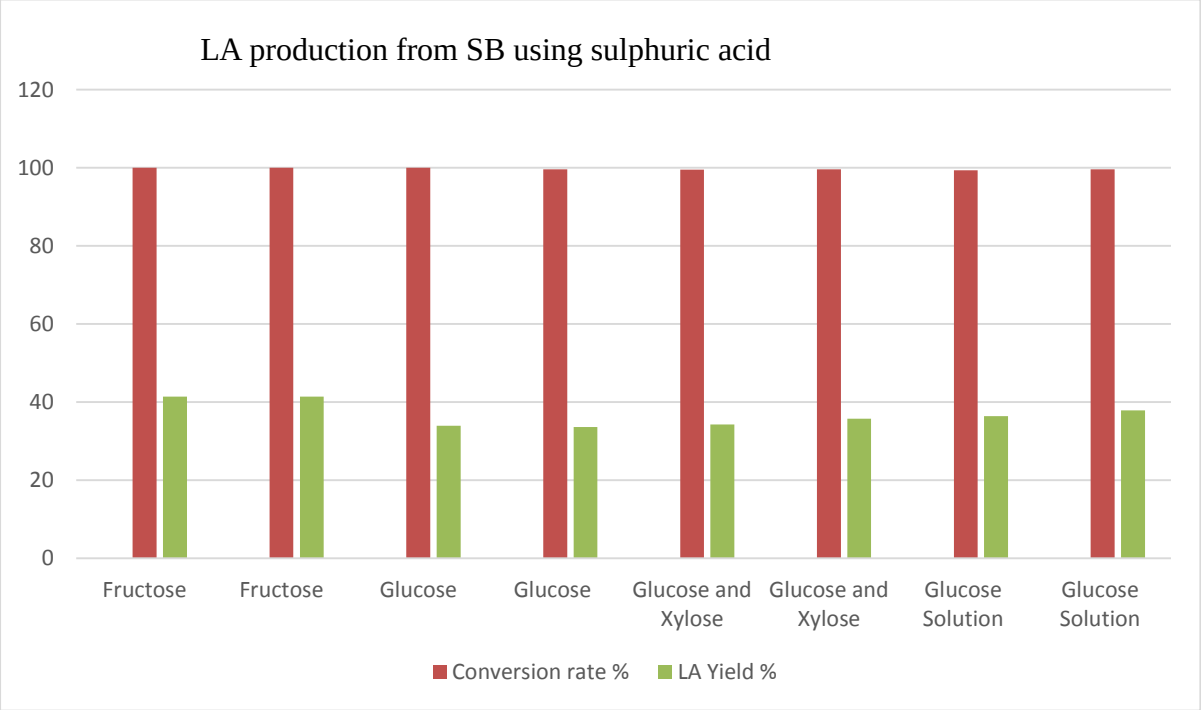


Figure 4.53 Graph for the conversion and LA yield using sulphuric acid

Table 4.16: Glucose solution and other substrate hydrolysed with methanesulfonic acid (MSA) to produce LA

No.	Sample	Cellobiose	Glucose	Xylose	Arabinose	Formic Acid	Acetic Acid	Levulinic acid	HMF	Furfural
		mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
1	Fructose	< 50	< 50	60	< 50	8150	350	17500	< 50	< 50
2	Fructose	< 50	< 50	80	60	7900	360	17400	< 50	< 50
3	Glucose	< 50	1150	70	< 50	6700	220	13700	50	< 50
4	Glucose	< 50	300	60	< 50	6750	240	13900	< 50	< 50
5	Glucose and xylose	< 50	100	100	< 50	7100	300	13800	< 50	240
6	Glucose and xylose	< 50	220	110	< 50	7000	290	13500	< 50	280
7	Glucose Solution	< 50	340	220	< 50	8200	2750	15100	< 50	310
8	Glucose Solution	< 50	210	230	< 50	8150	2800	14700	< 50	270

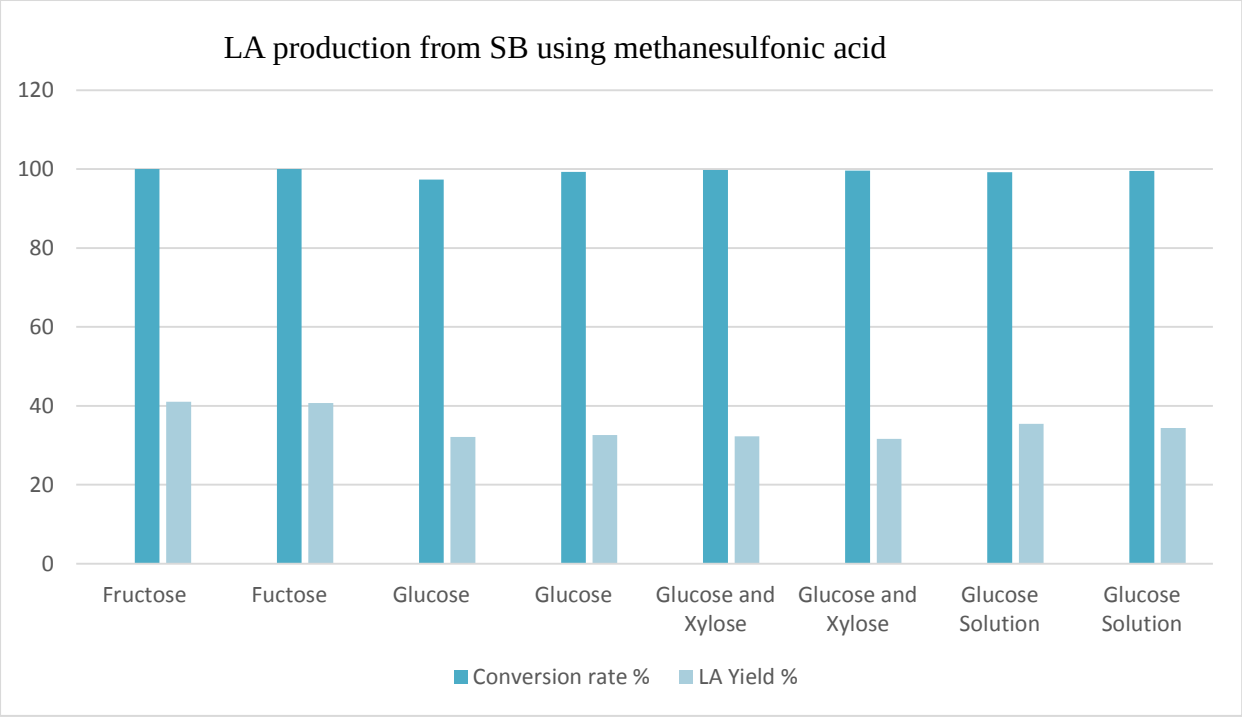


Figure 4.54 Graph for the conversion and LA yield using sulphuric acid

The production of LA was also done from cellulignin using MSA compared with pure cellulose, table 4.17 shows the result for those experiments and a small amount of LA was formed in these reactions and for the cellulignin it was worse this may be due to the presence of lignin and that not all glucose was converted to produce the products. Figs. 4.55 - 4.60 are the HPLC chromatogram for the acid hydrolysis result in table 4.15 and 4.16.

Table 4.17: Production of LA from cellulignin and pure cellulose using MSA

No.	Sample	Cellobiose	Glucose	Xylose	Arabinose	Formic Acid	Acetic Acid	Levulinic acid	HMF	Furfural
		mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
1	Cellulignin (LHW)	< 50	1000	< 50	< 50	900	220	1760	< 50	< 50
2	Cellulignin (LHW)	< 50	1150	< 50	< 50	980	210	1990	< 50	< 50
3	Cellulose (pure)	< 50	2600	50	< 50	1350	50	2600	< 50	< 50
4	Cellulose (pure)	< 50	2200	< 50	< 50	1900	80	3900	< 50	< 50

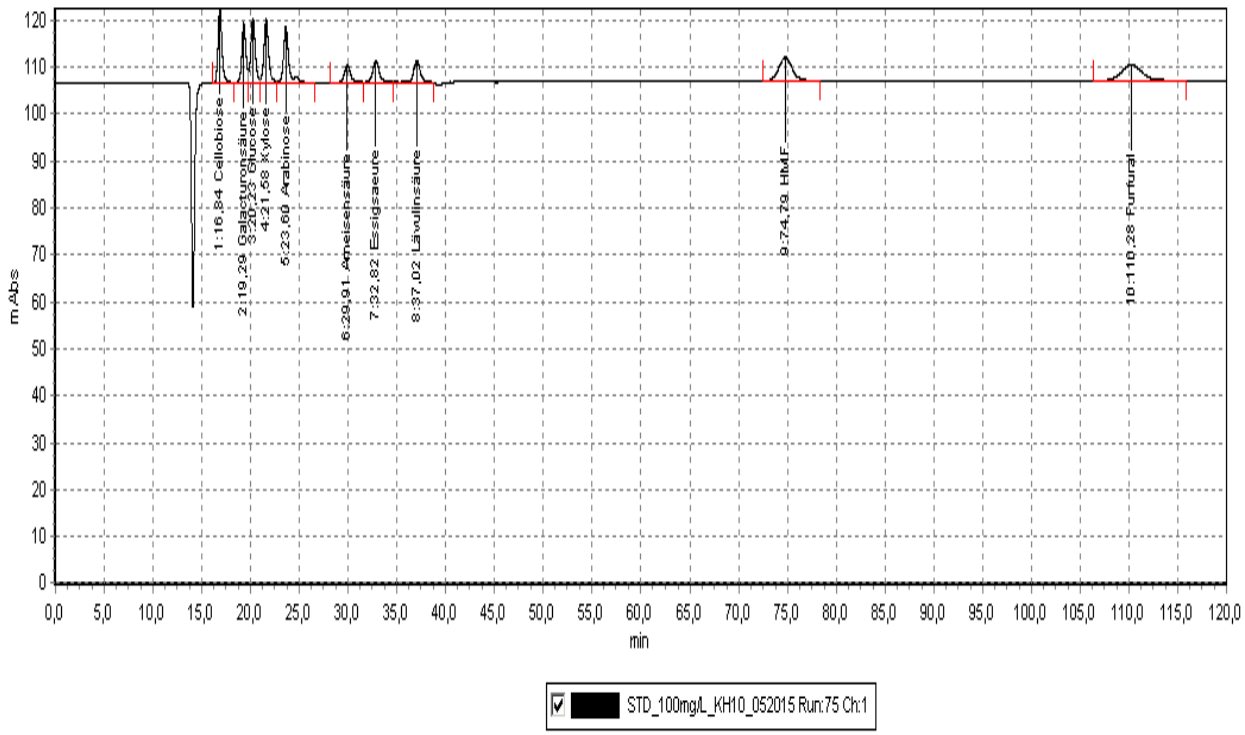


Figure 4.55 HPLC chromatogram for the standards of 100 mg/L

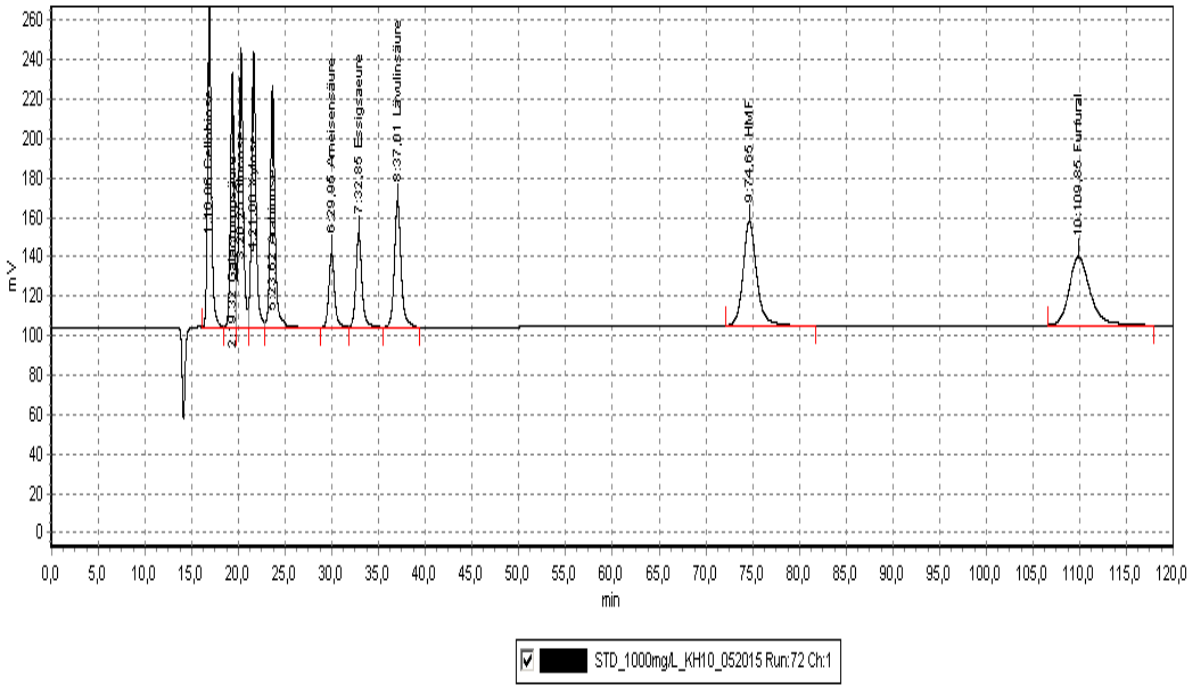


Figure 4.56 HPLC chromatogram for the standards of 1000 mg/L

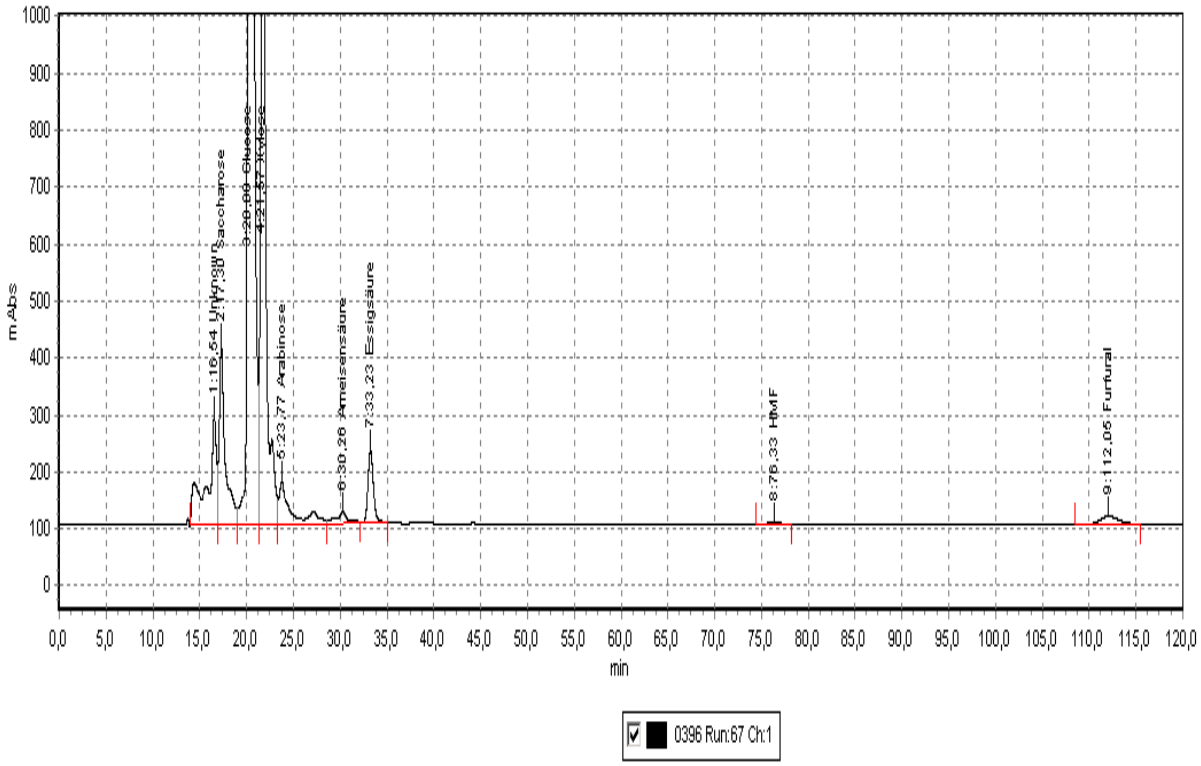


Figure 4.57 HPLC chromatogram for the sample 396, undiluted

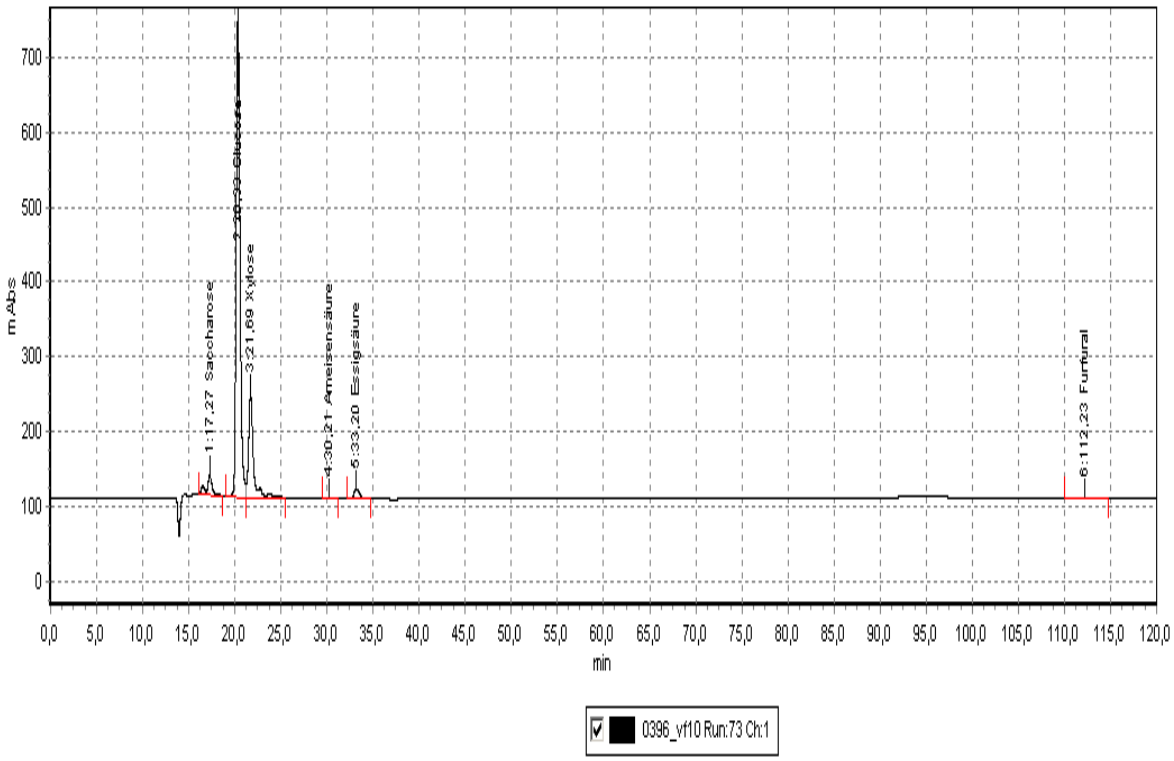


Figure 4.58 HPLC chromatogram for the sample 396, diluted 1:10

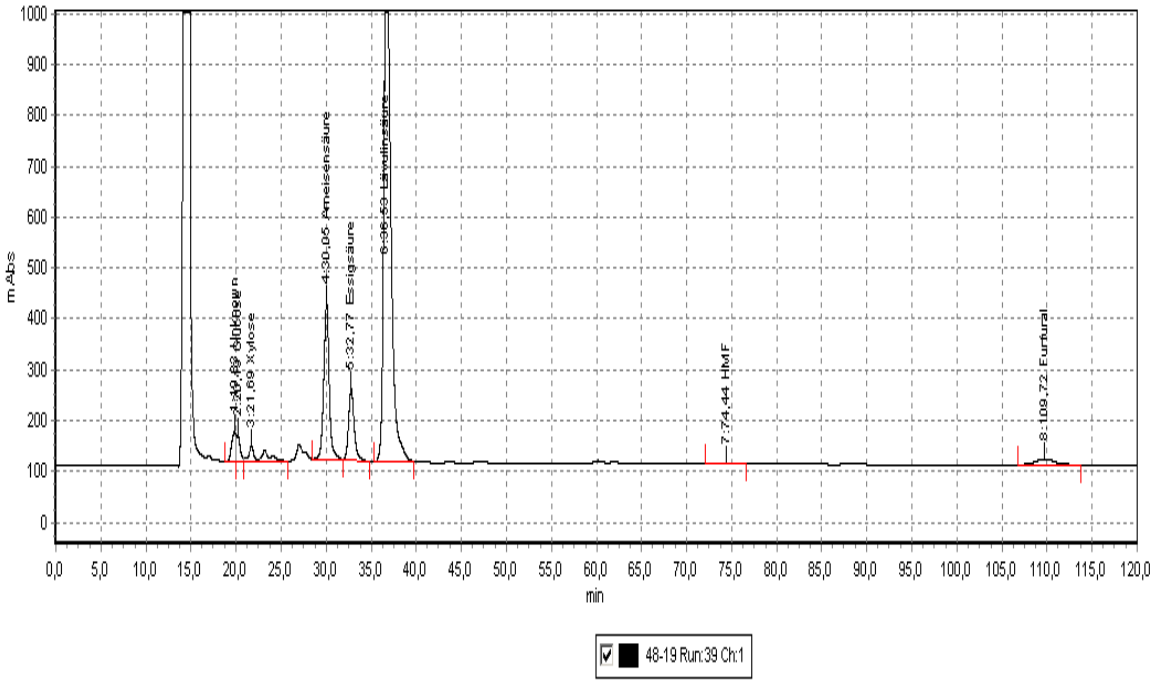


Figure 4.59 HPLC chromatogram for the sample 425, undiluted

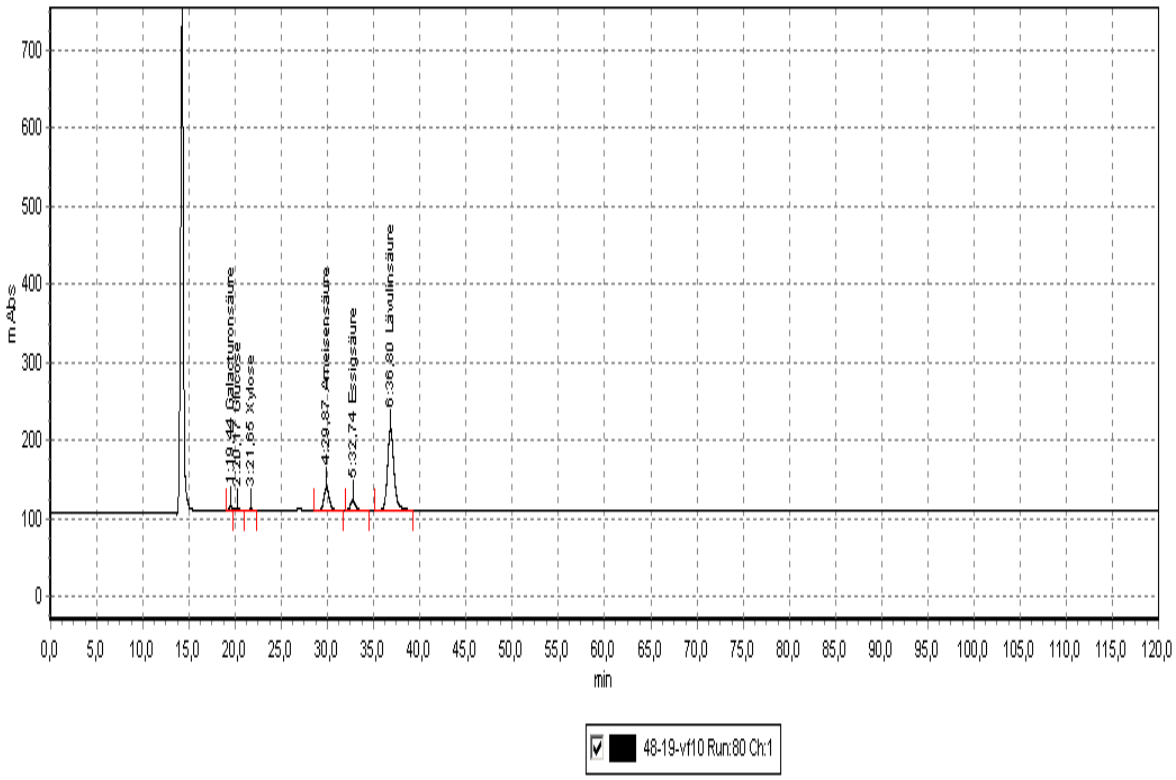


Figure 4.60 HPLC chromatogram for the sample 425, diluted 1:10

4.3.3 Optimization of LA Production

Fig. 4.61 shows the results for the optimization of LA production using a "Design-Expert Software", a central composite design. From the set of experiments that were done using central composite design, it showed that the acid hydrolysis of glucose solution with MSA (0.250 M) at 200 °C, for 90 minutes gave the highest yield of 19000 mg\L LA compared with other parameters (see table 4.18), these are the optimum parameters for the LA production.

When comparing the results for the LA production from the earlier experiments that were done (see tables 4.15 and 4.16), when sulphuric acid (0.5 M) was used an average of 15900 mg/L LA was produced and when MSA (0.5 M) was used an average of 14900 mg/L LA was produced, these acid hydrolysis reactions were carried out at 180 °C, for 45 minutes, it is observed that the difference between the parameters used in the earlier experiments (see table 4.15 and 4.16) and the optimisation experiments (optimum conditions, see table 4.18) is that the temperature increased by 20 ^{o}C, the acid concentration decreased by 0.25 M and the time

increased by 45 minutes, showing that increasing temperature, time and decreasing the acid concentration favours the increase in LA yield when a 42.4 g/L of glucose solution is used and the LA yield increased by 4100 mg/L, from 14800mg/L to 19000 mg/L.

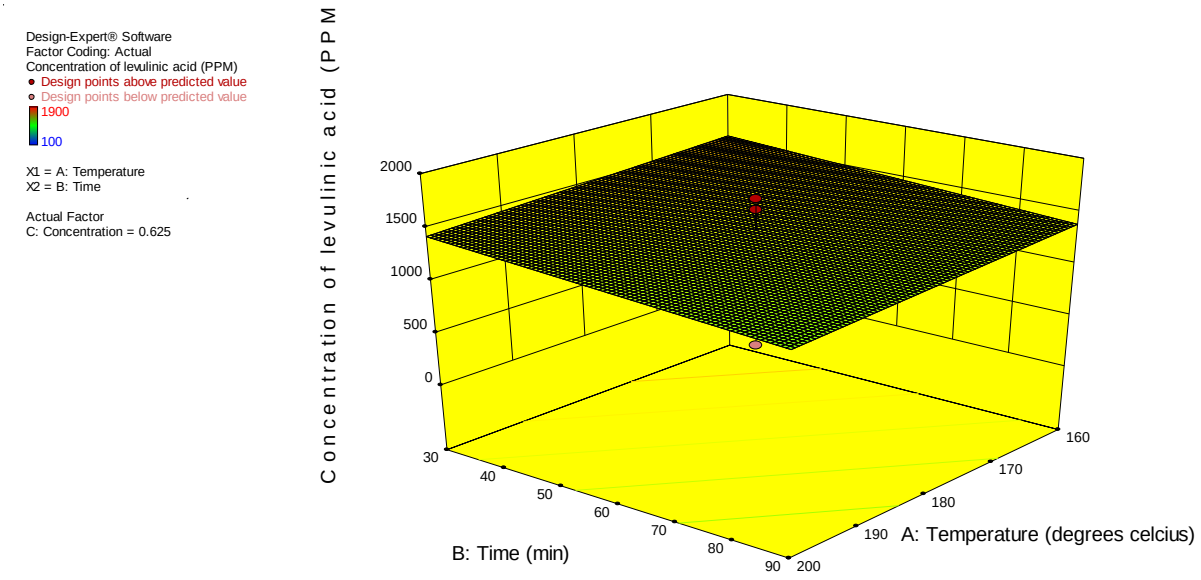


Figure 4.61 Central composite design for the optimization of LA production

Table 4.18: HPLC analysis of the acid hydrolysis sample for the optimization of the parameters

Sample	Cellobiose	Glucose	Xylose	Arabinose	Formic acid	Acetic acid	Levulinic acid	HMF	Furfural
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
398	<100	3800	18500	600	2800	4000	1000	<100	1500
431	170	14500	930	< 50	5900	290	11000	810	2300
432	< 50	< 50	210	< 50	7400	290	16000	< 50	< 50
433	140	16500	930	< 50	6000	290	11000	560	1800
434	220	15000	800	< 50	5700	260	12000	410	1300
435	< 50	370	190	< 50	7900	280	17000	< 50	< 50
436	< 50	< 50	160	< 50	6400	300	16000	< 50	< 50
437	< 50	< 50	250	< 50	7400	280	16000	< 50	< 50

438	< 50	< 50	230	< 50	7600	300	18000	< 50	< 50
439	< 50	< 50	230	< 50	7800	290	17000	< 50	< 50
440	95	260	260	< 50	7800	300	16000	< 50	860
441	< 50	< 50	210	< 50	7900	300	16000	< 50	< 50
442	< 50	< 50	190	< 50	4700	320	16000	< 50	< 50
443	< 50	< 50	160	< 50	7800	290	16000	< 50	< 50
444	< 50	13000	670	< 50	5600	270	12000	360	1400
445	< 50	< 50	180	< 50	8500	300	19000	< 50	130
446	< 50	< 50	220	< 50	6500	310	15000	< 50	< 50
447	575	33500	5160	529	2300	270	3000	920	2400
448	< 50	< 50	170	< 50	7100	300	16000	< 50	< 50
449	< 50	< 50	130	< 50	3700	310	17000	< 50	< 50
450	< 50	< 50	170	< 50	7800	300	17000	< 50	< 50

CHAPTER 5

SUMMARY AND CONCLUSIONS

In part A, LA was produced from fructose so as to observe the reaction of producing LA, the results from the characterisation of the mixture and final product showed that there was LA, formic acid, acetic acid in the mixture.

Part B describes the production of glucose from SB, using acid/alkali pre-treatment to extract cellulose from SB and enzyme hydrolysis to hydrolyse cellulose into glucose. The surfactant was added and increased the glucose concentration when compared with other experiments that didn't include the surfactant. The pre-treated substrate (10 % w/v) when hydrolysed using 20 FPU/g with 0.4 % Tween 80 for 20 hrs. resulted in 609 mg/g (depithed bagasse) sugars, being the best condition to use, when compared with other conditions. Enzymatic hydrolysis is more environmentally friendly and non-toxic than acid hydrolysis reactions. In this work enzymatic hydrolysis was done in part B using *Cellulase* for 24 hrs. to produce glucose from cellulose using SB.

In part C, LA was produced from sugarcane bagasse, and when comparing parts A and part C it can be said that it is easier to produce LA from fructose compared to producing it from glucose from SB and that is because producing LA from fructose is a one-step reaction.

In part D liquid hot water (LHW) was used as a pre-treatment method to dissolves the hemicellulose from SB in hot liquid water, cellulignin was left in the reactor as a solid residue. This method is most preferred because it uses water and the reaction time was only 1 hr whereas in parts B and C acid treatment was used for the removal of hemicellulose in SB the latter being toxic chemicals which are more harmful to the environment .

In part D, LA was produced from glucose solution using both methanesulfonic acid (MSA) and sulphuric acid as a catalyst and the HPLC analysis showed that 34.9 % and 37.2 % of LA was produced from 42 g/L of glucose solution using MSA (0.5 M) and sulphuric acid (0.5 M) respectively at 180 ^{O}C for 45 minutes. Part D also showed that pure fructose produced slightly more LA when compared with pure glucose, pure mixture of glucose and xylose and glucose solution from bagasse for both MSA and sulphuric acid.

The optimization for LA production showed that the acid hydrolysis of glucose solution with 0.250 M of MSA for 90 min at 200 ^{o}C, gave the highest LA yield of 19000 mg\L compared with other parameters that were used. The above parameters are the optimum conditions for the LA production from a 42.4 g/L of glucose solution.

In part C, where comparative studies was done for the production of LA from mill-run and depith bagasse, the HPLC result for the mixture that resulted after acid hydrolysis of cellulose to glucose shows that there is no major difference in the amount of glucose that was produced from both the bagasse samples.

When comparing the use of methanesulfonic acid and sulphuric acid for the acid hydrolysis of glucose to produce LA for parts C and D it was observed that there is no much difference therefore MSA can be used for the production of LA because it is more environmentally friendly, less toxic and less corrosive compared to H_2SO_4.

LA can be produced from SB in a design that complies with some of the green chemistry principles, namely using LHW, enzyme hydrolysis, and MSA for acid hydrolysis.

All the qualitative and quantitative analysis that was done showed that LA and other compounds such as formic acid and acetic acid were produced. Analytical techniques used included: FTIR, HPLC, ^{1}H NMR. And other test such as TGA/DSC and SEM which showed the changes in the structure of SB as pre-treatment was done, the pre-treatment showed to be successful since the pore size was observed to increase in the SEM images. This led to the accessibility of the substrate to cellulase enzyme for hydrolysis.

CHAPTER 6

RECOMMENDATIONS

From the information that was found during this project for the production of levulinic acid from sugarcane bagasse it is recommended that for the sustainable production of levulinic acid, liquid hot water pretreatment should be used for the removal of hemicellulose since it only uses water. Enzymatic hydrolysis is also recommended to be used for the removal of lignin and converting cellulose into glucose solution in cellulignin because it can also be used extract lignin and it is also environmentally friendly.

The depithed bagasse is the recommended bagasse to use when the compound of interest is cellulose because the handling is better than the mill-run. The mill-run also has a very low bulk density compared with the depithed bagasse. In this work in part C the bagasse that was used was pelletized because of low bulk density, because of pelletizing 1 kg of bagasse was able to fit in a 3 L reactor, which would have been impossible if the bagasse was not pelletized first. The study between two different type of bagasse namely: (i) mill-run bagasse and (ii) depithed bagasse showed that there is not much difference between the bagasse in terms of producing reducing sugars (part B) or glucose (part C) because depithed bagasse produced a slightly more glucose or reducing sugars compared to mill-run bagasse.

When comparing two acids namely: (i) sulphuric acid (mineral acid) and (ii) methanesulfonic acid (MSA), (organic acid), which were used in this work, it is recommended that MSA should be used for the production of LA since it is less corrosive (which is very important for the reactors when producing products on a pilot scale or small scale because corrosive compound destroys reactors) and it is environmentally friendly.

The optimisation of LA production showed that the optimum conditions are:

(i) Temperature (200 ^{o}C)

(ii) Acid concentration (0.250 M)

(iii) Time (60 minutes)

The above conditions are the recommended conditions for the production of LA from a glucose solution with a concentration of 42.4 g/L.

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APPENDIX

Articles in preparation:

1) Enzymatic saccharification of acid/alkali pre-treated mill-run and depithed sugarcane bagasse

   Thandeka Mkhize, Lethiwe Debra Mthembu, Rishi Gupta, Amandeep Kaur, Ramesh Chander Kuhad, Prashant Reddy and Nirmala Deenadayalu

   Submitted to Bioresource

2) Production of levulinic acid using from bagasse using liquid hot water treatment

   Lisa M. Schmidt, Lethiwe D. Mthembu, Prashant Reddy, Nirmala Deenadayalu, Martin Kaltschmitt, Irina Smirnova

3) Production of levulinic acid from depithed and mill-run sugarcane bagasse

   Lethiwe Debra Mthembu, Nirmala Deenadayalu, Prashant Reddy