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ETHANOL PRODUCTION FROM LIGNOCELLULOSIC SUGARCANE LEAVES AND TOPS

By

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A dissertation submitted to the Faculty of Science and Agriculture in conformity with the requirements for the degree of Master of Chemistry

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Declaration

The work contained in this thesis is original research carried out by myself. Where contributions were made by others, these are clearly indicated and referenced. The work has not been submitted for the attainment of any degree, diploma or award at any other university.

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I also take this opportunity to thank my family for their understanding and support in the pursuit of this research.

Abstract

Various methods for the production of bioethanol using different feedstocks have been researched on. In most work on bioethanol synthesis from sugar cane, tops and leaves have been regarded as waste and generally removed and thrown away. In this work, lignocellulosic sugarcane leaves and tops were not discarded but instead used as biomass to evaluate their hydrolyzate content. The leaves and tops were hydrolysed using different methods, namely concentrated acid, dilute acid pre-treatment with subsequent enzyme hydrolysis and compared with a combination of oxidative alkali pretreatment and enzyme hydrolysis. Subsequent fermentation of the hydrolyzates into bioethanol was done using the yeast *saccharomyces cerevisae*. Acid hydrolysis has the problem of producing inhibitors, which have to be removed and this was done using overliming with calcium hydroxide and compared to sodium hydroxide neutralization.

Oxidative alkali pre-treatment with enzyme hydrolysis gave the highest yields of fermentable sugars of 38% (g/g) using 7% (v/v) peroxide pre-treated biomass than 36% (g/g) for 5% (v/v) with the least inhibitors. Concentrated and dilute acid hydrolysis each gave yields of25% (g/g) and 22% (g/g) yields respectively although for acid a neutralization step was necessary and resulted in dilution. Alkaline neutralization of acid hydrolyzates using sodium hydroxide resulted in less dilution and loss of fermentable sugars as compared to overliming. Higher yields of bioethanol, 13.7 (g/l) were obtained from enzyme hydrolyzates than 6.9 (g/l) bioethanol from dilute acid hydrolyzates. There was more bioethanol yield 13.7 (g/l) after 72h of fermentation with the yeast than 7.0 (g/l) bioethanol after 24h. However, the longer fermentation period diminishes the value of the increase in yield by lowering the efficiency of the process.

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List of Abbreviations

A_{540}.....Absorbance at 540 nm

AFEX.....Ammonia Freeze Explosion /Ammonia Fibre Explosion

CrI.....Crystallinity Index

DNS.....Dinitrosalicylic Acid

EDS/EDX.....Energy Dispersive X-ray Spectroscopy

FPA.....Filter Paper Assay

FPU.....Filter Paper Units

FID.....Flame Ionization Detector

FTIR.....Fourier Transform Infrared Spectroscopy

GC.....Gas Chromatography

GWP.....Global Warming Potential

HPLC.....High Performance liquid Chromatography

HCL.....Hydrochloric acid

HMF.....Hydroxy- methyl –furfural

IUPAC.....International Union of Pure and Applied Chemists

NaOH .....Sodium hydroxide

H_2SO_4.....Sulphuric acid

SHF.....Separate Hydrolysis and Fermentation

SSCF.....Simultaneous Saccharification and Co-fermentation

SSF.....Simultaneous Saccharification and Fermentation

SEM.....Scanning Electron Microscope

XRD.....X-ray Diffraction Spectrometer

Chapter 1

1. Introduction

The ever rising cost of fossil fuels, coupled with their being non-renewable has put a great impetus on the scientist community to look for alternative sources of fuel. Bioethanol production has the potential to support and maybe even surpass current depleting sources of fossil fuels.

The greatest possible source of sugars for ethanol production is starch based food sources such as that containing cellulose which is one of the main energy-providing food sources. However, overpopulation and droughts have led to severe food shortages and so alternative sources of cellulose other than the conventional ones have to be found to afford the use of cellulose for bioethanol production. Starch based crops such as sugarcane produce tonnes of waste in the form of plant leaves and tops. These leaves and tops contain a lot of lignocellulose which can be used to produce ethanol. As such, research is being carried out in order to find ways that are efficient, cost effective and environmentally friendly to produce lignocellulose-based bioethanol.

1.1 Background

Bioethanol is used in blends with gasoline in varying proportions such as 10 and 22% in the US and Brazil, respectively (Wyman, 1994). It is an oxygenated fuel, with 35% oxygen, thereby reducing particulate and NO_X emission from combustion reactions (Prasad et al., 2006). According to Lynd et al., (1991), bioethanol may be used directly (95% ethanol and 5% water) thereby benefitting the environment. Although ethanol has two thirds the heat and energy value of petrol, it has better combustion and acts as an octane booster in fuel blends. A similar booster role is played by the conventional additive, meta-tertiary butyl ethanol, in diesel which has unfortunately been identified as a potential health hazard (S. Prasad et al., 2006).Bioethanol offers the advantage of aiding in the reduction of global warming through the use of carbon dioxide by plants. The Global Warming Potential (GWP) in Brazil in the pre-mechanisation (100% burning of biomass residue), current (≈ 50% burning) and future (0% burning) scenarios were calculated. Galdos et al., 2013 indicated that current ethanol production has a GWP of 46% and is projected to be 70% smaller with complete mechanised

harvesting of food crops residues with subsequent reduction in black carbon (found in soot) emissions being produced.

The United States and Brazil together account for 89% of the world's bioethanol production (Limayem et al., 2012). In the United States, the main raw material for bioethanol production is corn with up to 48.52 billion litres of bioethanol produced, whilst in Brazil it is sugarcane juice and molasses. Across the world different feedstocks are being researched on including crops such as rice and sugar beet. However the current production of bioethanol is not enough to replace part of the one trillion gallons of fossil based fuels in use globally each year (Limayem et al., 2012).

The use of food sources for bioethanol production brings with it the challenge of whether it is ethical to use food to produce fuel whilst there are food shortages and increasing food prices in the world. This is also in addition to land use challenges where bio-fuel crops could be given priority over basic food crops. Hence research has been focused on food crop residues such as corn stover and sugarcane bagasse which are lignocellulosic materials.

The use of lignocellulosic materials has its own challenges one of which is the recalcitrance of the biomass. Steps involved in bioethanol production include pre-treatment, hydrolysis, fermentation and distillation. Different pre-treatment methods are being researched on from milling, through the use of liquid hot water, chemical treatment to biological treatment and mechanised treatments such as microwave treatment. The development of environmentally friendly, efficient and cost effective methods is the basis of modern research in lignocellulose bioethanol production.

1.2 Problem Statement

One of the major problems facing the world today is population growth and with it comes food shortage. This has had a profound effect on the available sources of sugar and starch based crops as these account for a large fraction of food crops. Such crops however, produce large quantities of waste materials in the form of lignocellulosic matter. The components of a sugar cane plant are illustrated in Figure 1.1. (www.bing.com)

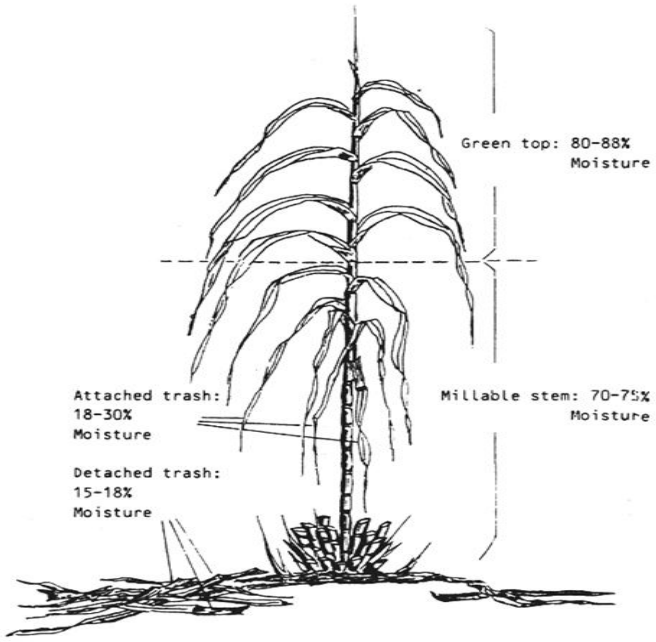


Figure 1.1 Composition of a sugar cane plant (Alexander, 1985)

Sugar cane leaves are either burnt to enable manual harvest (da Silva et al., 2010), adding to environmental pollution and greenhouse gases or they are left in the field as part of manure, aiding in provision of soil nutrients. Use of mechanised harvesters can avoid air pollution concerns and a percentage of waste can be worked into the soil as compost but these are attainable at significant costs. It would be beneficial to convert most of the sugar cane waste into biomass to be used for bioethanol production. Use of waste as a raw material for bioethanol production would not compete with food sources such as the sugar stem from the sugar cane plant.

1.3 Rationale and Motivation

The use of mechanised harvesters can produce huge amounts of waste materials that can be put to use in bioethanol production. Thus, bioethanol production using sugarcane leaves and waste materials will be beneficial through increasing utilisation of the whole sugarcane plant. The fact that the cellulosic material used is not from food based sources will avoid competition with the traditional sources of fuel.

Lignocellulosic materials are renewable, unlike the current fossil based fuels and they aid in environmental management through their use of the greenhouse gas carbon dioxide.

1.4 AIM AND OBJECTIVES

The major aim of this research was to produce ethanol from sugarcane waste materials.

To achieve the above aim, the project sought to achieve the following objectives

- Carrying out acid and alkali/oxidative pre-treatment
- Varying of reaction temperatures during acid pre-treatment
- Carry out enzyme hydrolysis of the products of pre-treatment

Combining chemical and enzymatic hydrolysis for efficient bioethanol production

Comparing yields of bioethanol from processes using different pre-treatment methods

Evaluation of the bioethanol product.

Chapter 2

2. Literature Review

2.1 Composition of lignocellulose

Lignocellulosic materials generally consist of 40% cellulose, 30% hemicellulose, and about 20% lignin (Carrasco et al., 2009). Hemicellulose serves as a connection between lignin and cellulose and can be hydrolysed by acid or base as well as hemicellulose enzyme (Talebnia et al., 2010). This lignin/cellulose/hemicellulose connection is a very strong one (Talebnia et al., 2010) so pre-treatment is therefore necessary to make cellulose accessible. In addition to this connection, the crystalline nature of cellulose is so intense that there is a low surface area available to hydrolysis enzymes once exposed. The degree of crystallinity of cellulose can therefore be a hindrance to achieving high sugar yields. The composition of lignocellulose is shown in Figure 2.1

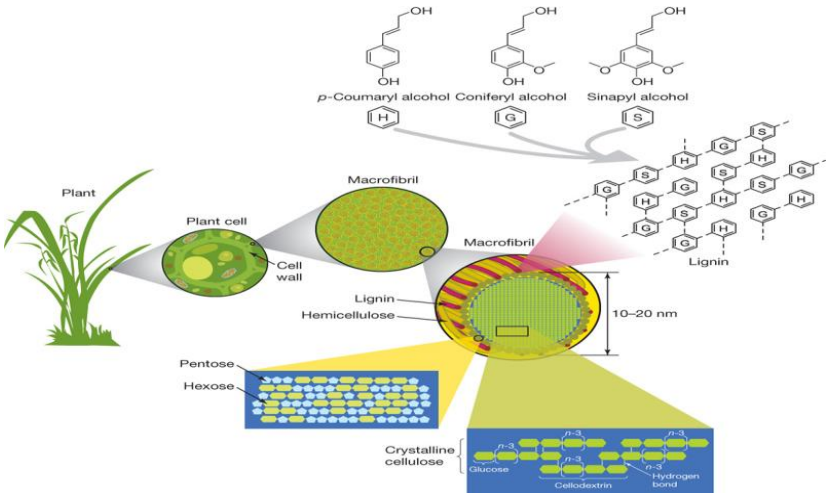


Figure2.1 Plant showing the composition of lignocellulose (Hurst, 2008)

2.2 Pre-treatment of lignocellulose

Generally, production of lignocellulosic ethanol involves four steps, namely, pre-treatment, saccharification/hydrolysis, fermentation and distillation (Margeot et al., 2009).Various

methods have been extensively used for the pre-treatment of cellulose. These can be classified as physical, chemical, biological, or at times a combination of these processes can be used with good results as reported by Sun et al., (2002) and Rabelo et al., (2011).The pre-treatment methods include super-critical explosion by carbon dioxide, ammonia freeze explosion (AFEX) (Sun et al., 2002), solvent delignification (using ethanol, butanol and/or acetic acid), as well as thermal and mechanical processes (Buaban et al., 2010). The purpose of pre-treatment is to increase accessibility of cellulose to hydrolysis enzymes and can be achieved through the disruption of the lignocellulose-cellulose-hemicellulose complex. Pre-treatment should therefore; improve the formation of sugars or the ability to form sugars by enzymatic hydrolysis; help to avoid the loss or degradation of substrate; avoid the formation of inhibitors to subsequent procedures; and be cost effective. A schematic of the overall pre-treatment process is illustrated in Figure 2.2 below:

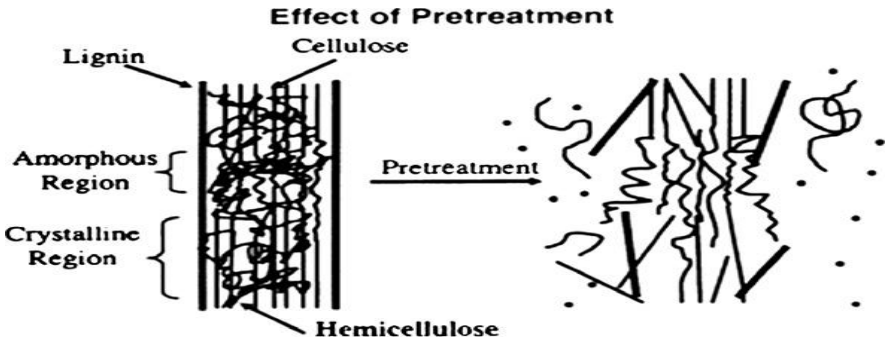


Figure 2.2 A schematic showing the effect of pre-treatment (Balat, 2011)

2.2.1 Pre-treatment by Steam Explosion

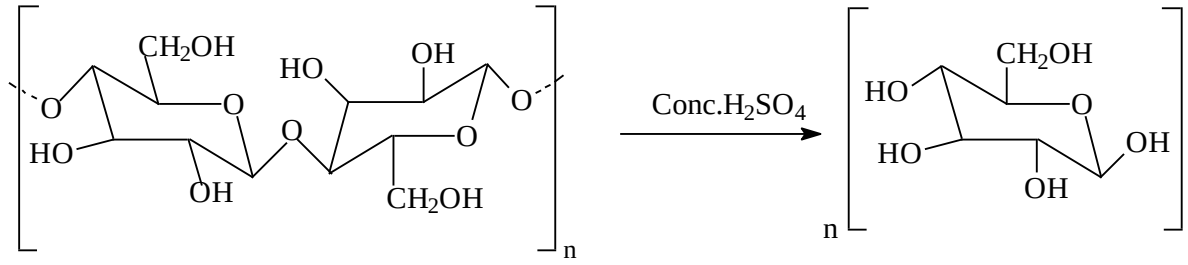
Steam explosion is usually carried out at temperatures of 160 – 260^{o}C and pressures of 0.69 – 4.83 MPa (Sun et al., 2002). Chipped biomass is treated with high pressure saturated steam followed by a swift reduction in pressure by exposing the reactor to atmospheric pressure. The relatively lower temperatures of about 160°C and longer residence times (10min) are more favourable than higher temperatures of 270°C and shorter times (1min) (Sun and Cheng, 2002).

Materials that have been reportedly treated and used as biomass include bagasse, corn stalk, wheat straw, rice straw, barley straw, sweet sorghum bagasse, olive stones, reed canary grass(Sun et al 2002). Addition of dilute sulphuric acid was found to improve the efficiency of further enzymatic hydrolysis. Lignin was not solubilised but redistributed (Belkacemi et

al., 1997, de Bari et al., 2002, Hamelinck et al 2005, Nakamura et al., 2001, Negro et al., 1999, Sanchez et al., 2008).

2.2.2 Acid Pre-treatment

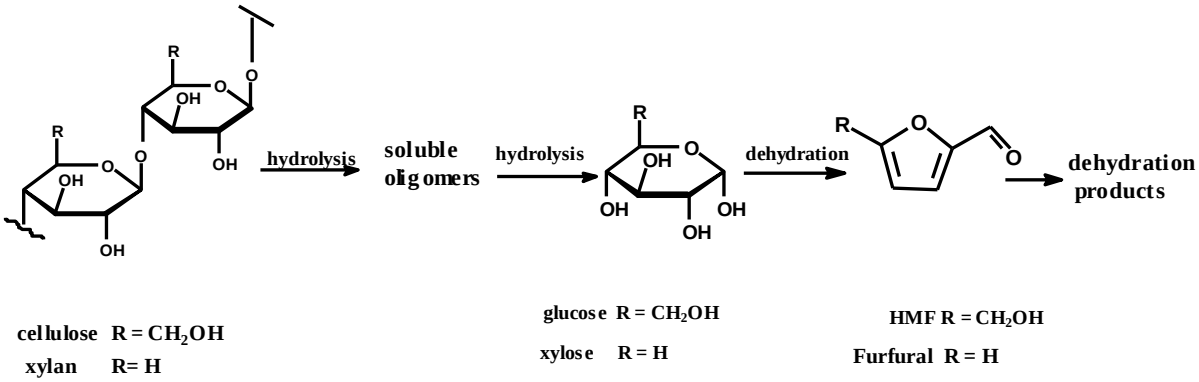
Concentrated acids such as H_2SO_4 and HCl have been used for treatment and hydrolysis at ambient temperatures. They have the advantage of eliminating the use of enzymes for the saccharification step. However, they have disadvantages of requiring expensive resistant material equipment and result in the formation of inhibitors (Olsson and Hahn-Hagerdal, 1996; Galbe and Zacchi, 2002, Sun and Cheng, 2002). The Scheme 2.1 below illustrates the functional groups of cellulose that are hydrolyzed using acid:



Scheme 2.1 Schematic acid hydrolysis of cellulose

Dilute acid treatment

Dilute acid pre-treatment at moderate temperatures frees hemicellulose and cellulose, disrupting the lignin in the process without solubilising it (Knappert et al, 1981). Low acid concentrations of 0.75 – 5% H_2SO_4, HCl, or HNO_3 have been used for pre-treatment. However it was found that at low temperatures below 160°C, direct saccharification suffered from yields lower than 75% because of sugar decomposition (Sun and Cheng, 2002; Hamelinck et al, 2005) as shown in Scheme 2.2 below:



Scheme2.2: Formation of degradation products (Sun and Cheng 2002)

This suggests that higher temperatures of 160 to 200°C may be preferred to lower temperatures because they lead to further cellulose hydrolysis. Although high temperatures result in higher yields of over 75% and lower reaction times, they accelerate the rate of hemicellulose decomposition, producing inhibitors, and increasing equipment corrosion (Galbe and Zacchi,2002; Taherzadeh and Karimi,2007).

There is also a need for a pH neutralization step when using acid pretreatment in preparation for enzyme saccharification and fermentation.

2.2.3 Alkaline/ oxidative pre-treatment

Different types of alkali have been researched on for pre-treatment purposes. Alkali pre-treatment serves the purpose of removing lignin, increasing the porosity of the lignocellulosic materials, leading to swelling and increase in the internal surface area. This results in a decrease in polymerization and crystallinity as well as a disruption of the lignin structure (Fan et al., 1987; Bjerre et al., 1996). However experiments have shown that addition of hydrogen peroxide can greatly improve the sugar yields (Sanchez et al, 2008).

In their research, Boopathy et al (2007) used alkali delignification in order for cellulose to become readily available for the enzymes, allowing the yeast to convert the glucose into ethanol (Wyman, 1996).

There are different factors that can affect the success of enzyme hydrolysis. The two main factors are the nature of the substrate and the type of enzyme used. The presence of more sites on the substrate for the enzyme to attach itself can mean the difference between high and

low sugar yields. Hence pre-treatment needs not be severe and there should be as few inhibitors as possible or none at all. Another factor affecting successful enzyme hydrolysis is the type, dosage and efficiency of the enzyme used (Alvira P. et al., 2010). Rabelo S.C. et al., (2011) reported higher glucose yield of 691mg g^{-1} glucose after alkali hydrolysis with 7.35% v/v of peroxide. In this research, the aim was to use sodium hydroxide in combination with hydrogen peroxide to hydrolyse the hemicellulose at various concentrations and time periods so as to obtain optimal conditions.

2.2.4 Organosolv Process

Use of organic solvents such as methanol, ethanol, acetone, ethylene glycol, triethylene glycol or their mixture with 1% HCl or H_2SO_4 at temperatures of 185- 198°C have been shown to produce near total hydrolysis of hemicellulose and near total solubilisation of lignin (Sun and Cheng, 2002). However the solvent needs to be recovered to reduce costs as well as reduce inhibition (Sun and Cheng,2002).

2.2.5 Physico- Chemical Methods

Other pre-treatment methods besides those already described above are given in this section.

2.2.5.1 Mechanical

Mechanical methods include chipping, grinding and milling for example with a ball mill pulveriser, knife or hammer mill. Materials pre-treated using these methods include corn stover, cane bagasse, wood and forestry wastes such as hardwood straw (Sun and Cheng, 2002, Yu and Zhang, 2003, Sanchez et al., 2008).

2.2.5.2 Pyrolysis

Pyrolysis at temperatures of 300°C and above followed by cooling and condensing is another pre-treatment method. It can be carried out in conjunction with dilute acid (1N H_2SO_4)

hydrolysis at 97°C for 2,5h to produce about 80% reducing sugars(Sun and Cheng, 2002). Materials worked on include corn stover, wood, and waste cotton Sanchez et al. (2008).

2.2.5.3 Liquid Hot Water

This method of pre-treatment makes use of hot water at temperatures of 170 – 230°C and high pressures of more than 5MPa for about 1 – 46 minutes to treat the lignocellulose material. Solid loading is less than 20%. This method achieves 80 -100% hemicellulose hydrolysis, 88 – 98% xylose recovery, >50% oligomers, and has low or no formation of inhibitors (Sanchez et al, 20008). Further cellulose conversion with enzymes will achieve more than 90% conversion. There is partial solubilisation of lignin at about 20 – 50%. Treated materials include bagasse, corn stover, olive pulp and alfalfa fibre (Sanchez et al, 2008).

2.2.5.4 Ammonia fiber explosion (AFEX)

In the Ammonia Fibre Explosion method, 1 – 2 kg of ammonia is used per kg of dry biomass at a temperature about 90°C. The mixture is refluxed for 30 minutes to pre-treat the lignocellulosic materials. Pressures of 1.12 – 1.36 MPa are used. The method requires an ammonia recovery step in order to make it cost effective and between 0 -60% hemicellulose hydrolysis can be achieved depending on moisture content of the substrate. It results in more than 90% oligomers being produced and there are no inhibitors formed. Cellulose depolymerisation occurs to a certain extent. Further cellulose conversion can be more than 90% for low lignin biomass and >50% for high-lignin biomass. Approximately 10- 20% lignin is solubilised. Treated biomass includes aspen woodchips, bagasse, wheat straw, barley straw, rice hulls, corn stover, switchgrasss, coastal burmudagrass, alfalfa, newsprint (Dale et al., 1996, Lynd et al., 2002, Sun and Cheng et al., 2002).

2.2.5.5 Carbon dioxide Explosion

This method involves the use of 4 kg CO_2 per kg of fibre, at a pressure of 5.68 MPa to pre-treat the lignocellulose material. There are no inhibitors formed and further conversion of cellulose can be >75%. Treated materials include bagasse, alfalfa and recycled paper (Sun and Cheng, 2002)

2.2.5 Ozonolysis

Ozonolysis is a method carried out using ozone at room temperature and pressure to disrupt the lignin-cellulose-hemicellulose matrix. There are no inhibitors formed and further cellulose conversion can be more than 57% even though there is lignin degradation. Pre-treated materials have included poplar saw dust, pine, bagasse, wheat straw, cotton straw, green hay, peanut, Sun and Cheng (2002).

2.2.6 Biological Methods of pre-treatment

These methods include the use of fungal and enzymatic pre-treatment. An example of these methods is the use of microbes such as brown-, white- and soft-rot fungi to attack the lignin and hemicellulose in lignocellulosic materials. Brown-rot fungi mainly degrade cellulose whereas white- and soft-rot fungi attack both cellulose and lignin. According to Fan et al., (1987), white-rot fungi are the most effective basidiomycetes for biological pre-treatment of lignocellulosic materials. The disadvantage of these methods is that the reactions or conversions take a long time, up to a few weeks.

2.2.7 Summary of pre-treatment methods

Various pre-treatment methods are being researched on and the best method to apply depends on the material to be treated, and the subsequent steps of hydrolysis and fermentation. Those methods that result in few to no inhibitors are the ones that have received the most attention in further research. There is a need for consideration of other constraints or variables such as conversion period, cost of chemicals, materials, equipment and yield to ensure lowering of costs. A combination of treatment methods can be used to ensure high yields in subsequent steps.

2.3 Saccharification/Hydrolysis

Hydrolysis of cellulose into glucose (saccharification) can be carried out using acids or enzymes.

2.3.1 Acid hydrolysis

Acid hydrolysis can be achieved using either dilute acid or concentrated acid (Sanchez et al., 2008). High temperatures of 200 to 240°C are usually used when dilute acids are used for hydrolysis of cellulose but there is subsequent degradation of glucose to hydroxyl-methyl

furfural (HMF) under these conditions, with xylose also forming furfural. A 50% glucose yield is possible using this method (Hamelinck et al., 2005). Using concentrated acid of 30 – 70% H_2SO_4 results in higher glucose yields of about 90% and is relatively rapid (10- 12h) (Sanchez et al., 2008). The drawback with concentrated acid is the huge cost of acid and a recovery step is necessary to lower the cost.

Dilute acid hydrolysis has been successfully used to pre-treat lignocellulose material followed by fermentation. It has also been shown to result in high yields using batch methods with high solid loading, according to Schell et al., (2003), resulting in up to 77% xylose yield in their pilot plant. Dilute acid hydrolysis has a higher rate of hydrolysis than enzyme hydrolysis but has the disadvantage of decomposing the sugars by dehydration resulting in furfural, from pentoses, and hydroxymethylfurfural (HMF) from hexoses which are fermentation inhibitors (Lenthan et al., 2010). These compounds combine with the acetic acid formed during the hydrolysis of the acetyl groups in the hemicellulose decreasing the rate of fermentation. Hence a detoxification and neutralization steps are necessary.

2.3.2 Detoxification

Chemical detoxification such as overliming, ion exchange adsorption, addition of activated carbon, solvent extraction, or steam stripping are used to improve the fermentability of an acid hydrolyzate, but huge costs are incurred in these steps which can also result in considerable sugar loss (Olsson and Hahn-Hagerdal, 1996; Galbe and Zacchi, 2002; Sun and Cheng, 2002). Overliming involves the use of calcium hydroxide to raise the pH to between 9 to 10.5 and then lowering the pH to pH 5.5 to pH 7 with dilute acid. Organic solvents are used for solvent extraction in the ratio of 3: 1 organic solvent: aqueous phase respectively volumetric ratio. For ion exchange detoxification, weak base resins such as Amberlyst A20 can be used with ammonia being used for regeneration.

2.3.3 Enzymatic Hydrolysis

Enzymatic hydrolysis makes use of cellulase in reactions that convert cellulose into glucose-reducing sugars. Three steps are involved in enzyme hydrolysis of cellulose: the adsorption of cellulase onto the substrate surface; biodegradation of cellulose into reducing sugars and desorption of cellulase as illustrated in Figure 2.3.

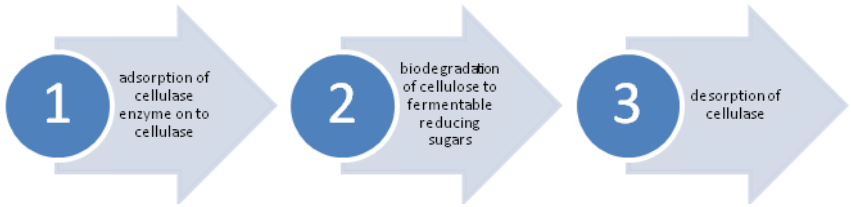


Figure 2.3: Enzymatic hydrolysis steps

The nature of the substrate, cellulase activity and the reaction conditions are the factors that affect enzymatic hydrolysis. Most of the commercially available enzymes are obtained aerobically from the fungus Trichodermareesei (Sun and Cheng 2002). The main drawback of cellulase is its inability to hydrolyse xylan. Hemicellulose which is comprised of mainly xylan, is converted into pentoses by xylan degrading enzymes produced from various fungi and bacteria such as Trichodermaspp, Penicillumspp, Talaromycesspp, Aspergyllusspp and Bacillus spp (Balat M. 2011)

2.4 Fermentation

Fermentation is usually carried out in industrial ethanol plants using the yeasts. (Rabelo et al., 2011). Fermentation can be carried out using a variety of methods. *Saccharomyces cerevisiae* is the commonly used yeast for fermentation. It has the advantage of being substrate specific. The efficiency of the yeast depends on the concentration, nature of substrate, methods followed and time taken for fermentation. The presence of inhibitors is of great concern especially for acid hydrolyzates. Care has to be taken that conditions of temperature and pH are at optimal for the specific yeast used. *Saccharomyces cerevisiae* has been shown to be tolerant to high sugar concentrations, resistant to adverse conditions generated by the presence of ethanol and stable at higher temperatures and other conditions in industrial operations (Rabelo et al., 2011). The drawback with this microorganism is that it can only ferment hexoses and cannot ferment pentoses. There are basically two types of methods employed for fermenting biomass hydrolyzates. In separate hydrolysis and fermentation (SHF), the hydrolysis is first carried out at its optimum conditions of temperature, followed by fermentation at conditions conducive for microorganisms involved in fermentation. In the other method, hydrolysis and fermentation are performed at the same time, with the same conditions, a method known as simultaneous saccharification and fermentation(SSF). The SSF method has the advantage of increasing the hydrolysis rate through conversion of sugars

that inhibit the cellulase activity. The diagrammatic representation of steps involved in converting lignocellulose material to ethanol through Simultaneous saccharification and fermentation (SSF)/simultaneous saccharification co-fermentation (SSCF) and separate hydrolysis and fermentation (SHF) is shown in Figure 2.4 below:

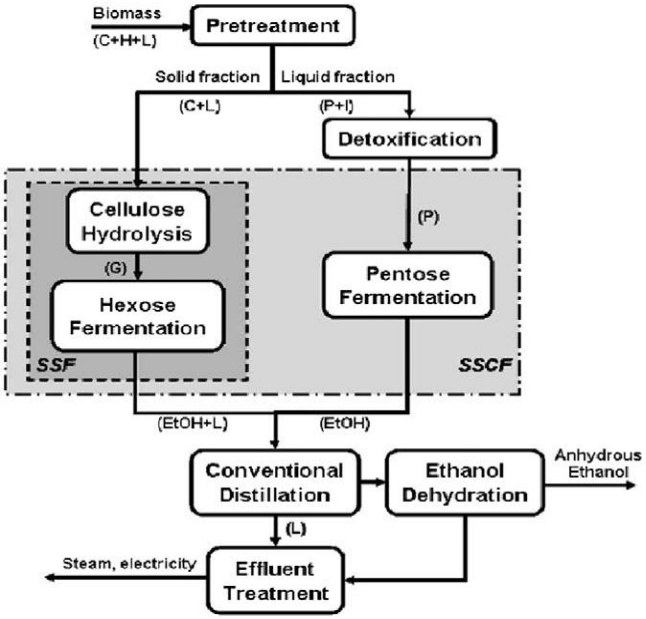


Figure 2.4: Steps leading to SSF and SHF/SSCF (Cardona et al., 2007)

Any raw materials containing sugary contents can be used as a raw material in the fermentation of glucose into ethanol (Prasad et al., 2007). Fermentation is carried out under anaerobic conditions leading to the glycosylation of one glucose molecule into two moles of ethanol and two moles of carbon dioxide as shown in Scheme 2.3 (Ingram et al., 1998).

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ + energy (stored as ATP)

Scheme 2.3: Conversion of glucose into ethanol and carbon dioxide

Chapter 3

3. Methodology

3.1 Introduction

In this chapter, the methodologies followed during pretreatment and treatment of the biomass through to bioethanol production, are laid out. The treatment processes are grouped into two basic categories, which are, pretreatment and treatment. Under pretreatment, the biomass handling, dilute acid and alkali-peroxide pretreatment procedures are outlined. Treatment methods that were used in this research include concentrated acid hydrolysis, and treatment with enzyme hydrolysis. Both dilute acid pretreatment and alkali-peroxide pretreatment were followed by enzyme hydrolysis. Hydrolysis steps were subsequently followed by fermentation and characterization steps. All these steps are summarised in the flow diagram Figure 3.1 below:

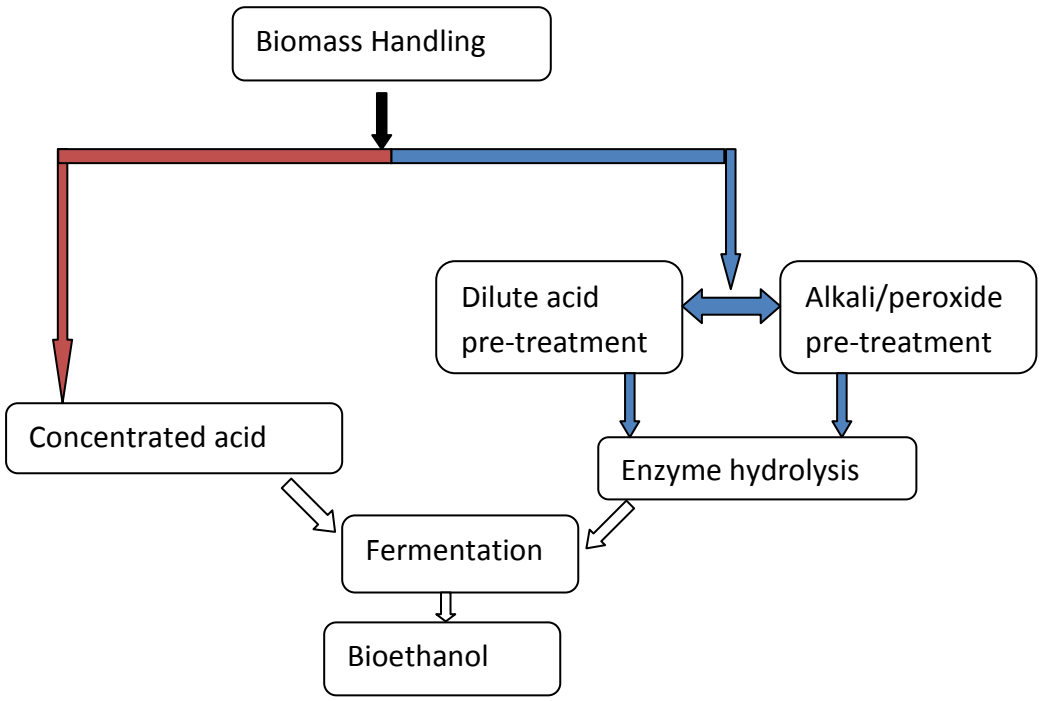


Figure 3.1: Flow diagram of the bioethanol production steps.

In Figure3.1 , characterisation is incorporated in all the processes and as such is not shown in the diagram but is never the less an integral part of this research. As such there is a chapter on characterisation.

3.2 Biomass handling

3.2.1 Materials:

The materials used in this research are sugar cane leaves and tops. In this research, sugarcane leaves and tops are the biomass material because they offer a low cost raw material which is readily available as food residue (Hsu, et al., 2011). The materials also offer the advantage of not being in competition with food sources and can result in bioethanol being produced cost effectively when combined with efficient enzymes for a high yield. The chemicals and reagents used include the following:

Sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), Hydrogen peroxide (H_2O_2), Dinitrosalicylic acid (DNS), cellulose enzymes, baker's yeast,(saccharomyces cerevisae), glucose, ethanol, propanol, citric acid monohydrate, Rochelle salts(Na-k tartrate), Whatman no 1 filter paper. All chemicals used are analytical grade unless stated otherwise.

3.2.2 Pre-treatment of biomass

Pre-treatment is an important step in lignocellulosic bioethanol production. The main aim of pre-treating the lignocellulosic material before hydrolysis was to remove the lignin and hemicellulose. It also helps in reducing the crystallinity of cellulose resulting in the more amorphous cellulose which is more accessible to hydrolysis (Sun and Cheng, 2002).

Successful pre-treatment should also result in little or no inhibitors present in subsequent steps of hydrolysis and fermentation. As mentioned earlier in Section 2.2, there are several steps that have been researched on regarding pre-treatment.

3.2.3 Experimental Procedures

The lignocellulosic material was washed by triple rinsing with deionised water followed by drying in an oven at 60°C for 24h and left at room temperature for 48h in sealed glass bottles. After drying, a blender was used to grind the materials into fine particles. The resultant material was then stored frozen in closed plastic bag container to be subjected to pre-treatment and subsequent hydrolysis and fermentation procedures. This was done in order to ensure the materials remain in a more or less similar condition as they are subjected to different preatreatment and treatment conditions.

It is important to have a very smooth and fine powder for XRD samples so as to increase the accuracy of results. A pestle and mortar were used to grind the sample down to particles of not more than about 0.002 mm to 0.005 mm cross section by using a sieve. The ideal sample has to be homogeneous so that the crystallites are randomly distributed.

3.3 Concentrated acid hydrolysis method

Sulphuric acid concentrations of 30 and 72% v/v were used in this research, for purposes of comparing whether there is a difference in the overall effect on . Acid blanks were used as controls. A range of residence times from 1h to 12h were studied using 0.300mg samples which was weighed and placed into 100 ml glass bottles. This was followed by the addition of 3ml of the 30% acid concentration. The same procedure was repeated for the 72% acid concentration as well as blanks. All experiments were carried out in triplicate and left at room temperature for the required residence time of either 1h or 12h.

After the prescribed residence time, 84ml of deionised water were added and left to stand for 2h. Acid hydrolysis requires a neutralization step and this was carried out using alkali neutralization and overliming.

3.4 Neutralization

Two methods of neutralization were carried out, i.e., use of 1M NaOH for neutralization and overliming. 1M NaOH was used to adjust the pH until it was in the region of pH 5.5 to 6.5. The solution was left to settle for 1h. In overliming, the hydrolyzates were placed in beakers on a magnetic stirrer equipped with a hot plate. They were then heated to 60°C and the temperature maintained while solid calcium hydroxide was added until pH

was 10.5 to 11. After 30 minutes of continuous stirring the pH was then brought down to between 5.5 to 6 with 1M H_2SO_4.

3.5 Dilute acid hydrolysis and enzyme fermentation method

Different concentrations of H_2SO_4, 1.6M and 2M, were prepared and used for hydrolysis. Of the dry substrate, 3 g were weighed and placed into 9 x 300ml glass bottles. To each of three bottles, 200ml of 1.6 M sulphuric acid was added and the process repeated with 2M sulphuric acid and a deionised water control experiment. They were then left to soak for 2h at room temperature. After 2h, they were autoclaved at 121°C for 20 min then left to cool at room temperature.

For inhibitors removal/detoxification of lignocellulosic hydrolyzates we made use of calcium hydroxide for overliming followed by sulphuric acid neutralization, or sodium hydroxide for direct neutralization.

3.6 Alkali/oxidative pre-treatment method

Solutions of hydrogen peroxide of concentrations 0,5%, 3%, 5%, and 7% (v/v) were prepared. To each concentration, NaOH was added to adjust the pH to 11, then 4g samples of the dry matter where added into each 500 ml Erlenmeyer flask with subsequent addition of 100ml of H_2O_2 solution. The mixture was then stirred for 48h with samples taken at 24h intervals. In addition, a deionised (DI) water control was conducted without adjusting the pH. Each H_2O_2, pH, and time treatment combination was repeated three times. After the allotted amount of time for stirring, the mixture was filtered and the residue collected. The residue was triple rinsed for 30 minutes in DI water and oven dried at 100°C for approximately 12h. To prepare for the next step of hydrolysis, the dried residue was then soaked in a buffer solution of pH 4.8.

3.7 Enzyme hydrolysis method

The enzyme cellulase was used to hydrolyse products of dilute acid pre-treatment and alkali/oxidative pre-treatment to give reducing sugars whose concentrations were determined using the Dinitrosalicylic Acid (DNS) method. A buffer of pH 4.8 was prepared according to the International Union of Pure and Applied Chemistry (IUPAC)

method (Ghose, 1987). A solid loading of 15% (w/v) of pre-treated hydrolyzates was used. Enzyme hydrolysis was conducted at mild conditions of temperature (45 -50°C) and pH 4.8. The advantage of enzyme hydrolysis over acid hydrolysis is that it does not have a corrosion problem (Duff and Murray, 1996). A cellulase dosage of 10FPU/g was used since it provides high levels of glucose in a reasonable time (48 -72h) (Gregg and Saddler, 1996).

3.8 Fermentation method

Fermentation was carried out using the yeast *saccharomyces cerevisae* obtained from bakers' yeast. The growth medium consisted of 20 g/l of saccharose, 5 g/l of yeast extract, 5 g/l K_2HPO_4, 1.5 g/l NH_4CL, 1.15 g/l of KCl and 0.65 g/l of $MgSO_4.7H_2O$. A 10% (v/v) yeast inoculum was charged into 250 ml conical flasks together with nutrients to make a 150 ml sample. Reaction temperatures of about 30°C were maintained throughout the fermentation period. A 30 ml sample was taken every 24h, 5 days and 7 days and microcentrifuged at 5000 rpm for 15 minutes then filtered. The supernatant was analysed by a Gas Chromatography (GC) spectrometer equipped with a FID detector at 250°C for detection of ethanol. In this research separate hydrolysis and fermentation (SHF) was used to ferment the hydrolyzates and all fermentations were carried out in triplicate. The batch fermentation method was followed to ferment hydrolyzates into bioethanol.

CHAPTER 4

4. CHARACTERISATION

4.1 Introduction

The lignocellulose material and products from acid and peroxide treatments were characterised using Fourier Transform Infrared Spectroscopy (FTIR) to obtain infrared spectrum of the material and hydrolyzates. X-Ray diffraction (X-RD) was done to obtain the crystalline arrangement within the untreated lignocellulose material and compared to the pre-treated material by calculating the crystallinity indices of the material. The surface micrographs of untreated and pre-treated material were taken using Scanning Electron Microscopy (SEM) to assess effectiveness or lack thereof of pretreatment methods in disrupting the existing bonds and linkages within the lignocellulose material. In addition to FTIR, XRD and SEM, Ultra Violet Spectroscopy (UV-Vis), Gas chromatography and other experimental techniques such as the IUPAC Dinitrosalicylic acid method of reducing sugars determination were applied to help analyse and understand the characteristics of the results of hydrolysis and fermentation procedures.

4.2 FTIR analysis

FTIR spectra with a resolution of 4 cm^{-1} were obtained from a Perkin Elmer spectrometer equipped with a CSI window and beam splitter. Scans were run per each sample between 370 cm^{-1} and 5000 cm^{-1}. Each sample (calibration and validation) was analyzed in triplicate and the resulting spectra were averaged.

4.3 X-ray diffraction

X-ray diffraction was used to analyse the samples' crystallinity. The ideal sample for X-ray diffraction analysis has to be homogeneous so that the crystallites are randomly distributed. The sample was then pressed into a sample holder and analysed by XRD on the D8 advance diffractometer (Bruker AXS, Germany) The XRD had CuKα radiation, wavelength of 0.1542 nm at 40 kV and 40 mA with goniometer radius of 280mm. The

diffraction intensity was measured in the range of 2θ = 10 – 45 degrees with a step size of 0.02 degrees per second. The method of Segal et al. (1959) was used to calculate the crystallinity indices (CrI). Nickel-filtered Cu Kα radiation (ʎ =0.1542 nm) was used at 40kV and 40mA. The diffraction intensity was measured in the range of $2\theta = 10 - 60^{\circ}$, with a step size of 0.02° at a rate of 2°/min. The CrI was calculated based on the Segal et al. (1959) method.

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

Equation 1: Equation for crystallinity index calculation.

Where I_{002} is the intensity of the 002 crystalline peak at $2\theta = 22.4^{\circ}$ and I_{am} corresponds to the amorphous cellulose region for cellulose, hemicellulose and lignin at $2\theta = 18.0^{\circ}$ (Sinhu et al., 2011).

4.4 Gas Chromatography

Gas chromatography was used for the determination of the products of fermentation. Bioethanol presence was characterised by using gas chromatogram spectrometer equipped with a FID detector. The initial oven temperature was 65°C and it was held there for 5 minutes. It was then increased to 150°C at a rate of 4°C/ min and held for 5 min before being raised to 250°C at a rate of 4°C/min and held for a further 5 min and the set on split. The injector temperature was set at 175°C and detector temperature set at 250°C.

4.5 UV-Vis Spectrophotometry

A UV-Vis Spectrometer was used for dinitro-salicylic acid **(DNS)** assays and was set at a wavelength of 540nm to analyse the hydrolyzates.

The Filter Paper Assay for saccharifying cellulase (FPU Assay) from the IUPAC Applied Chemistry Division Commission on Biotechnology was used. This method was be used to estimate the amount of reducing sugars in the hydrolyzates. The DNS reagent was prepared according to IUPAC method. Once mixed with the hydrolyzates, the DNS reagent would attach itself to glucose molecules. Glucose standards of 0.2 – 5.0mg of

glucose per ml were prepared together with a water blank for 100% transmittance. The results were used to plot a graph of mg of glucose against A_{540}.

The resultant graph was then used to estimate the amount of glucose produced. An X-ray deffractometer was used to analyse the change in crystallinity of the material. The method of Segal et al., (1959) was then used to calculate the crystallinity indices.

4.6 Enzyme activity –Filter paper assay for saccharifying cellulase (FPU)

There are a number of challenges faced when assessing the activity of cellulase. Some of these challenges are the varying nature of the substrate and its insolubility; very little literature and understanding of processes and activities involved when endo- and exo-glucanases coordinate to break the substrate; and generation of a variety of end products. (Ghose, 1987). Hence a series of empirical assays were developed to try and standardise the assessment procedures. The Filter Paper Assay for saccharifying cellulase (FPU Assay) from the IUPAC Applied Chemistry Division Commission on Biotechnology was used (Ghose, 1987). A UV-Vis-spectrophotometer was used to measure absorbance at 540nm (A_{540}) the wavelength at which the reducing sugars can be detected.

In order to determine the filter paper activity (FPA), 1ml of 50mm sodium acetate buffer pH 5.0, Whatman filter paper No. 1 (1cm x 6cm) was mixed with 0.5ml of enzyme solution at the appropriate dilution. This mixture was incubated at 50°C for 60 min. The aim was to determine the amount of enzyme that would produce 2mg of glucose as filter paper units (FPU) per ml.

4.7 Reducing Sugar Estimation by Dinitrosalicylic Acid (DNS) Method (Ghose, 1987)

DNS reagent was prepared by mixing 1416 ml of distilled water with 10.6g of 3,5-dinitrosalicylic acid and 19.8 g of NaOH. Once the mixture was dissolved, 306g of Rochelle salts (Na-K tartrate), 7.6 ml of phenol (melted at 50°C and 8.3g of Na metabisulfite were then added. When the above mixture is titrated with 3 ml of 0.1N HCl in the presents of phenolphthalein indicator, it should take 5-6 ml of HCl to

neutralize. NaOH was added (2g = 1ml 0.1N HCl).Standards solution of glucose of 0.2 – 5.0mg per ml were prepared. A water blank was also used for 0% absorbance. The results were used to plot a graph of mg of glucose against A_{540}. This graph was then used to estimate the amount of glucose produced. To a 2ml sample of hydrolyzates, 3ml of DNS was added and the mixture boiled in a water bath followed by cooling. These were then diluted with 20 ml of distilled water, just like the standards as per the IUPAC recommendations.

Chapter 5

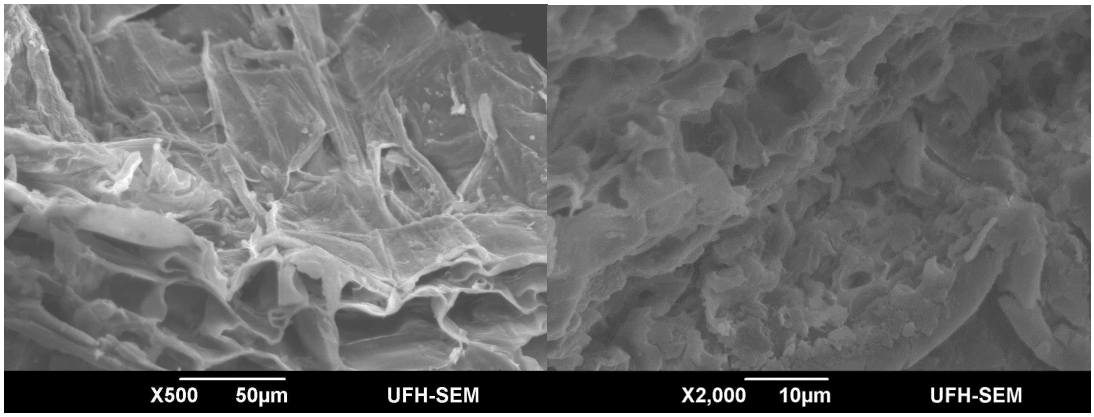
5. Results and discussion

5.1 Introduction

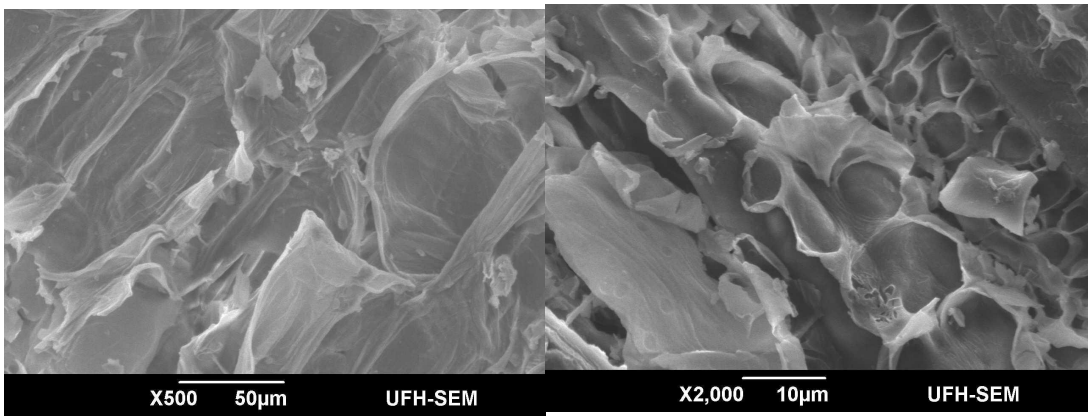
This chapter contains the results of pretreatment, treatment/hydrolysis and fermentation processes. Pretreatment processes used in this research were mechanical communition followed by acid and alkali-oxidative pretreatment. In order to investigate the effectiveness of pretreatment SEM, EDS and XRD were used and the results are shown in this chapter. The results of concentrated hydrolysis and cellulase hydrolysis follow thereafter. DNS method was used for the amount of reducing sugars present after hydrolysis with gas chromatography results for ethanol content determination following thereafter. A summary of the processes involved can be seen in the flow diagram fig 3.1 in the previous chapter.

5.2 Pretreatment and treatment

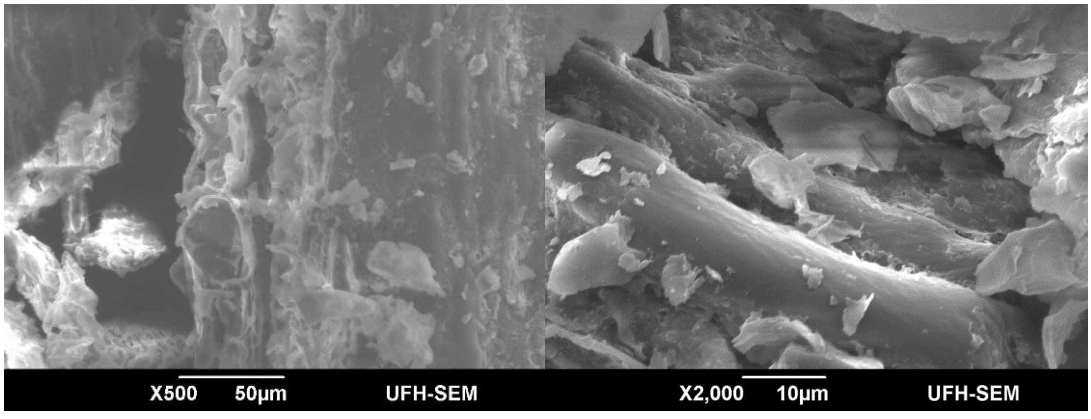
To show the effects of pre-treatment, Scanning electron microscopy imagery complemented by EDS and X-ray diffraction was used. Fig 4.1 below shows the resultant images after different pre-treatment and treatments of the biomass from raw, crushed lignocellulose compared to acid treated alkali-oxide treated lignocellulose material.



a)b)



b) d)

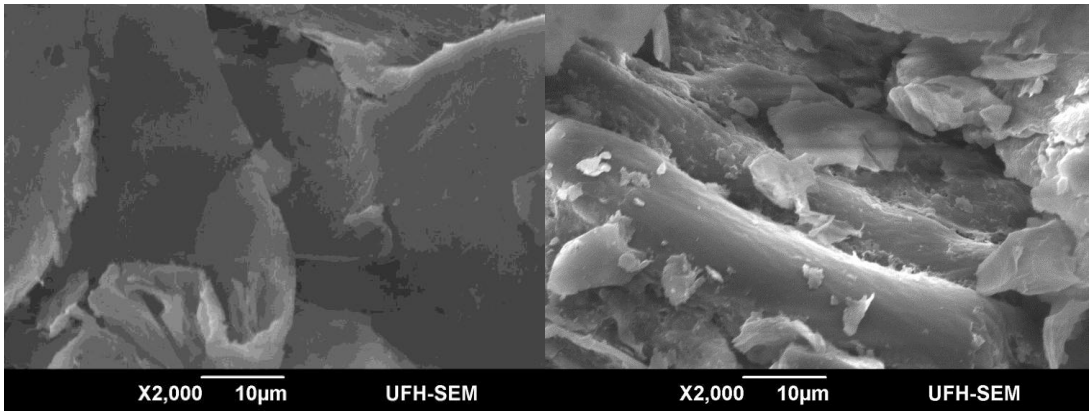


e) f)

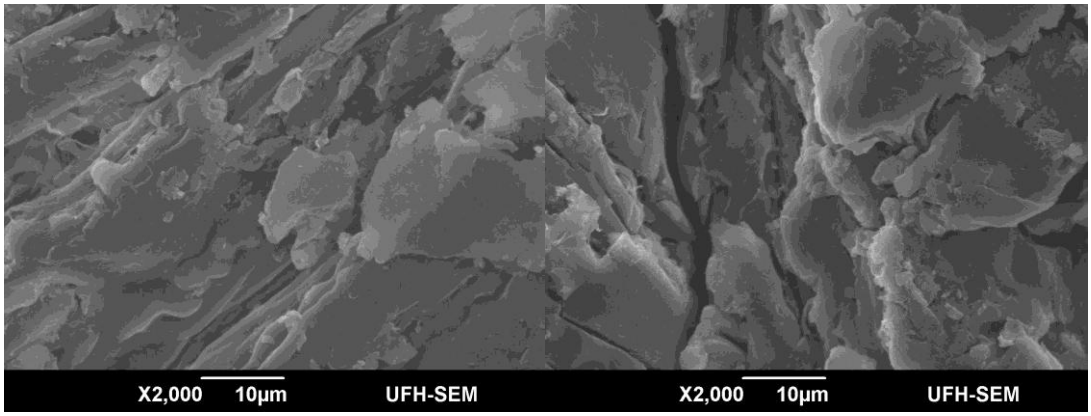
Figure 5.1 SEM micrographs a) – b) 72% sulphuric acid hydrolysed lignocellulose S.E.M. image at X500 and X2000 respectively; c) – d) 30% Sulphuric acid hydrolysed lignocellulose images at X500 and X2000 respectively; and e) – f) Raw untreated but blended

SEM images in Figure 5.1 above indicate 72% H_2SO_4 resulted in the highest disruption and destruction of the lignin-hemicellulose-cellulose complex. Flakes and rough surfaces can be seen in a) and b). 30% H_2SO_4 also resulted in nearly as much disruption and part destruction of lignin. Some of the SEM images are in Appendix A.

SEM was also used to assess the effectiveness of dilute acid hydrolysis and there were disruptions to the lignocellulose chains as can be observed on the images in figure 5.2 below.



a) Raw /Untreated b) 1.6M H_2SO_4 treated



c) H_2O_2 treated d) 2M H_2SO_4 treated

Figure 5.2: SEM micrographs of a) Raw untreated material, b) 1,6M Sulphuric acid hydrolysed lignocelluloses, c) H_2O_2 treated and d) 2M sulphuric acid hydrolysed lignocellulose.

Although the untreated material had some degree of flaking and rupturing due to blending, this became even more pronounced for peroxide and 2M acid treated material as shown in Figure 5.2 above. What do the SEM results suggest?

5.3 Energy dispersive spectroscopy (EDS)

In addition to SEM imagery, EDS was also used to analyse the elemental composition of the material. Whilst after hydrolysis, b) indicates a drastic shift downwards in both C and O. Upon hydrolysis with acid much of the sugars and part of the lignin are broken down and washed with the hydrolyzates for fermentation. As a result when the residue was run for EDS, much of it was insoluble lignin. The tables below indicate the compound percentages. Of main interest were the elements C, N and O which were present in higher amounts in the unhydrolysed sample than in the hydrolysed sample as indicated in Tables 5.1 and 5.2.

Table 5.1: Table of EDS results before hydrolysis of samples

Element	Amount (% weight)				
	Untreated / raw -1	Untreated 2	Untreated 3	Average	Standard deviation (σ)
C	34.65	30.26	31.11	32.00	1.9
N	10.29	10.6	11.37	10.75	0.45
O	44.98	50.09	51.35	48.81	2.75
Mg			0.39	0.39	0.39
Al	0.34	0.43	0.22	0.33	0.09
Si	4.20	0.25	1.62	2.02	1.64
S	0.18	6.03	0.49	2.23	2.67
K	1.72	0.04	0.74	0.83	0.69
Ca	1.14	1.34	1.13	1.20	0.10
Cu	2.77	0.94	1.58	1.76	0.76
Zn					

The results in Table 5.2 are calculated averages after analyses were done in triplicate similar to the original sample in Table 5.1 above.

Table 5.2: EDS results of untreated and treated material.

Element	Amount (% weight)					
	Untreated Average	1.6M Acid	2M Acid	H_2O_2 treated	30% Acid	72 % Acid
C	32.00	33.71	27.4	27.91	30.21	31.27
N	10.75	9.33	10.6	11.10	10.72	11.06
O	48.81	42.39	49.4	56.33	51.66	53.77
Mg	0.39	-	-	0.54	0.2	0.1
Al	0.33	0.28	0.20	0.41	0.45	0.31
Si	2.02	10.39	2.59	0.68	0.54	1.06
S	2.23	0.53	6.9	0.05	1.61	0.01
K	0.83	0.05	0.05	-	1.01	-
Ca	1.20	-	-	0.48	2.06	0.63
Cu	1.76	2.52	1.86	1.80	2.07	1.56
Na			0.14	0.88		

More EDS results can be seen in Annexure B.

The EDS results indicate that there was a change in the amount of exposed oxygen on the surface of the material. This change was more pronounced for peroxide and 72% acid treated material with averages of 56.33% and 53.77% respectively up from 48.81% for the untreated material. This indicates that more glucose and oligosaccharides were perhaps present on the surfaces of the treated material. SEM results also indicate that more cellulose surface area was made available for hydrolysis after crushing. This is crucial for subsequent enzyme hydrolysis and yeast fermentation. Dilute acid treatment

did not appear to have significantly altered the surface of the material in favour of enzyme hydrolysis steps, with 1.6M acid treated material decreasing in oxygen amounts as evidenced by the lower amounts of oxygen detected after treatment.

In addition to SEM and EDS analyses, XRD was also employed in this research to investigate the effectiveness of treatment methods.

5.4 X-ray Diffractograms of pre-treated and treated material

XRD was used on finely ground material to investigate changes in crystallinity and hence spacing before and after treatment. X-ray diffraction has the disadvantage of requiring the material to be finely ground so as to get consistent results. This makes it difficult to use x-ray diffraction on the unblended material hindering comparative analysis of effectiveness of treatment..

However treating the raw blended material significantly reduced the crystallinity of the lignocellulose resulting in more amorphous cellulose being made available.

5.4.1 Results of Concentrated acid hydrolysis

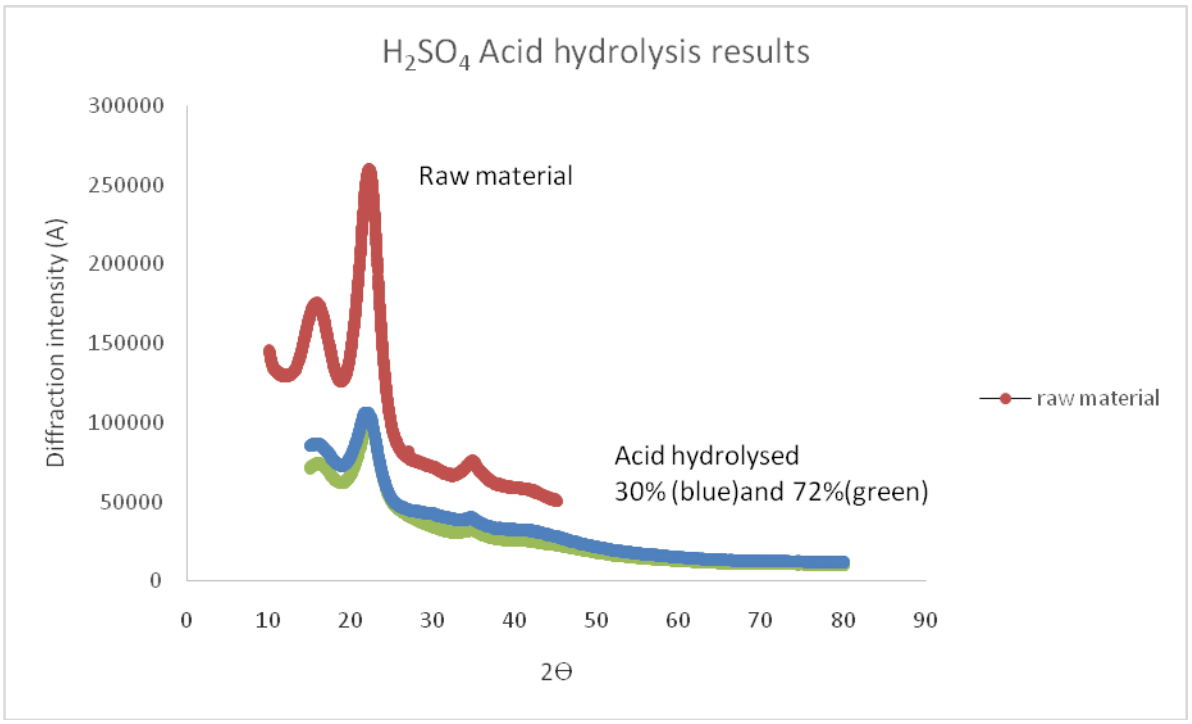


Figure 5.3: X-ray diffraction of concentrated acid hydrolysis residue.

Acid hydrolysis significantly reduced the crystallinity of the lignocellulose material as shown in Figure 5.3 and72% sulphuric acid gave the resultant residue with the least crystallinity.

5.4.2 Results of dilute acid hydrolysis

Although both 1.6M and 2M sulphuric acid concentrations were able to reduce the crystallinity of the lignocellulose, there was not much of a difference in the overall effects of 1.6M compared to 2M H_2SO_4. Figure 5.4 below shows the XRD graphs.

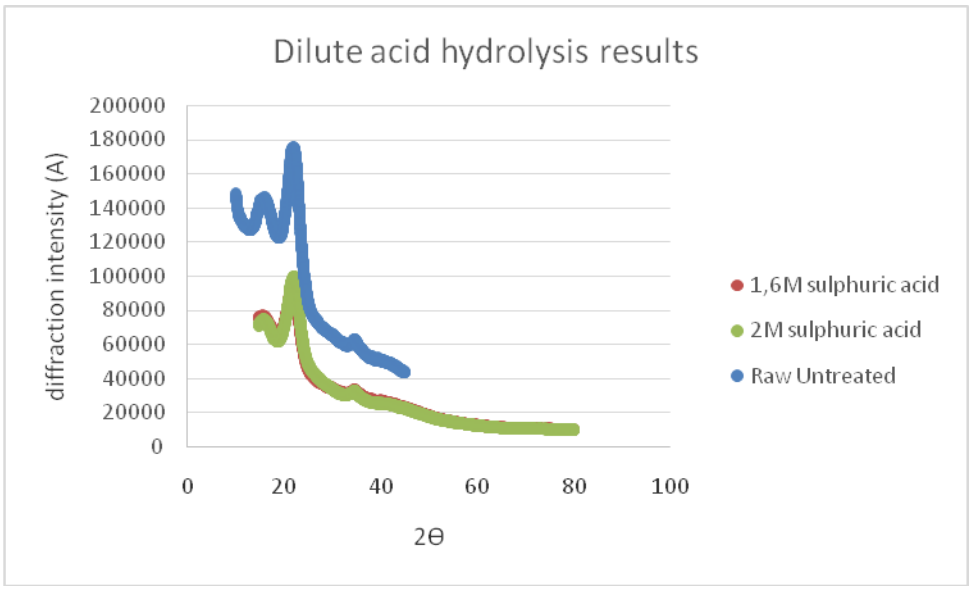


Figure 5.4: X-ray diffraction analysis of effects of dilute acid

5.4.3 Results of Alkali peroxide pre-treatment

X-ray diffraction results indicate there was a significant increase in the crystallinity of the material after pre-treatment. There was a little difference between pre-treatment for 24h and 48h. The increase in crystallinity was interpreted as due to hemicellulose solubilisation and partial lignin degradation as reported by Sun and Cheng (2002). This means cellulose was now more exposed in preparation for enzyme hydrolysis. Figure 5.5 on the next page shows X-ray diffraction graphs of these results.

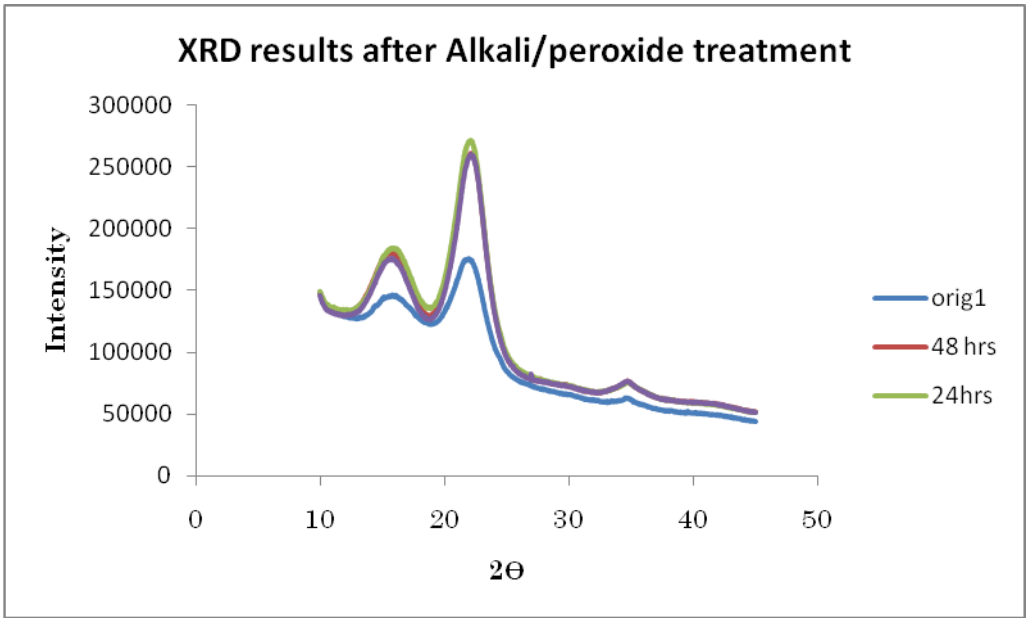


Figure 5.5 X-ray diffraction graphs of the raw untreated material and after treatment for 24 h and 48 h

The increase in crystallinity is confirmed by a calculation of crystallinity indices (CI)using the Segal method as mentioned earlier. The CI values indicate a sharp increase upon pre-treatment from CI values of 28% for the raw untreated material to 45.6% and 46.4% after 24h and 48h treatment respectively(See Annexure C).

5.5 FTIR results of Peroxide pre-treatment

Fourier transform infrared spectroscopy was carried out on both acid and peroxide pre-treated lignocellulose material. The effectiveness of each pretreatment concentration was analysed by observing the different absorption spectral band positions that were formed. Lignin, hemicellulose and cellulose each have specific absorption peaks and with each pretreatment bonds are rearranged, broken or in some instances not disturbed at all. The results of acid pretreatment area indicated in figure 5.6 on the next page.

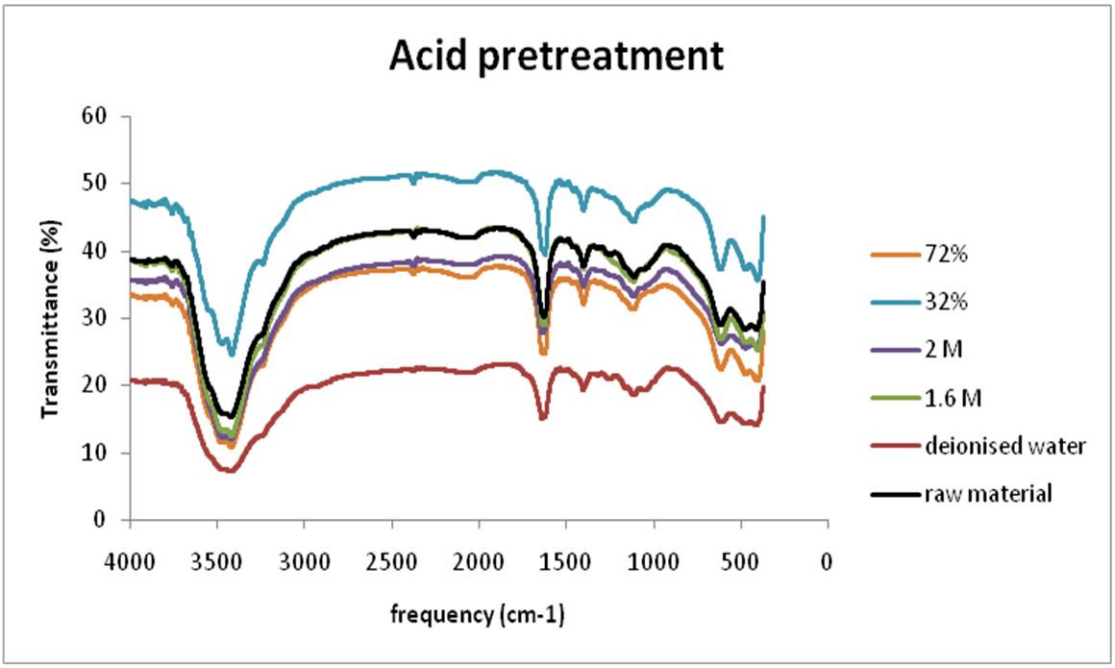


Figure 5.6 : FTIR results for acid pretreatment.

FTIR results indicate acid pretreatment has very little effect on lignin concentrations. This is evidenced by the peaks that were observed at 1400 and 1620 cm^{-1}. These peaks can be seen consistently irrespective of the pretreatment administered. Hemicellulose on the other hand was removed with each pretreatment. The peak at 1252 cm-1 in particular appears in the raw material and the deionised material but is quite insignificant the higher the acid concentration. This peaks at 1252 cm-1 and 1319 cm-1 are attributed to hemicellulose and are gradually deteriorating with subsequent increases in acid concentrations. On the raw material spectrogram there is a peak observed at 897 cm-1 which can be attributed to both cellulose and hemicelluloses and this is consistent with Chen et al, 2012. It is however more pronounced after deionised water pretreatment due to more of the hemicelluloses having been washed away. The peaks at 1058, 1152 and 1158 cm^{-1} are consistent with cellulose absorption spectra. Cellulose concentration was significantly reduced at higher acid concentrations with the only the peak at 152 remaining. This disappearance of the other two peaks has been attributed to the success of acid hydrolysis in disrupting the lignin-cellulose-hemicellulose complex resulting in hydrolysis and subsequent removal of part of the cellulose especially the amorphous part of cellulose.

FTIR was also used to analyse the results of peroxide treatment and these are shown in Figure 5.7 below.

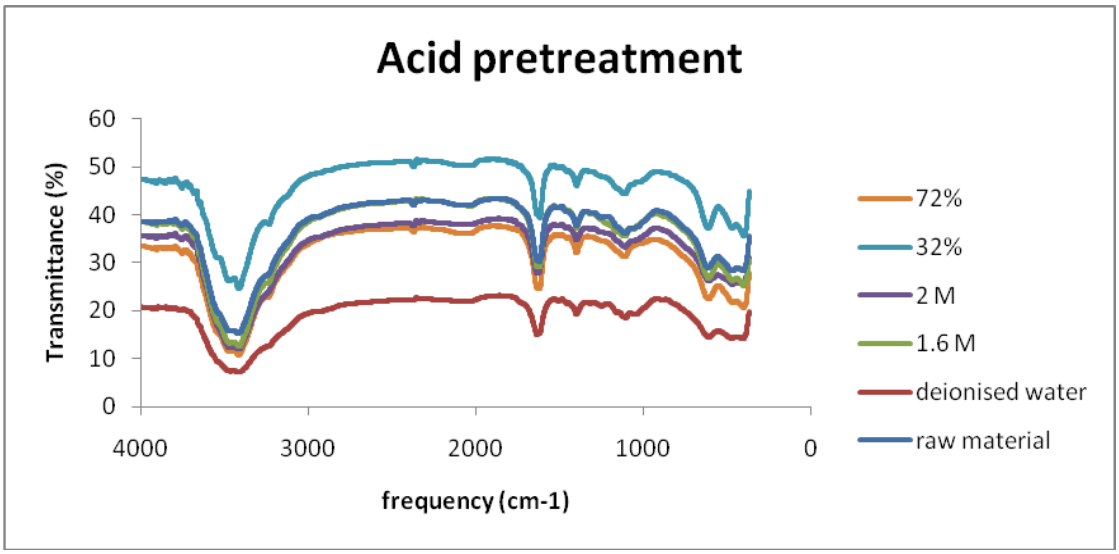


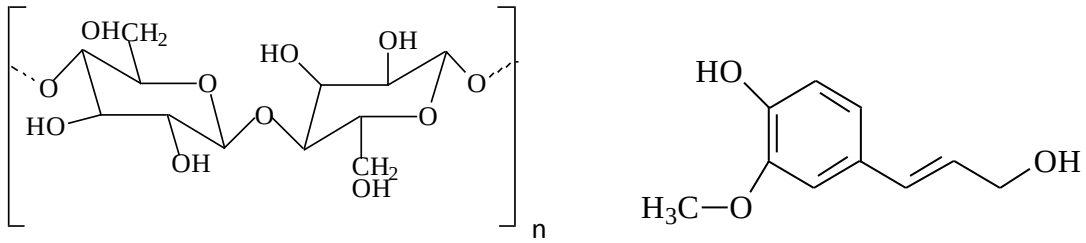
Figure 5.7: FTIR results for alkali peroxide treatment.

The peak assignments shown in table 5.3 below were made to corresponding infrared absorption at the given frequencies(Silverstein et al., 1981, Adapa et al., 2011):

Table 5.3: FTIR peak assignments

Wavenumbers (cm^{-1})	**Peak assignment**
3800 - 2800	A.3414 cm^{-1} O – H stretch, intermolecular hydrogen bonding. B. 2854 and 2925 - C – H stretch
1900 – 1500	C.1737 aromatic ring stretch(typical lignin) B. 1619 –very strong aromatic ring stretch, aromatic –C – O stretch. C = C, C = O, C = N , aromatic skeletal vibration
1500 – 1300	1459 – C – H deformation, 1376 – weak C – O stretching, acetylated hemicelluloses, 1320 $–CH_2–$
1300 – 1100	1161 – glycosydic linkage, C – O – C – Hemicellulose ring vibrational stretching, 1717 – cellulose– C – O stretch
1050 – 1200	E. 1060 – β- polysaccharide, 1045- C – OH bending, 1035 – C – O , C= C and C – C – O vibrational stretching

The structure of cellulose is shown in Figure 5.8 below



a) **b)**

Figure 5.8 a) Cellulose structure b) Coniferyl alcohol – unit structure of part of lignin

There was a significant change at C and D corresponding to normal C=O stretch peak and conjugated C=O stretch respectively. Also the C-H stretch at B decreased significantly with treatment, due to the removal of hemicellulose. There was a gradual reduction in the intensity of $-CH_2$ stretching between 2800 and 3000 cm^{-1}. This was coupled with a rearrangement and significant reduction in the amount of lignin as evidenced by the change in peak intensity at C. 1737 cm^{-1}. This was the case also for hemicelluloses with reductions in the peaks between 1050 – 1200 cm^{-1}.This was interpreted to imply that after treatment there was a significant increase in the amount of cellulose present with part of the lignin removed as seen on SEM images and alluded to in the literature review, an increase in peroxide concentration from 0% to 7% results in structural damage leading to exposure of cellulose. The peak at 1050 cm^{-1} did not appear to have been significantly affected. Hence the β-glycoside linkages in the cellulose part of lignocelluloses were still present. More detailed FTIR graphs are in Annexure D.

5.6 DNS Results

DNS assay results for concentrated acid hydrolysis shows that there was a reduction in the amount of reducing sugars present in the hydrolyzates. This is probably due to the dilution that takes place during detoxification. The formula used for calculation of glucose is as follows:

$$\text{Glucose yield} = \frac{glucoseobtainedinenzymatichyrolysis(g)}{glucoseinrawmaterials\ (g)} \times 100\%$$

Equation 2: Glucose yield calculation equation.

All experiments were carried out in triplicate and the results are as shown in the table 5.4 below:

Table 5.4: Results of acid hydrolysis

	Amount of glucose % (g/g)in pretreated biomass	
H_2SO_4 Concentration	**NaOH Neutralized**	**CaOH(overliming)**
30 %	16,9	9,0
72 %	76.5	59,7

5.7 Results of Enzyme Hydrolysis

DNS assay method was used to calculate the amount of reducing sugars present in the hydrolyzates. The graph for standards that was used for calculations of FPU is in Annexure D. Different enzyme concentrations were prepared in order to find the concentration of enzyme that would be equivalent to one filter paper unit as per the IUPAC method. The results of these tests are indicated in Table 5.5 below.

Table 5.5: Enzyme dilutions and glucose amounts produced

Enzyme concentration(g/v)	**Glucose(mg) produced in 1h**
0.15	1.67
0.30	2.64
0.60	3.60

All enzyme concentrations were prepared and analysed in triplicate and the results averaged to give the figures shown in Table 5.5. The results were then used to estimate the concentration of enzyme that would produce 2mg of glucose in 1h from the appropriate substrate. This concentration is equivalent to one filter paper unit (FPU).

Subsequent enzyme hydrolysis experiments were carried out using an enzyme concentration of 10 FPU.

5.7.1 Acid pretreatment and enzyme hydrolysis results.

Lignocellulose material was pre-treated with dilute acid, neutralized and detoxified in preparation for enzyme hydrolysis. The two methods of detoxification applied are sodium hydroxide neutralisation and overliming. The glucose produced from dilute acid samples is indicated in Table 5.6 below.

Table 5.6: DNS results of combined acid and enzyme hydrolysis.

H_2SO_4 Conc.	**Detoxification Method used and glucose % (g/g) produced after enzyme hydrolysis**	
	NaOH	**Overliming**
1.6 M	22,0	12,0
2.0 M	25,0	10,0

Results in table 5.6 indicate there is a higher concentration in the amount of glucose after sodium hydroxide detoxification than there is upon overliming. Overliming exhibited greater sensitivity to pH fluctuations during neutralization. Samples from overliming are more dilute and glucose residues could also be entrapped within lime deposits, thereby reducing the concentration of glucose obtained. A glucose recovering and concentration step is required in order to make overliming more viable.

5.7.2 Peroxide and Enzyme hydrolysis results.

Lignocellulose material was pre-treated with 0.5%, 3%, 5% and 7% concentrations of peroxide. The residue was washed and triple rinsed with deionised water before enzyme hydrolysis. The results of the amount of glucose produced per each concentration are indicated in table 5.7 on the next page.

Table 5.7: Glucose produced from peroxide pre-treated lignocellulose material.

Peroxide concentration % (v/v)	Glucose (mg/g) biomass
0,5	198,8
3,0	326,0
5,0	367,9
7,0	380,7

5.8 Detoxification Results and DNS analysis

Acid pretreatment and hydrolysis result in the production of fermentation inhibitors. In addition, hydrolysis enzymes and fermenting yeasts have optimum pH and temperature conditions which need to be followed for best results to be obtained. Thus detoxification of acid hydrolyzates was carried out using alkali neutralization and overliming methods.

The results of neutralization were assessed using the DNS assay method as mentioned earlier under characterization. There was higher dilution of the hydrolyzates that were detoxified using overliming than NaOH neutralization. Some of the sugars may also have been removed together with the cake that forms during overliming and is filtered.

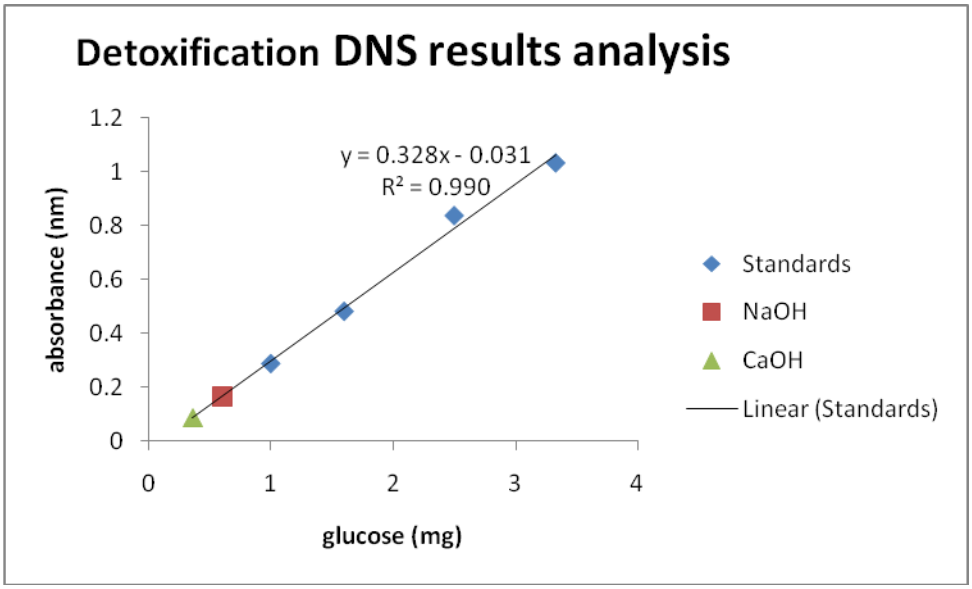
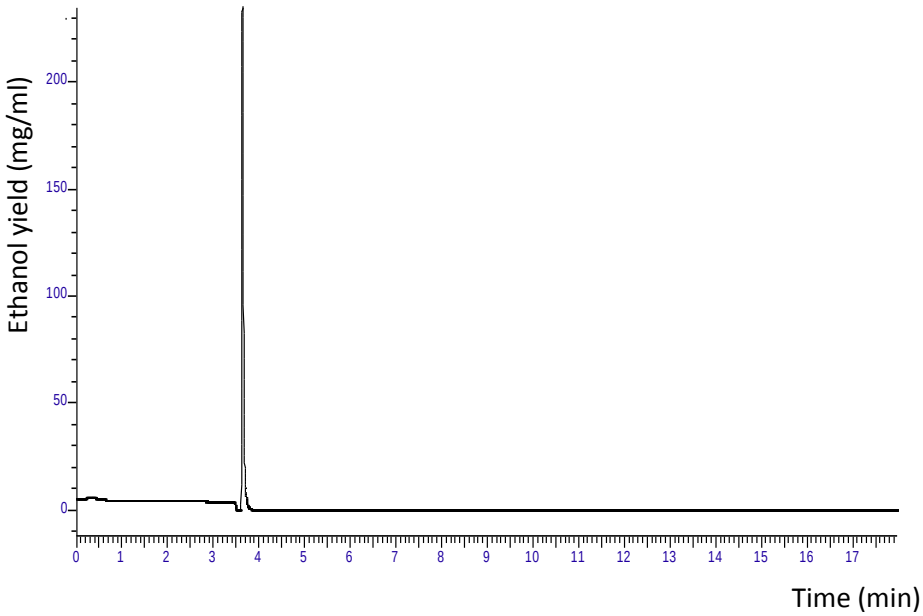


Figure 5.9: Graph of acid detoxification and neutralization results by Overliming and neutralization

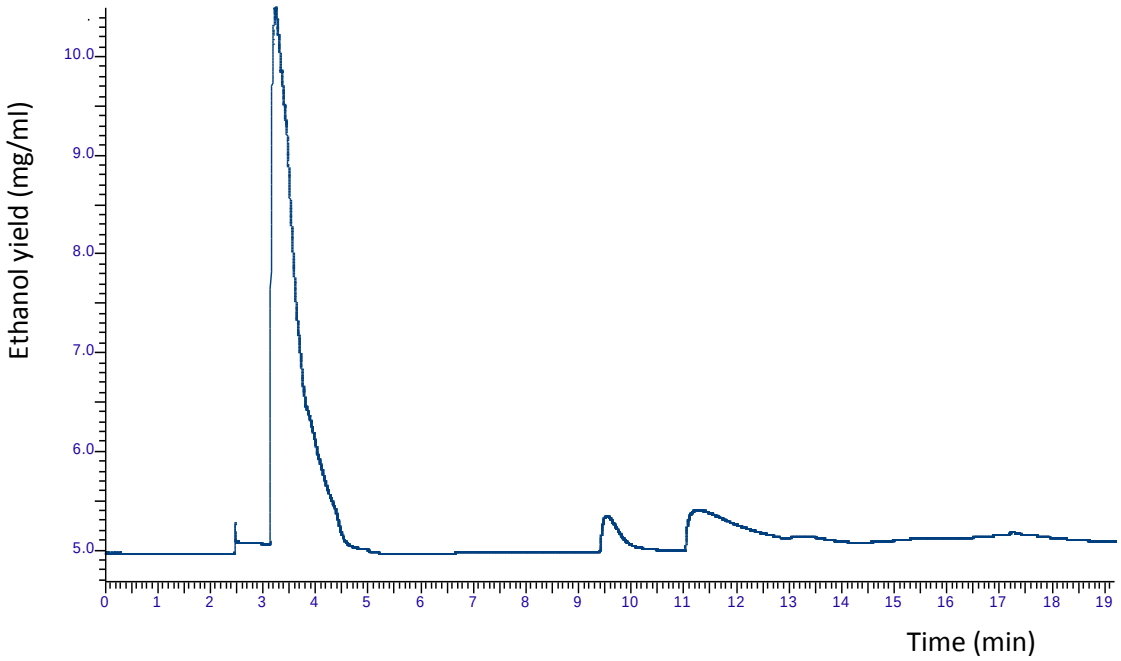
As shown in figure 5.9 above overliming resulted in more loss of reducing sugars than neutralising with alkali.

5.9 Fermentation Results

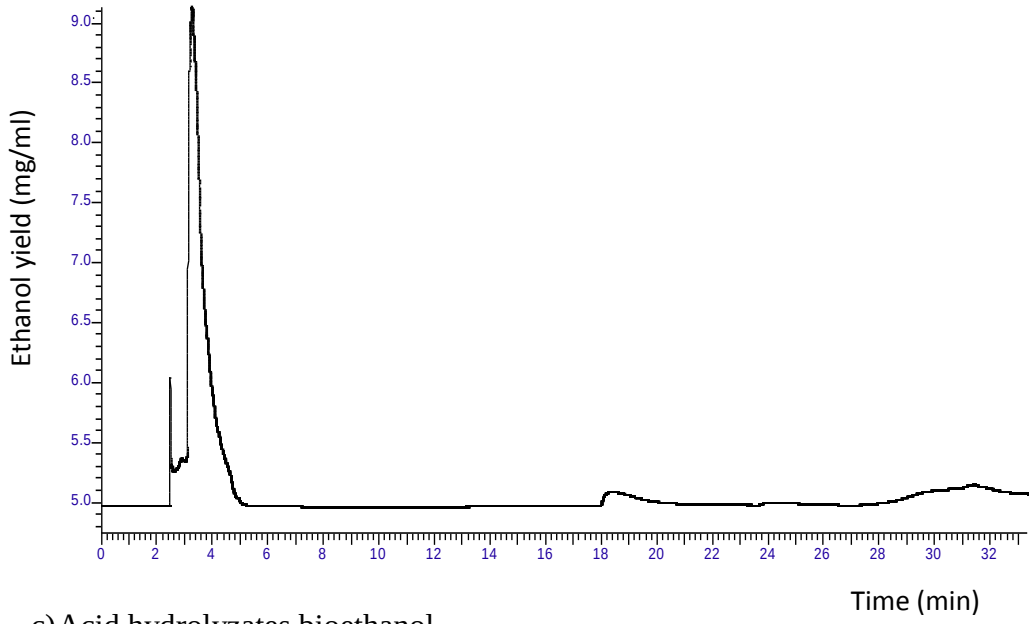
The results of fermentation were obtained using a GC equipped with a FID detector set at 250°C. There was a gradual increase in the amount of ethanol produced with increase in fermentation time from one day to five days. The results of fermentation are shown in figure 5.10 below.



a)Pure ethanol



b)**alkali- peroxide hydrolyzates bioethanol**



c)Acid hydrolyzates bioethanol

Figure 5.10: Bioethanol from a) pure ethanol b) alkali- peroxide and c) acid hydrolyzates after 72h of fermentation.

There was not much of a difference in terms of ethanol produced after 24h of fermentation. The difference was, however, seen after 72h of fermentation when peroxide treated hydrolyzates had slightly more ethanol than acid hydrolysed hydrolyzates as shown on figure 5.9 above. The retention time was 3.5 minutes for pure ethanol with the samples also showing retention times of about 3.5 minutes. Slight

variations were due to the presence of impurities in the samples. Other graphs for the pure ethanol and bioethanol after 24h are in Annexure E.

The following ethanol yields were obtained:

Table 5.8: Ethanol yield from fermentation of hydrolyzates

Source of fermentable glucose hydrolyzates	**Period of fermentation (Hours)**	**Ethanol Yield (g/litre)**
1,6 M H_2SO_4	24	6.9
1,6 M H_2SO_4	72	11.7
Enzyme	24	7.0
Enzyme	72	13.7

Table 5.8 above also shows that the highest yield of bioethanol was obtained from hydrolyzates obtained using the enzyme hydrolysis method. As earlier noted, the disadvantage of this method is the time it takes when compared to acid hydrolysis.

Chapter 6

6.0 Conclusion and Recommendations

6.1 Conclusions

The aim of this research was to produce bioethanol using lignocellulosic sugarcane leaves and tops. Pretreatment procedures conducted are namely concentrated acid hydrolysis, dilute acid hydrolysis and alkali-peroxide pretreatment with enzyme hydrolysis. Acid hydrolysis offers the advantage of taking a shorter period than enzyme hydrolysis. However enzyme hydrolysis gave a high amount of hydrolyzates 380 (mg/g) biomass without requiring any neutralization. The absence of fermentation inhibitors resulted in more bioethanol being produced through enzyme hydrolysis of 13.7 (g/L) than from acid hydrolysis 11,7 (g/L) after 72h of fermentation. Ethanol yield could have been higher if the yeast *saccharomyces cerevisiae* could ferment both pentose and hexose sugars. FTIR result spectra indicated acid was more successful at disturbing and removing hemicellulose as well as breaking down cellulose into oligosaccharides. Alkali peroxide on the other hand is more effective at disrupting lignin chains.

Concentrated acid hydrolysis results in a high amount of reducing sugars being produced indicating high hydrolysis success. The only problem is that at such high concentrations, inhibitors are inevitably produced therefore requiring a detoxification step. A concentration by evaporation step is also required after the detoxification step in order to make the concentration of reducing sugars in the hydrolyzates viable for fermentation. SEM images also show there was more disruption of the lignin-hemicellulose – cellulose complex after hydrolysis at high concentration. It has the drawback of requiring a detoxification and neutralization step in preparation for the next step of fermentation. An acid recovery step is also necessary in order to lower the costs of production.

Dilute acid hydrolysis greatly improves the accessibility of cellulose to hydrolysis enzymes in the next step. It would be beneficial to use 1.6M H_2SO_4 than 2M H_2SO_4 since there was not much of a difference in the reduction of the degree of crystallinity and the yield of sugar with 22% (g/g) yield of glucose per gram of biomass. This will in turn reduce costs incurred in the neutralization step without affecting the sugar yield. Lower temperatures were employed in dilute acid hydrolysis. Alkali neutralization with sodium hydroxide resulted in the highest

amount of reducing sugars being present in the hydrolyzates without requiring a further step of concentration which would have increased the costs. Overliming requires a concentration step in order to increase the sugar concentration before the succeeding step of fermentation so as to make it viable.

Bioethanol production remains one of the most promising possible substitutes for fossil based fuels. There is a need to make available cost effective methods employed in its production if it is to be sustainable.

6.2 Recommendations

Future work in bioethanol production needs to be focussed on producing enzymes that are more tolerant to harsh acid conditions or produce reducing sugars faster. The same also applies to fermentation yeasts.

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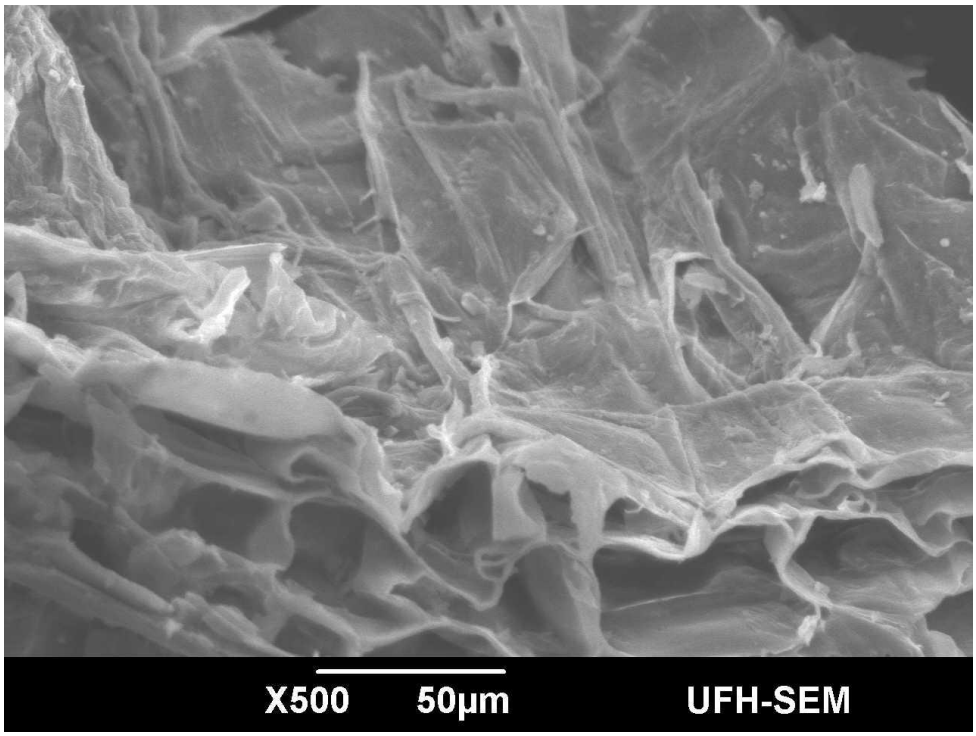
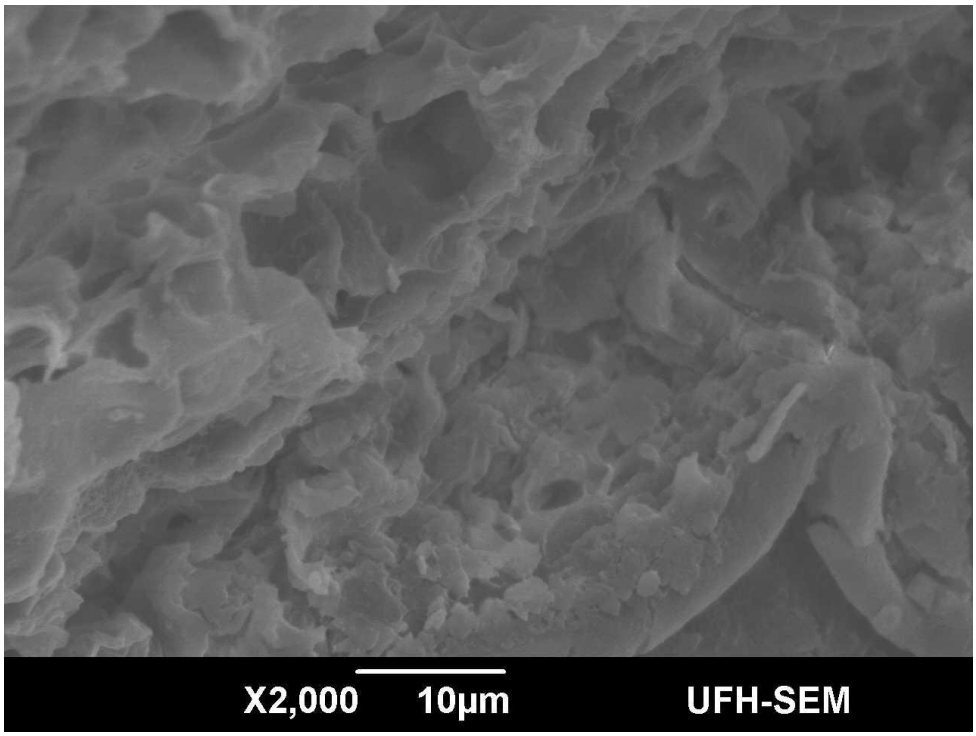
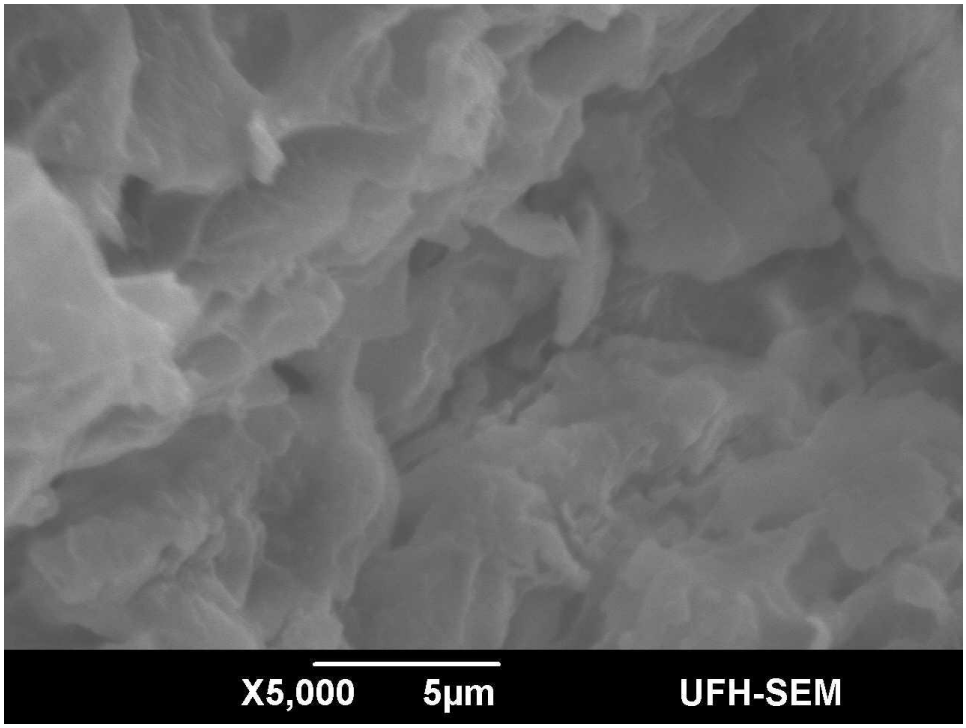
APPENDIX A: Concentrated H_2SO_4 treatment SEM result images



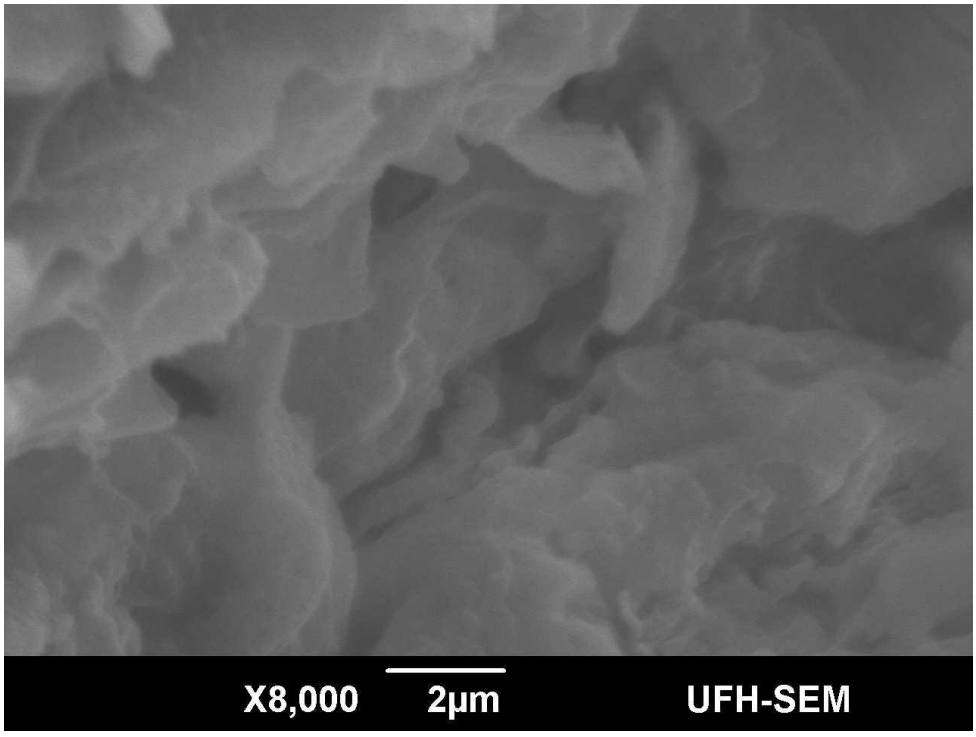
a)72% acid treated image at x500



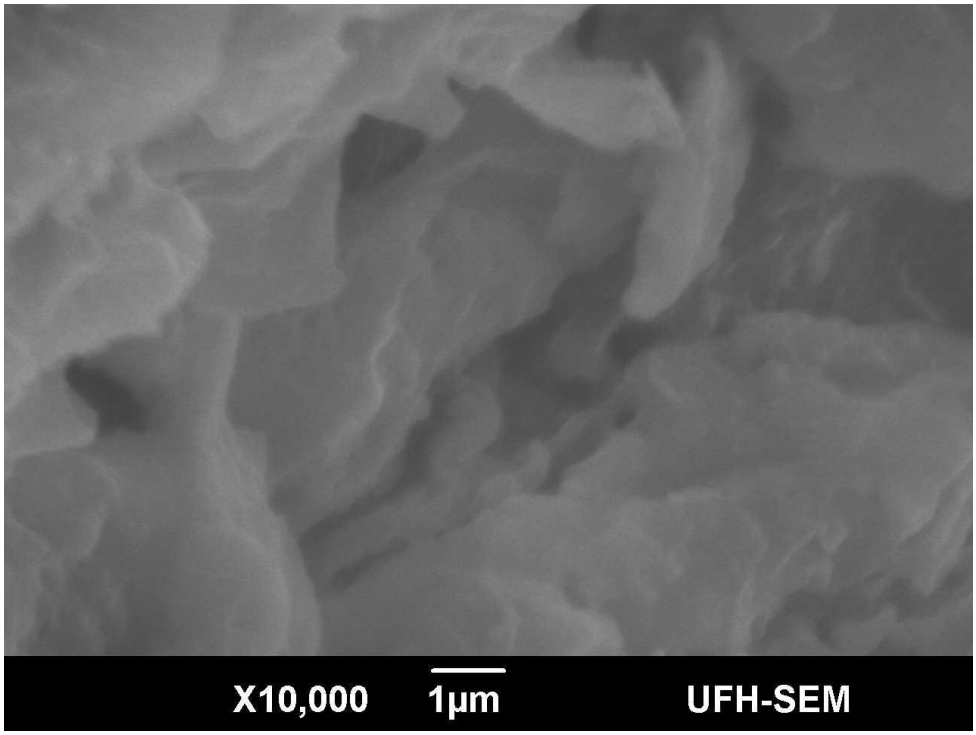
b)72 % sulphuric acid hydrolysed lignocellulose image.



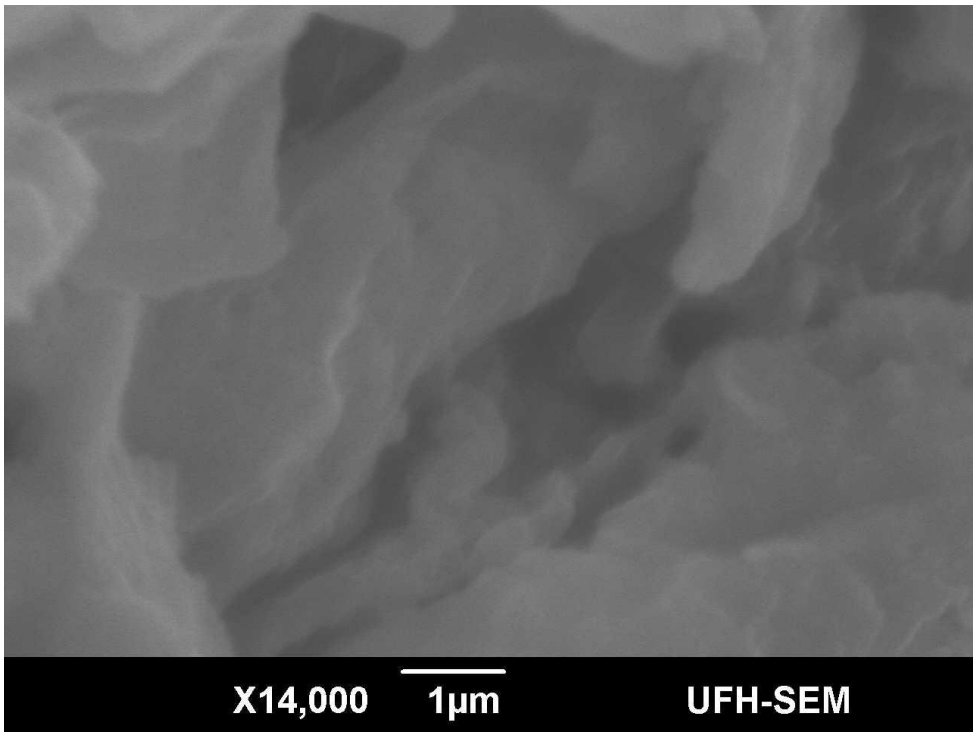
c)72% acid hydrolysed lignocellulose image at X 5000



d)72% acid hydrolysed lignocellulose at X8000

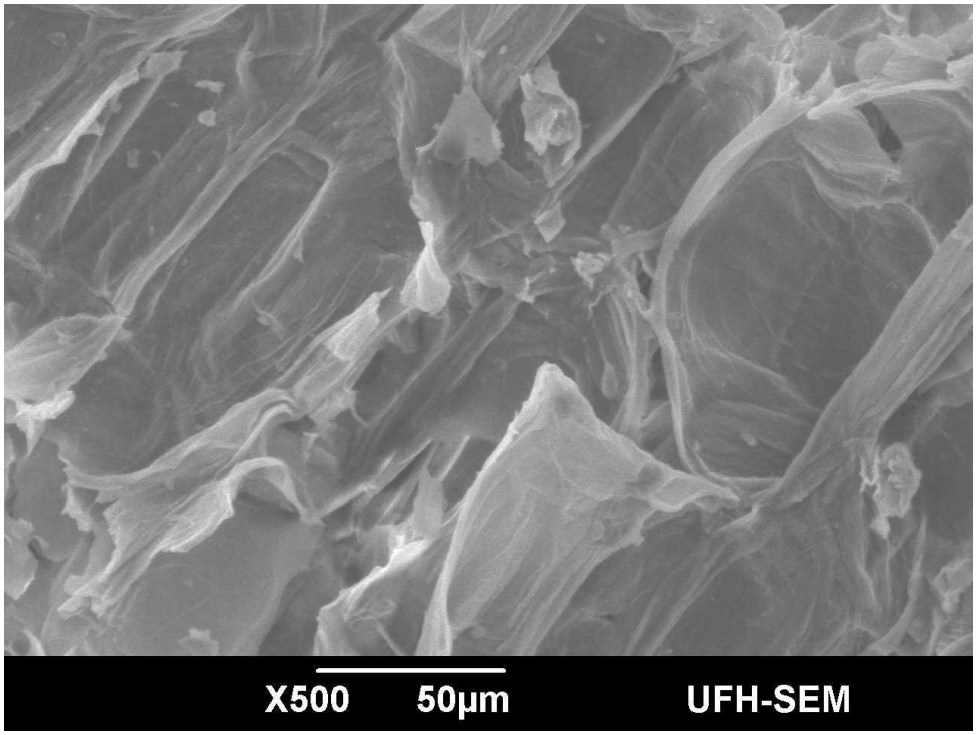


e)72 % acid treated lignocellulose image at X 10 000

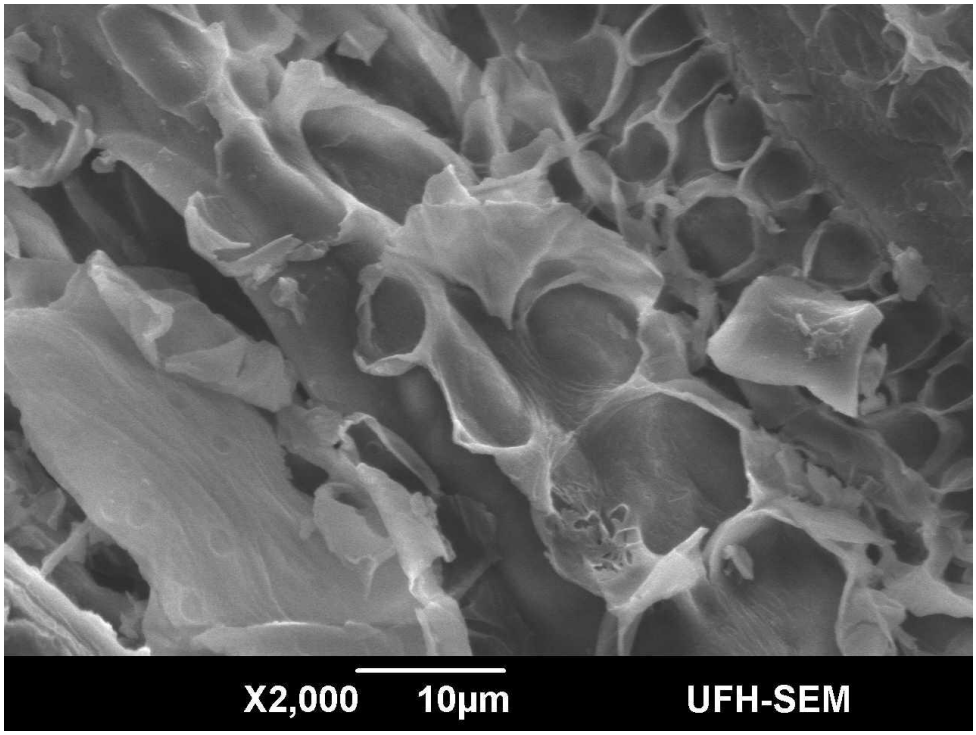


f)72 % sulphuric acid hydrolysed lignocellulose image

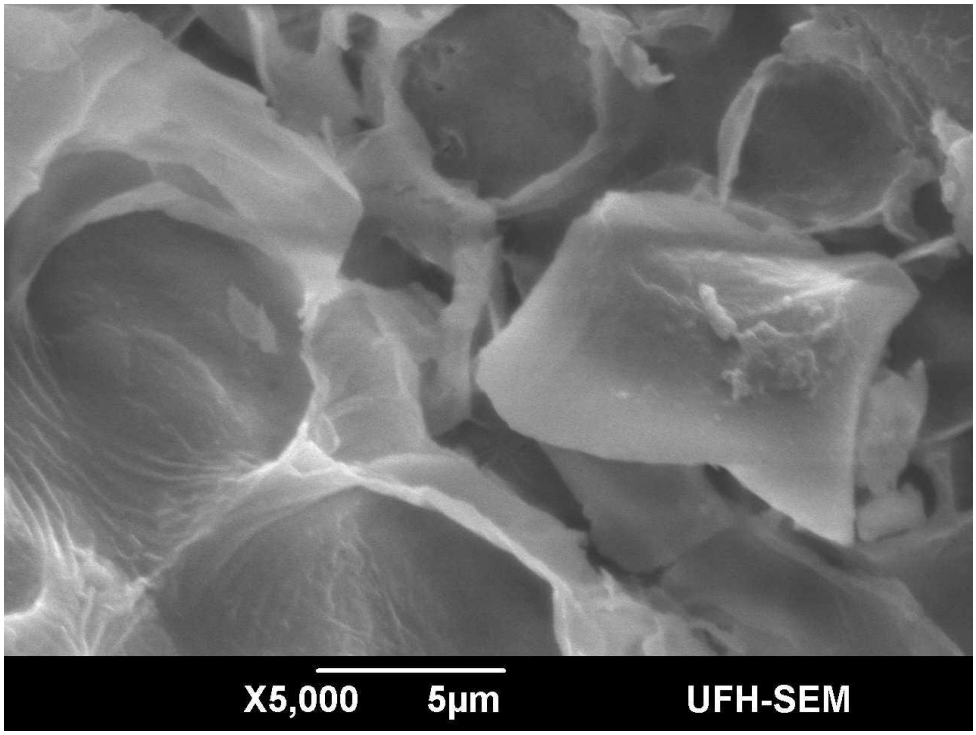
30% H_2SO_4



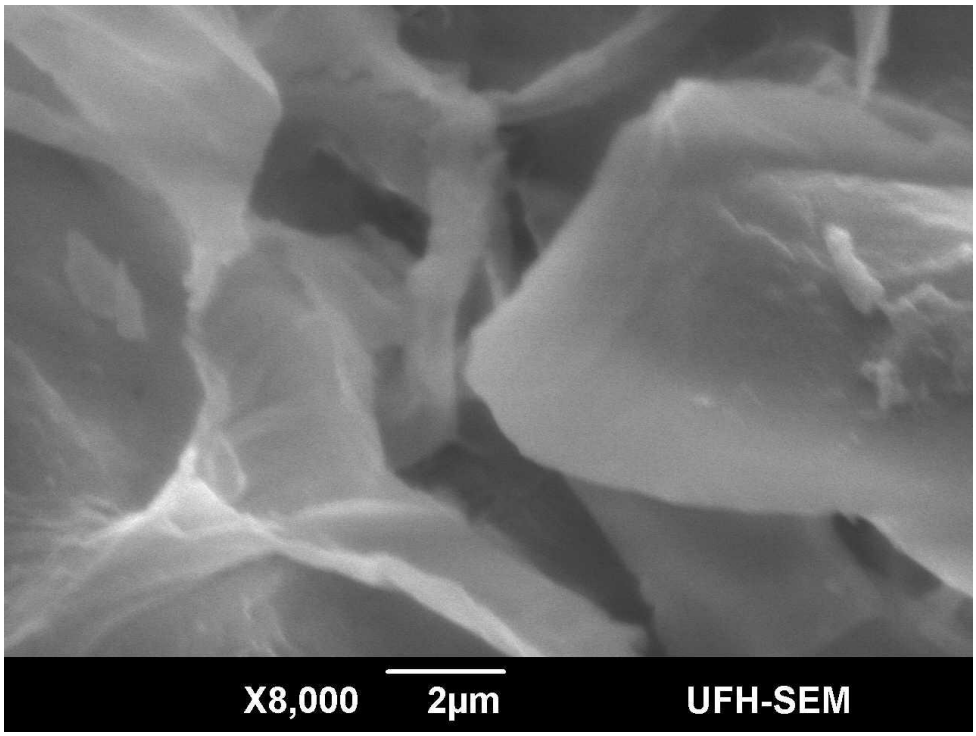
a)30 % sulphuric acid hydrolysed SEM image of lignocellulose



b)30 % sulphuric acid hydrolysed SEM image of lignocellulose

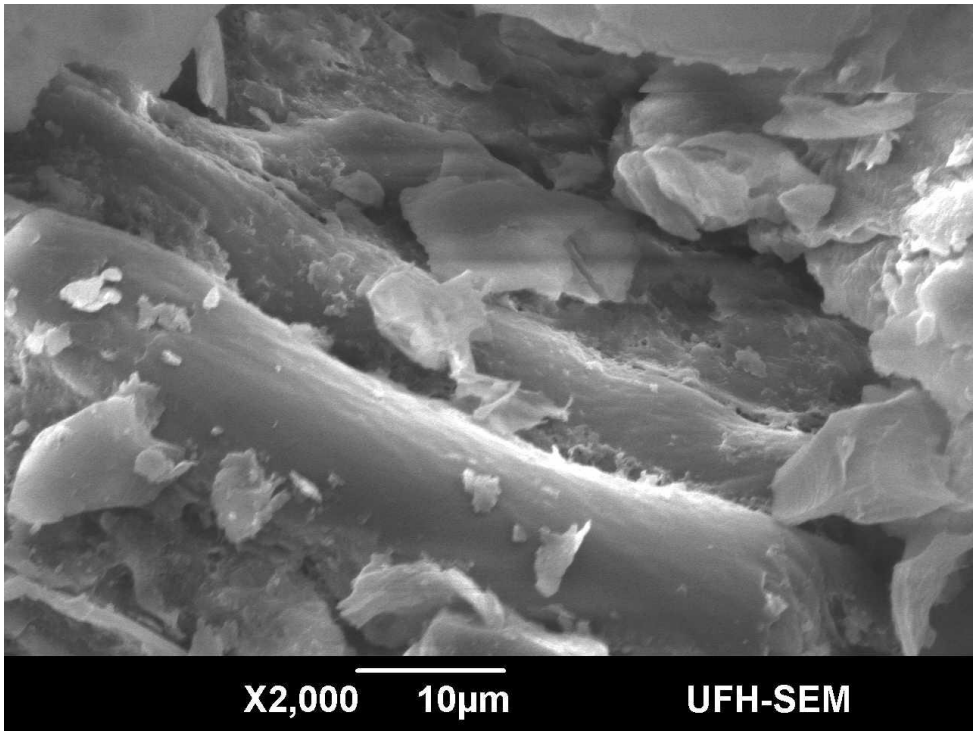


c)30 % sulphuric acid hydrolysed lignocellulose image at X 8000

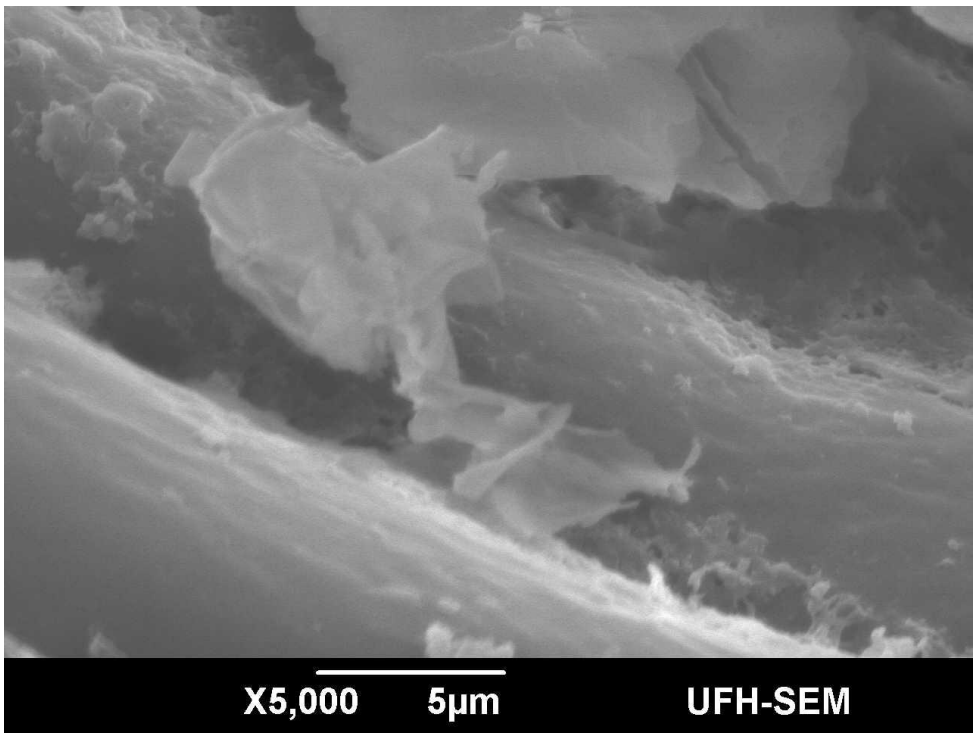


d)30 % acid hydrolysed lignocellulose SEM image at X 8 000

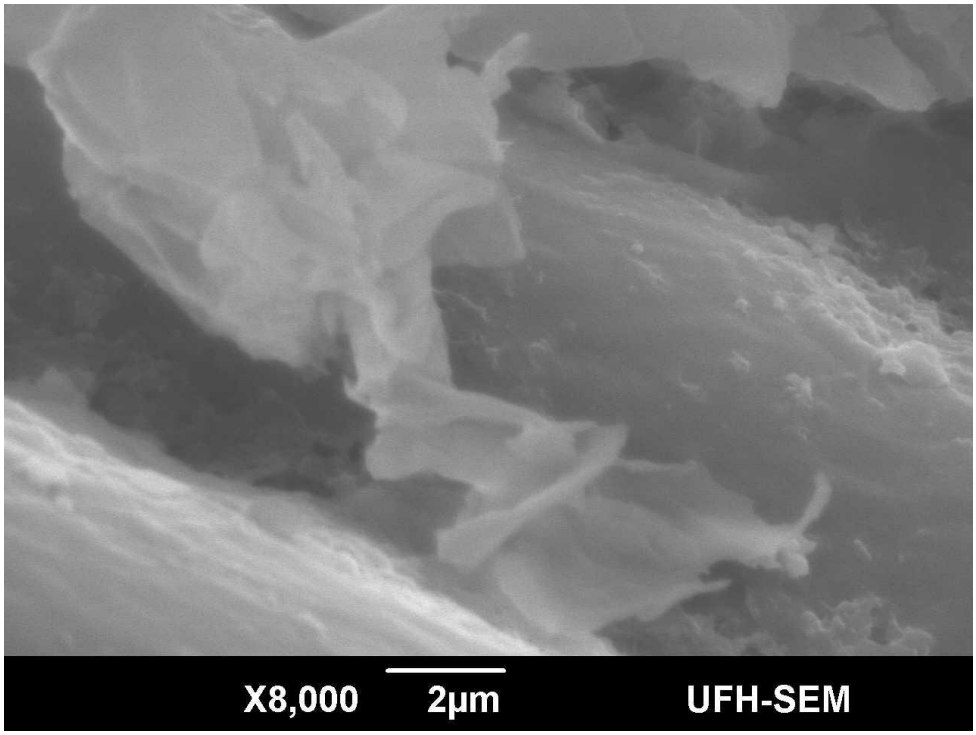
Untreated Lignocellulose material



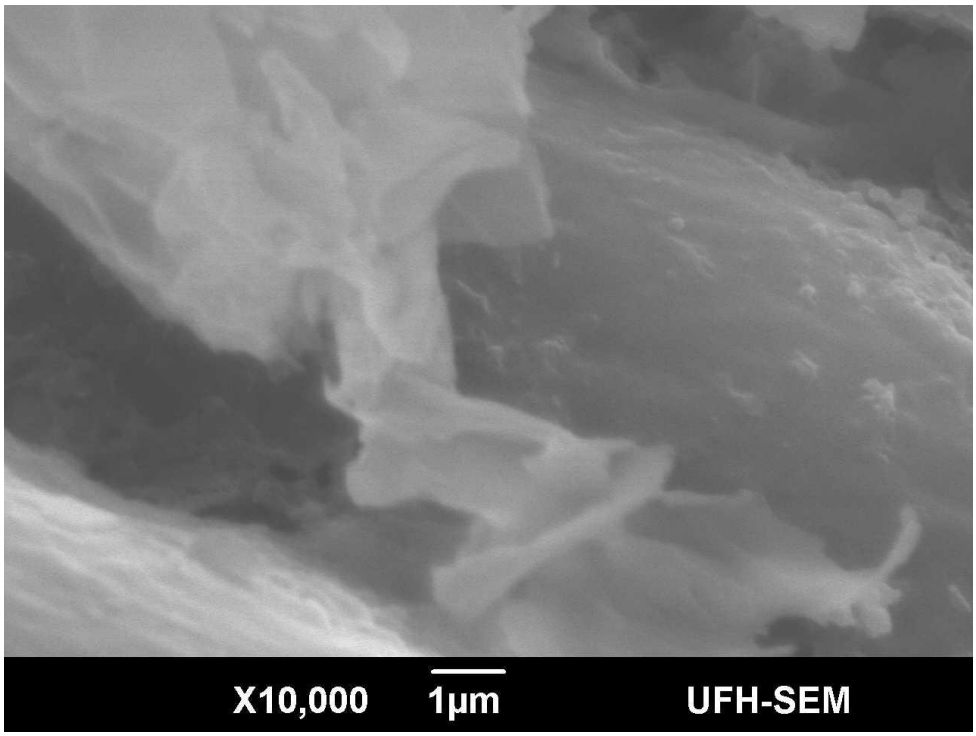
a)Original lignocellulose sample SEM image



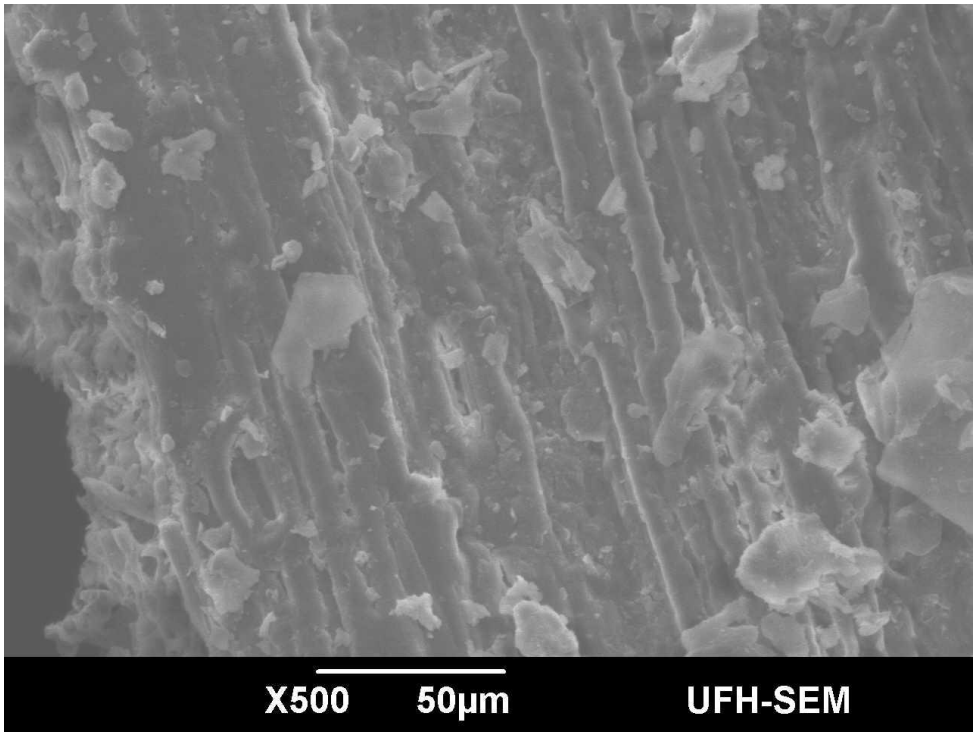
b)Untreated lignocellulose material at X5 000



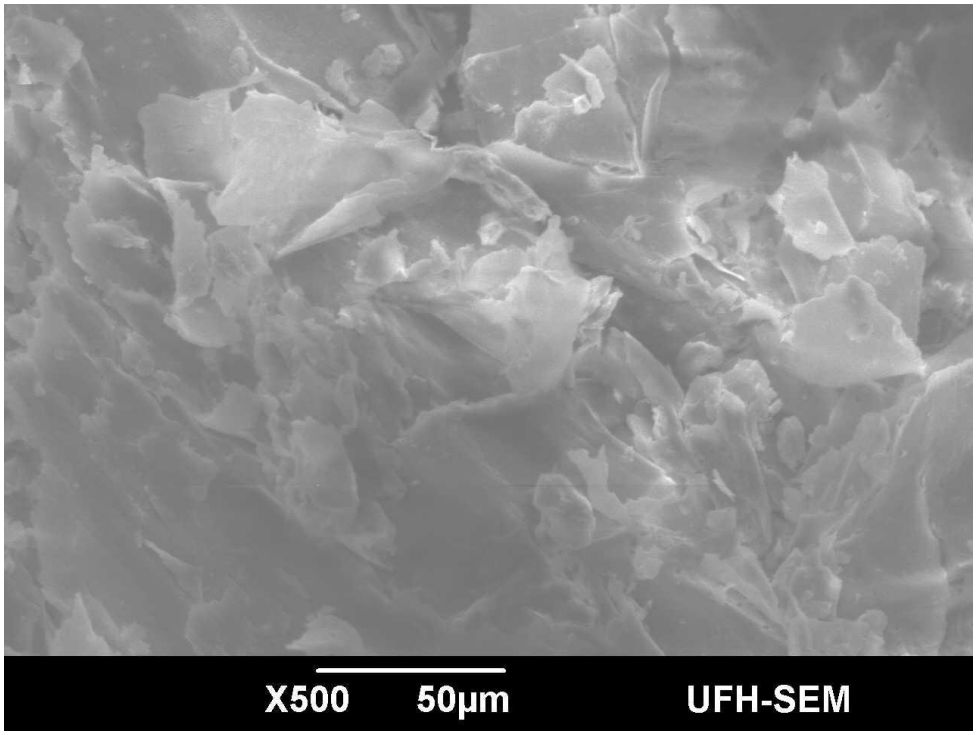
c)Untreated lignocellulose material at X8000



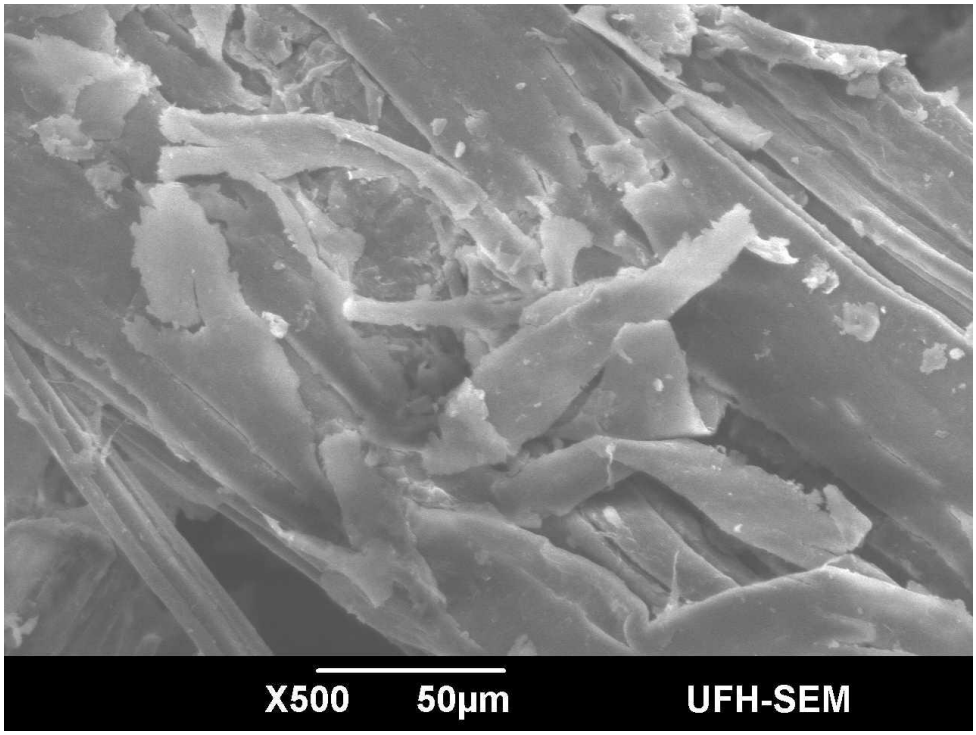
d)Untreated lignocellulose material SEM image at X 10 000



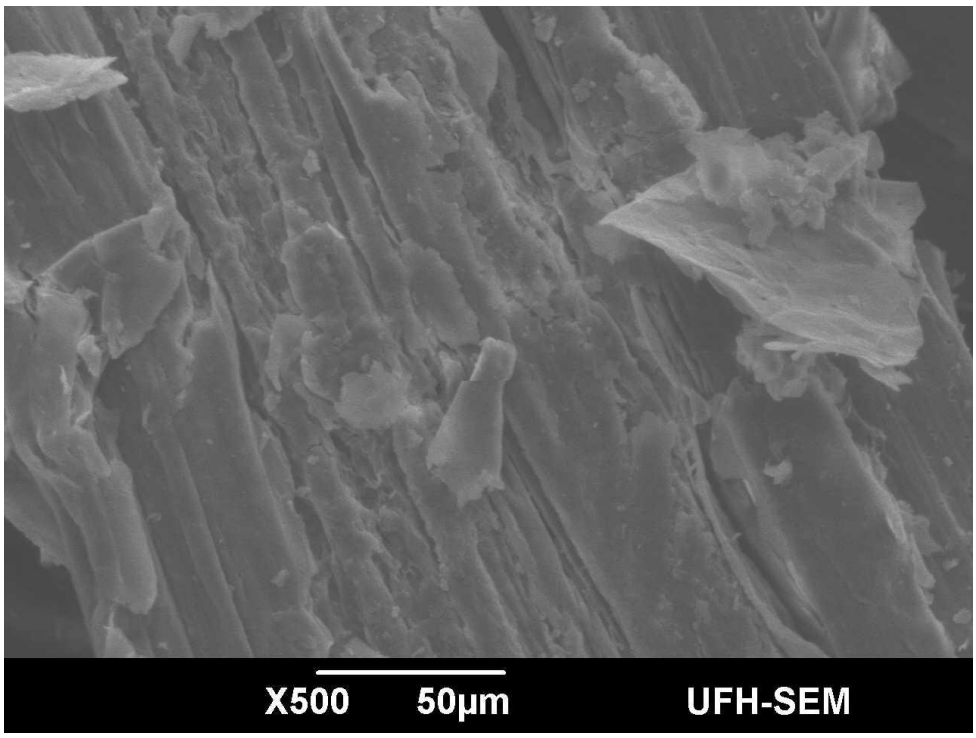
e)1.6 M sulphuric acid pre-treated SEM image



f)2 M sulphuric acid treated lignocellulose residue SM image



g)24 h peroxide pre-treated lignocellulose residue



h)48 h alkali-peroxide pre-treated residue

APPENDIX B EDS results

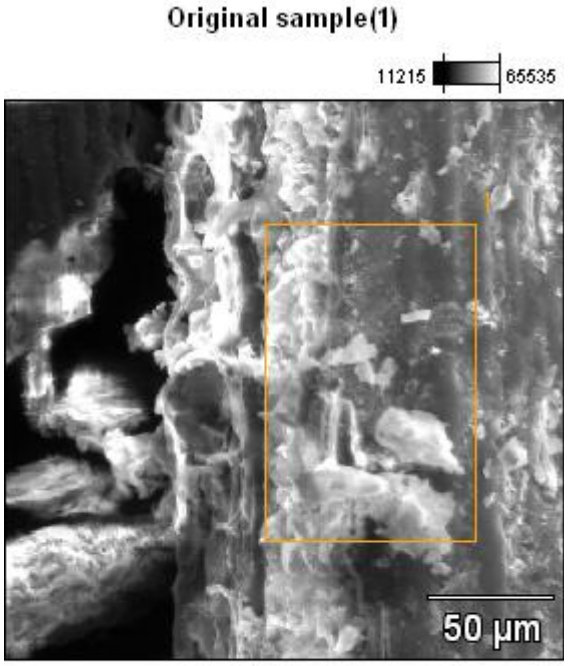
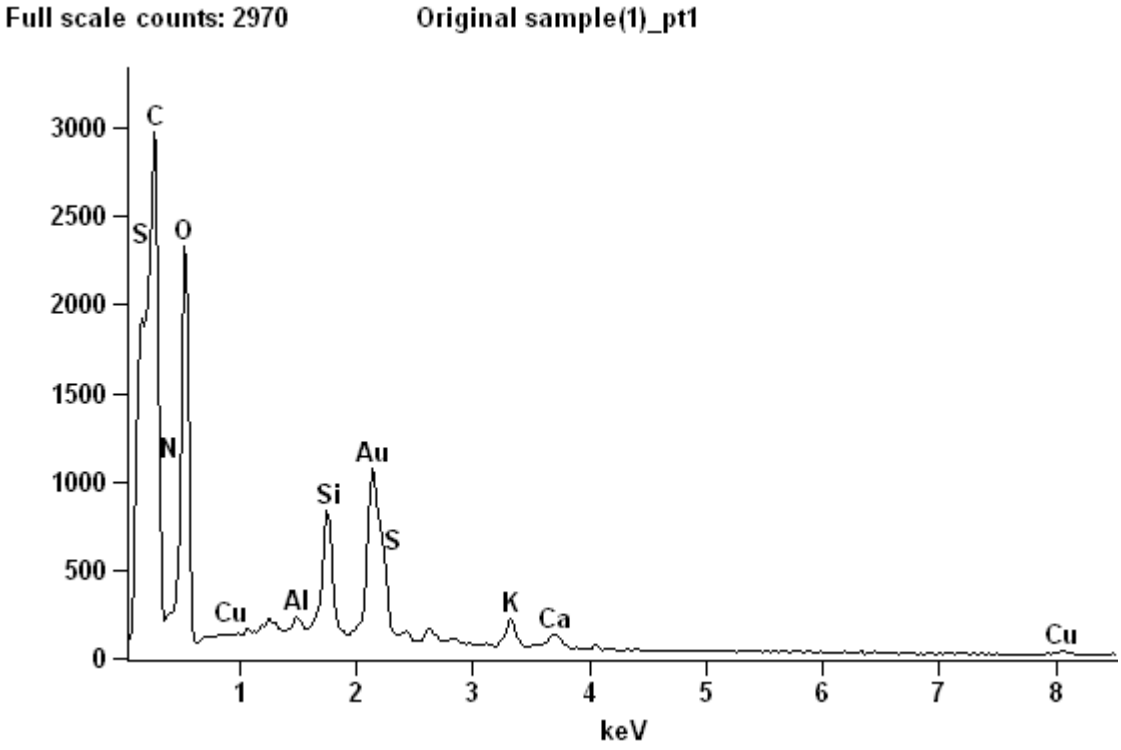


Image Name: Original sample (1)

Accelerating Voltage: 15.0 kV

Magnification: 500

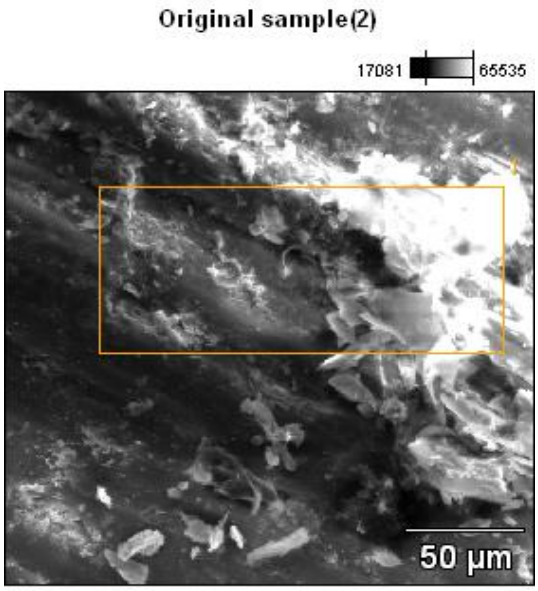


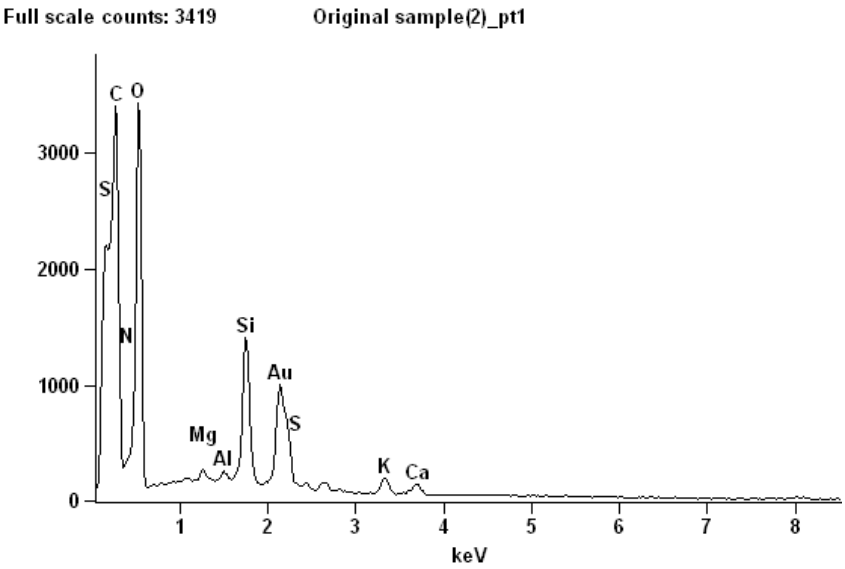
Filter fit Chi-squred value: 3.925 errors: ± 1 sigma

Correction method: Proza[Phi-Rho-Z}

Acc. Voltage: 15 Kv Take off angle: 35.7 deg

Element	Element wt %	Wt % error ±	Atom %	Atom % error ±	Compound formula	Compound Wt %
C	21.62	0.32	41.20	0.60	C	21.42
N	6.42	1.05	10.20	1.72	N	6.24
O	28.06	0.42	40.15	0.60	O	28.06
Al	0.24	0.03	0.20	0.03	Al	0.24
Si	2.62	0.04	2.14	0.03	Si	2.62
S	0.11	0.07	0.08	0.05	S	0.11
K	1.07	0.09	0.62	0.05	K	1.07
Ca	0.71	0.05	0.41	0.03	Ca	0.71
Cu	1.73	0.25	0.62	0.09	Cu	1.73
Au	37.61	2.89	4.37	0.34	Au	





Element	Element wt %	Wt % error ±	Atom %	Atom % error ±	Compound formula	Compound Wt %
C	22.55	0.32	36.62	0.52	C	22.55
N	7.90	1.09	11.00	1.52	N	7.90
O	37.32	0.46	45.49	0.56	O	37.32

Al	0.32	0,03	0.26	0.02	Al	0.32
Si	0.19	0.03	0.14	0.02	Si	0.19
S	4.49	0.07	3.12	0.05	S	4.49
K	0.03	0.03	0.02	0.04	K	0.03
Ca	1.00	0.08	0.50	0.04	Ca	1.00
Cu	0.70	0.04	0.34	0.02	Cu	0.70
Au	25.49	2.49	2.52	0.25	Au	25.49

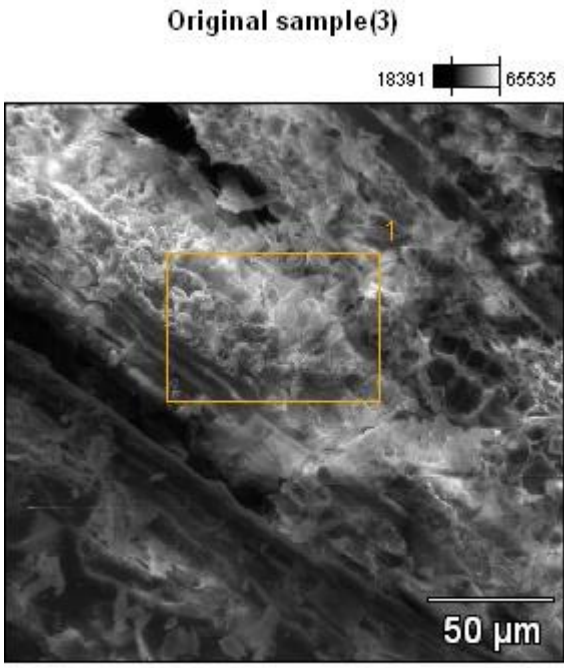
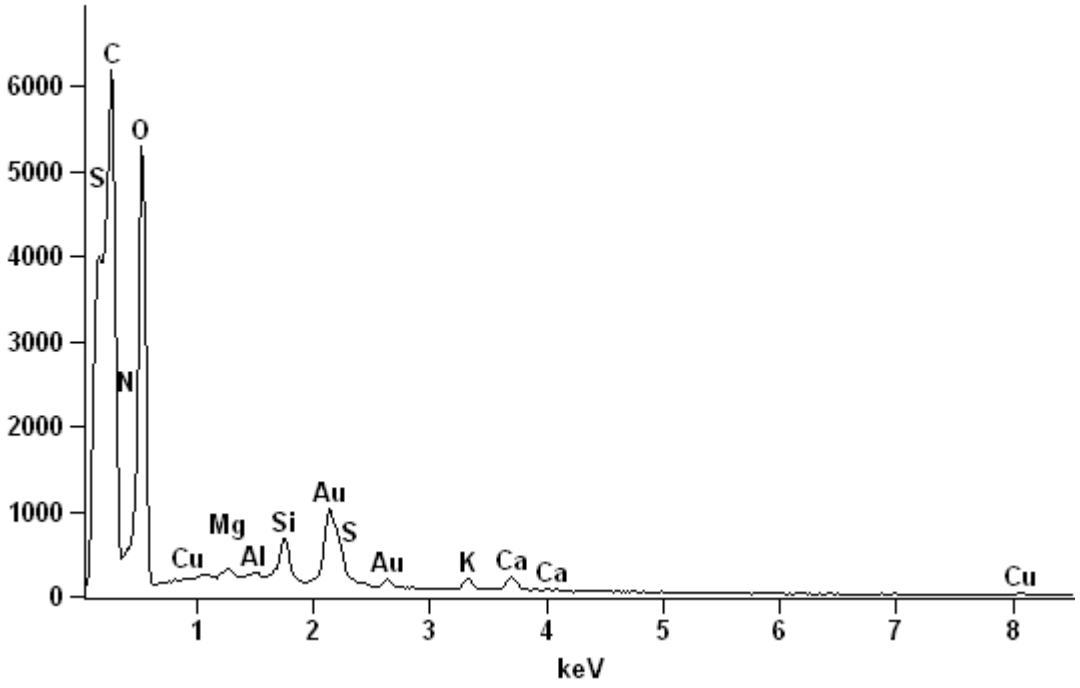


Image Name: Original sample (3)

Accelerating Voltage: 15.0 kV

Magnification: 500

Full scale counts: 6179 Original sample(3)_pt1



Thu Oct 04 11:02:07 2012
Filter Fit Chi-squared value: 4.425 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.7 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	25.77	+/-0.27	37.62	+/- 0.39	C	25.77
N K	9.42	+/-1.01	11.79	+/- 1.27	N	9.42
O K	42.53	+/-0.43	46.61	+/- 0.47	O	42.53
Mg K	0.32	+/-0.03	0.23	+/- 0.02	Mg	0.32
Al K	0.18	+/-0.02	0.12	+/- 0.02	Al	0.18
Si K	1.34	+/-0.03	0.84	+/- 0.02	Si	1.34
S K	0.41	+/-0.08	0.22	+/- 0.04	S	0.41
K K	0.61	+/-0.06	0.27	+/- 0.03	K	0.61
Ca K	0.94	+/-0.07	0.41	+/- 0.03	Ca	0.94
Cu K	1.31	+/-0.18	0.36	+/- 0.05	Cu	1.31

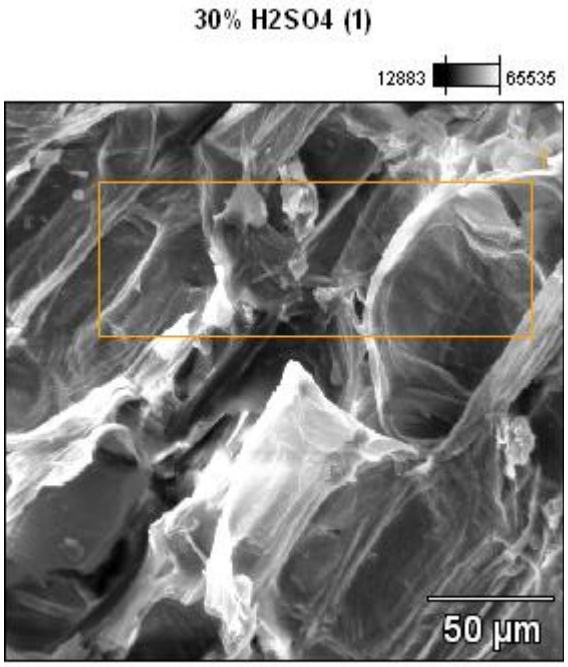
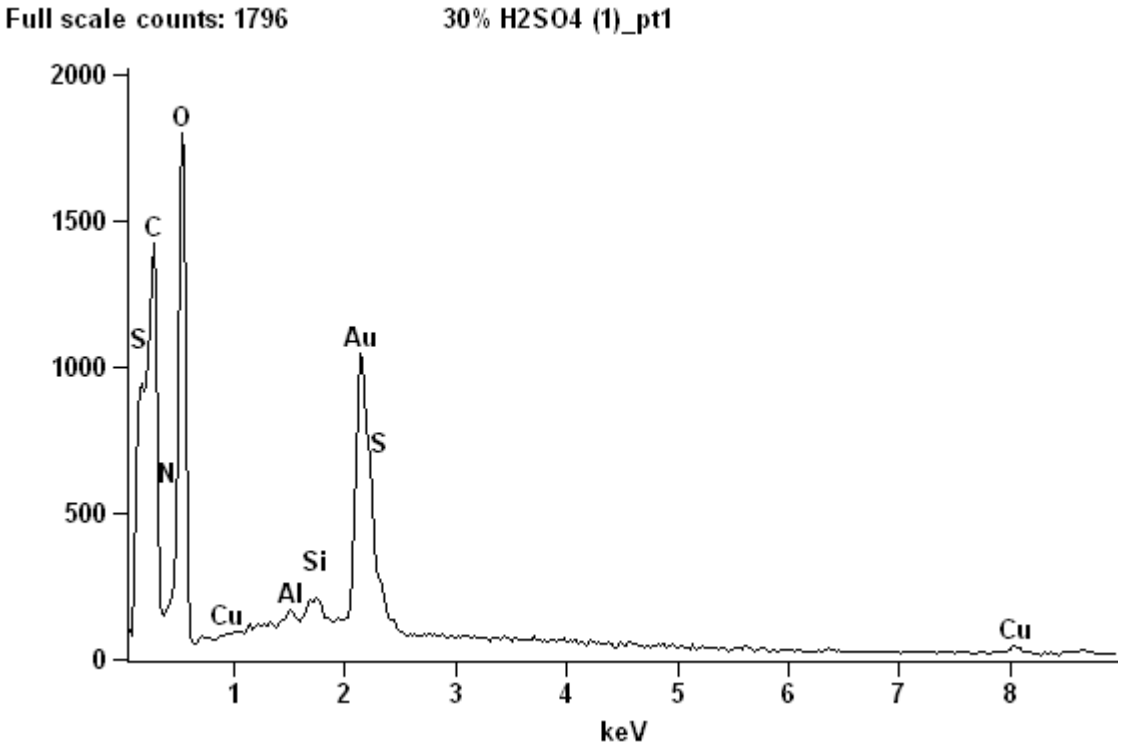


Image Name: 30% H2SO4 (1)

Accelerating Voltage: 15.0 kV

Magnification: 500



Thu Oct 04 10:44:58 2012
Filter Fit Chi-squared value: 1.787 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.6 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	14.36	+/-0.31	34.03	+/- 0.74	C	14.36
N K	4.99	+/-0.92	10.13	+/- 1.87	N	4.99
O K	25.51	+/-0.41	45.39	+/- 0.73	O	25.51
Al K	0.30	+/-0.05	0.32	+/- 0.05	Al	0.30
Si K	0.40	+/-0.03	0.40	+/- 0.04	Si	0.40
S K	1.39	+/-0.09	1.23	+/- 0.08	S	1.39
Cu K	2.73	+/-0.32	1.23	+/- 0.14	Cu	2.73
Au L	50.33	+/-5.34	7.27	+/- 0.77	Au	50.33
Total	100.00		100.00			100.00

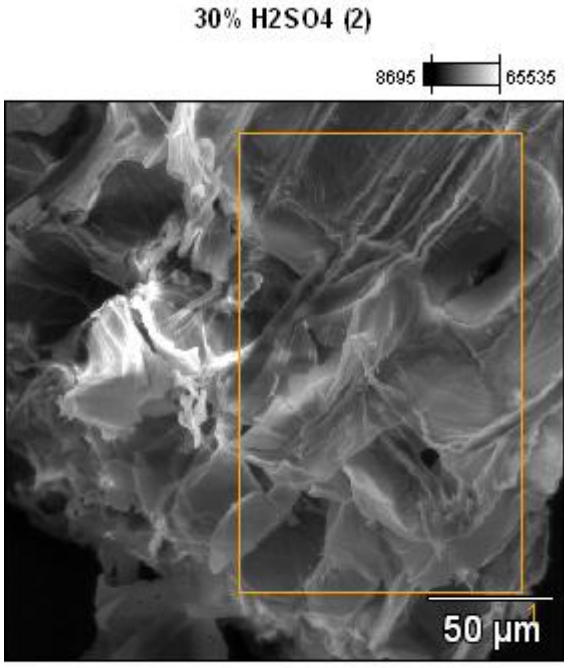
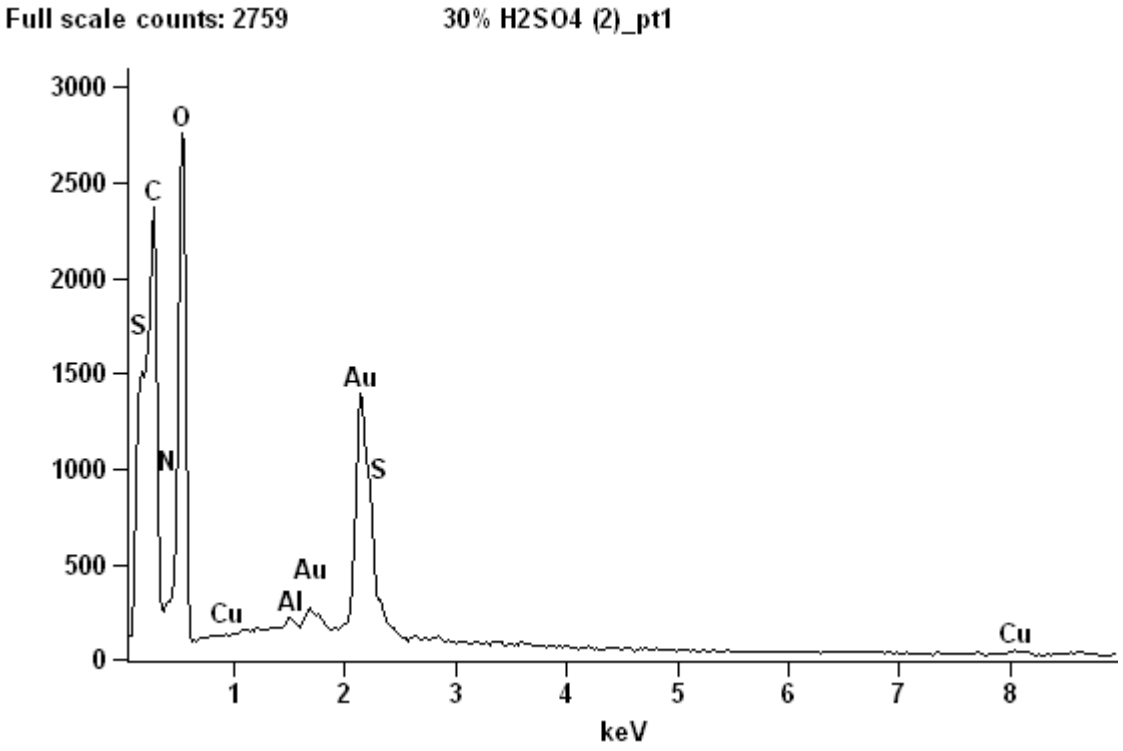


Image Name: 30% H2SO4 (2)

Accelerating Voltage: 15.0 kV

Magnification: 500


Full scale counts: 2759
30% H2SO4 (2)_pt1
3000
2500
2000
1500
1000
500
0
O
C
S
N
Au
S
Au
Al
Cu
Cu
1
2
3
4
5
6
7
8
keV

Thu Oct 04 10:47:15 2012
Filter Fit Chi-squared value: 2.640 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.7 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	16.46	+/-0.28	34.39	+/- 0.59	C	16.46
N K	6.27	+/-0.87	11.22	+/- 1.55	N	6.27
O K	30.00	+/-0.40	47.05	+/- 0.62	O	30.00
Al K	0.18	+/-0.03	0.17	+/- 0.03	Al	0.18
S K	0.91	+/-0.07	0.72	+/- 0.06	S	0.91
Cu K	2.14	+/-0.27	0.84	+/- 0.10	Cu	2.14
Au L	44.04	+/-3.11	5.61	+/- 0.40	Au	44.04
Total	100.00		100.00			100.00

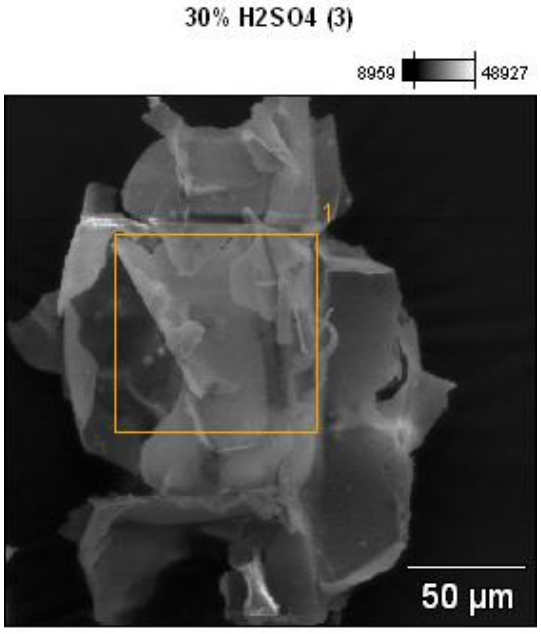
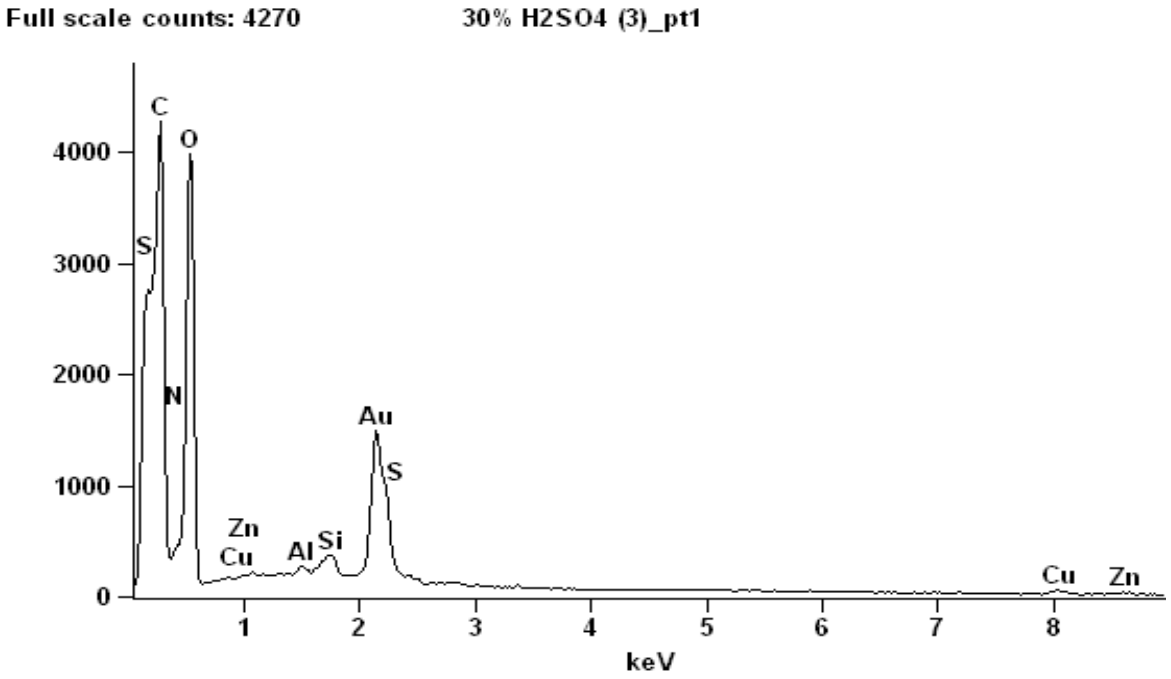


Image Name: 30% H2SO4 (3)

Accelerating Voltage: 15.0 kV



Thu Oct 04 10:50:25 2012
Filter Fit Chi-squared value: 2.768 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.7 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	21.94	+/-0.27	38.54	+/- 0.48	C	21.94
N K	7.43	+/-0.90	11.19	+/- 1.35	N	7.43
O K	34.08	+/-0.39	44.93	+/- 0.51	O	34.08
Al K	0.15	+/-0.02	0.12	+/- 0.02	Al	0.15
Si K	0.56	+/-0.03	0.42	+/- 0.02	Si	0.56
S K	0.28	+/-0.06	0.19	+/- 0.04	S	0.28
Cu K	2.07	+/-0.22	0.69	+/- 0.07	Cu	2.07
Zn K	1.61	+/-0.27	0.52	+/- 0.09	Zn	1.61
Au L	31.88	+/-3.63	3.41	+/- 0.39	Au	31.88
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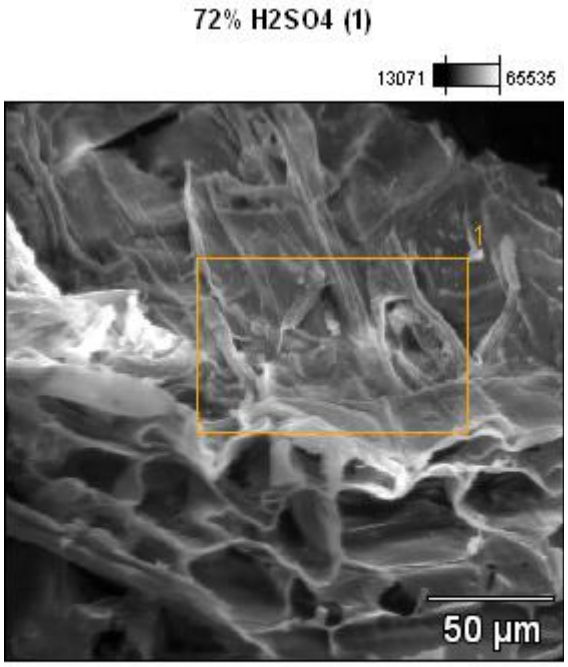
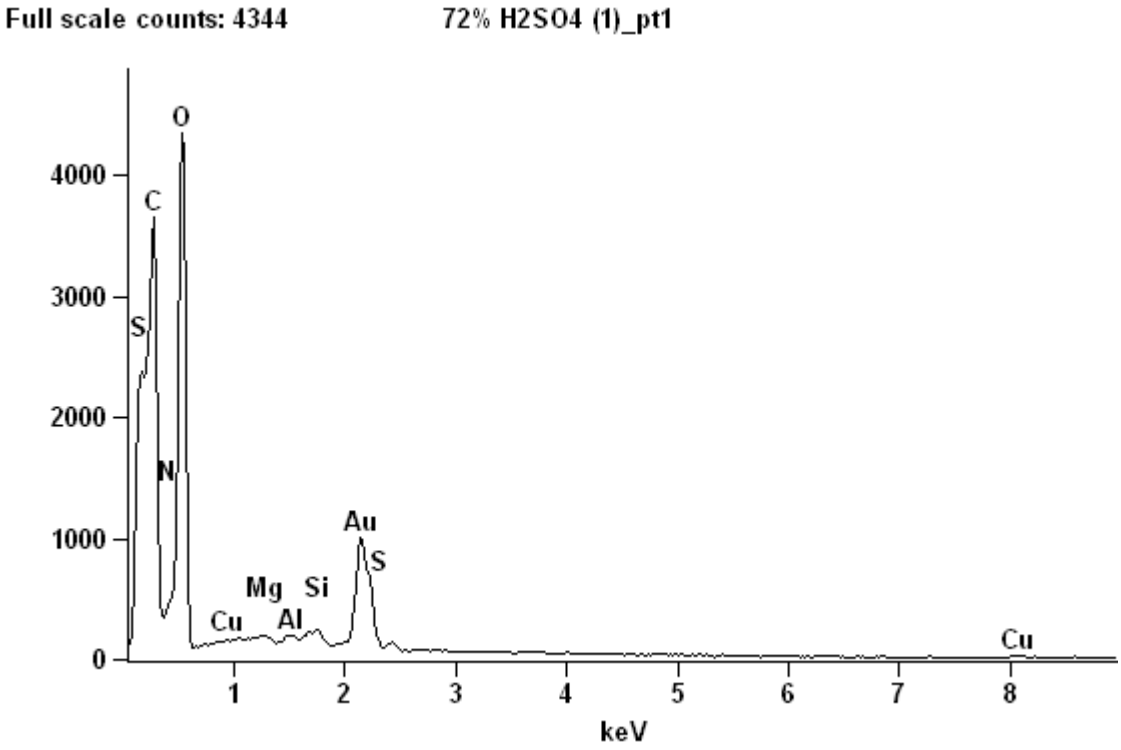


Image Name: 72% H2SO4 (1)

Accelerating Voltage: 15.0 kV

Magnification: 500



Thu Oct 04 10:35:40 2012
Filter Fit Chi-squared value: 2.772 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.6 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	20.40	+/-0.28	33.11	+/- 0.46	C	20.40
N K	8.53	+/-0.95	11.87	+/- 1.33	N	8.53
O K	42.06	+/-0.44	51.26	+/- 0.54	O	42.06
Mg K	0.22	+/-0.03	0.18	+/- 0.02	Mg	0.22
Al K	0.25	+/-0.05	0.18	+/- 0.03	Al	0.25
Si K	0.47	+/-0.05	0.33	+/- 0.03	Si	0.47
S K	0.00	+/-0.00	0.00	---	S	0.00
Cu K	1.40	+/-0.21	0.43	+/- 0.06	Cu	1.40
Au L	26.68	+/-2.35	2.64	+/- 0.23	Au	26.68
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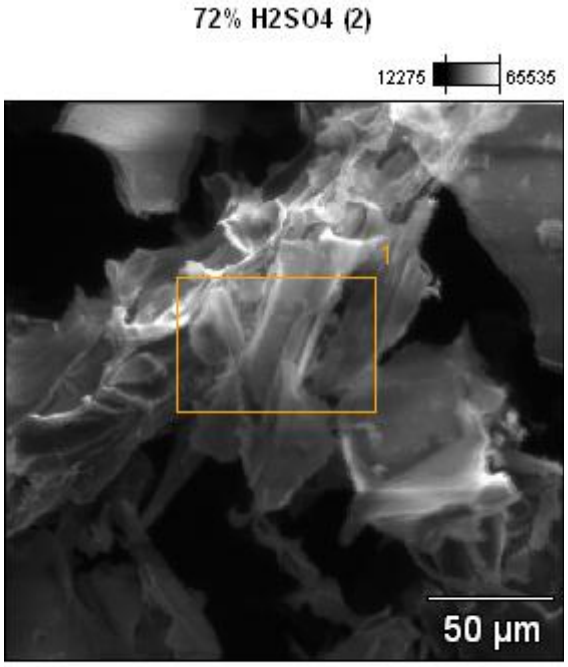
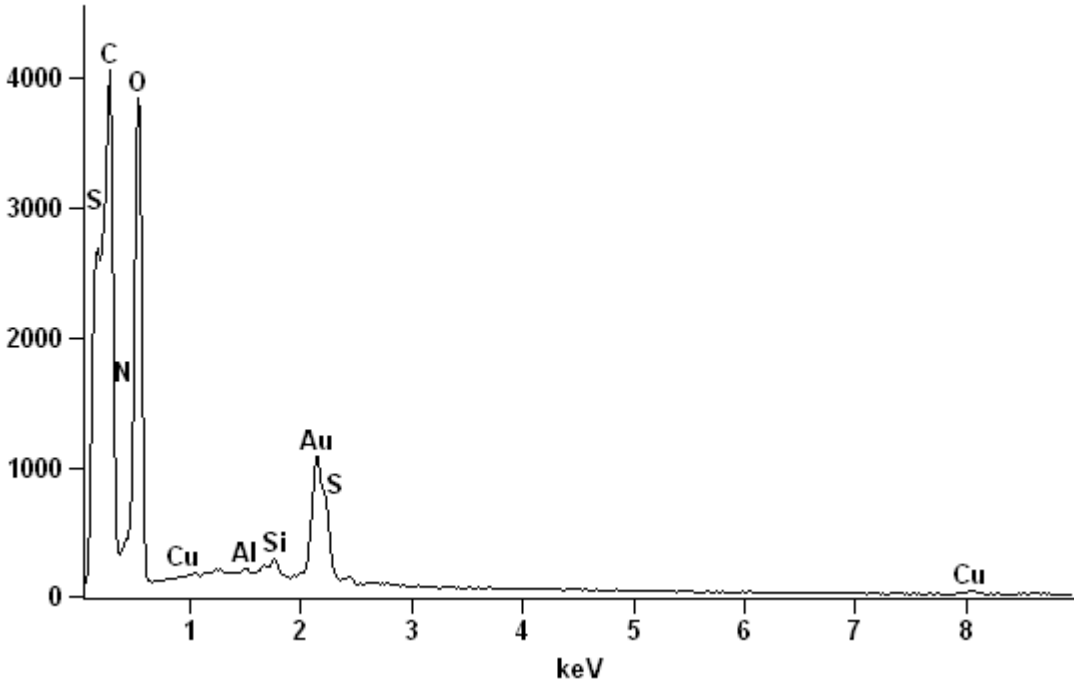


Image Name: 72% H2SO4 (2)

Accelerating Voltage: 15.0 kV

Magnification: 500

Full scale counts: 4052 72% H2SO4 (2)_pt1



Thu Oct 04 10:38:23 2012
Filter Fit Chi-squared value: 3.116 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.6 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	23.40	+/-0.30	37.54	+/- 0.47	C	23.40
N K	8.11	+/-1.06	11.16	+/- 1.46	N	8.11
O K	39.73	+/-0.45	47.84	+/- 0.54	O	39.73
Al K	0.14	+/-0.03	0.10	+/- 0.02	Al	0.14
Si K	0.39	+/-0.03	0.27	+/- 0.02	Si	0.39
S K	0.01	+/-0.01	0.01	+/- 0.04	S	0.01
Cu K	1.61	+/-0.24	0.49	+/- 0.07	Cu	1.61
Au L	26.61	+/-2.58	2.60	+/- 0.25	Au	26.61
Total	100.00		100.00			100.00

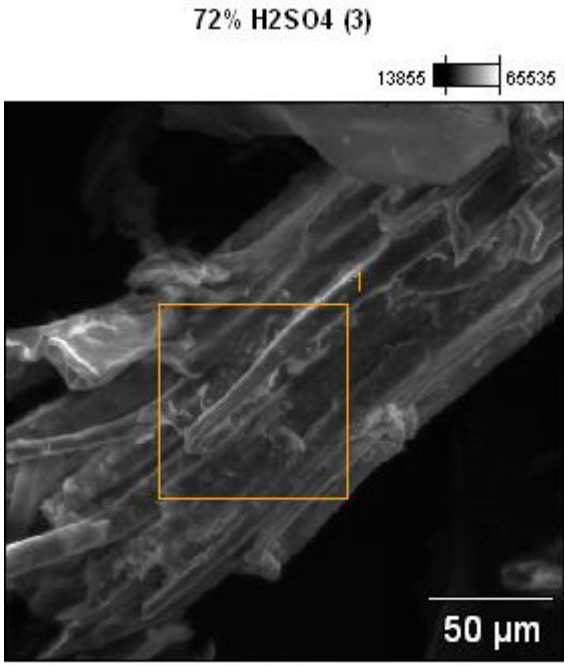
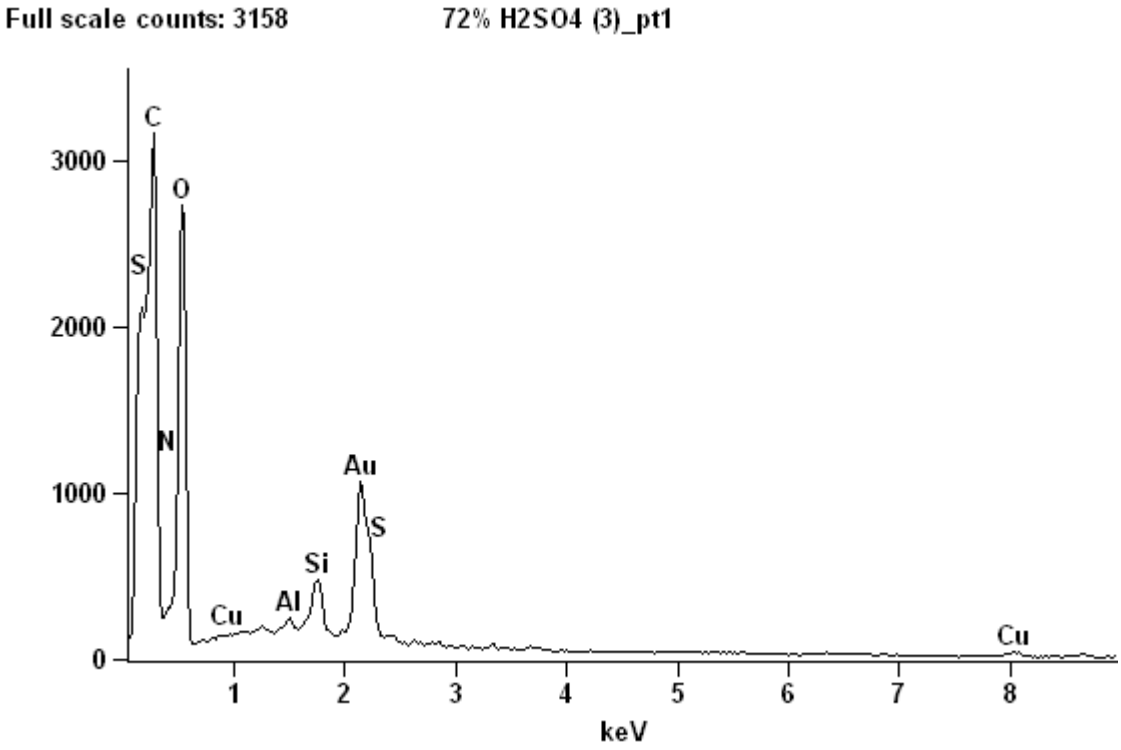


Image Name: 72% H2SO4 (3)

Accelerating Voltage: 15.0 kV

Magnification: 500



Thu Oct 04 10:40:36 2012
Filter Fit Chi-squared value: 2.500 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.6 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	22.14	+/-0.31	39.91	+/- 0.57	C	22.14
N K	6.84	+/-1.05	10.57	+/- 1.62	N	6.84
O K	32.32	+/-0.44	43.75	+/- 0.59	O	32.32
Al K	0.26	+/-0.03	0.21	+/- 0.02	Al	0.26
Si K	1.31	+/-0.04	1.01	+/- 0.03	Si	1.31
S K	0.02	+/-0.02	0.01	+/- 0.04	S	0.02
Cu K	2.00	+/-0.25	0.68	+/- 0.09	Cu	2.00
Au L	35.12	+/-2.84	3.86	+/- 0.31	Au	35.12
Total	100.00		100.00			100.00

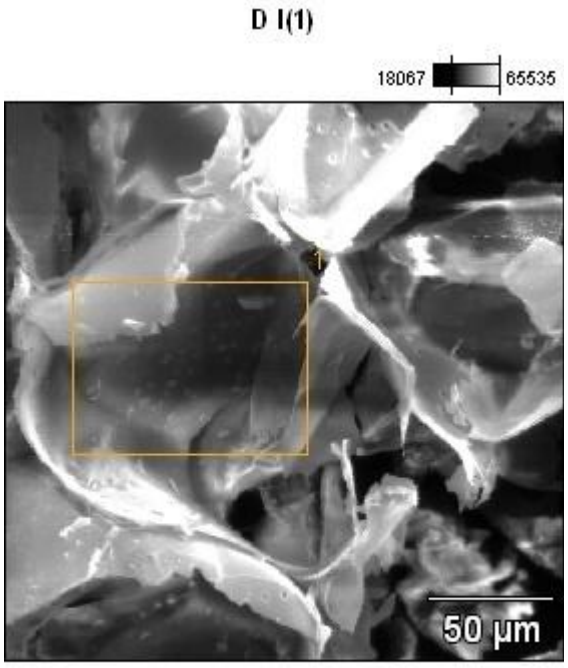
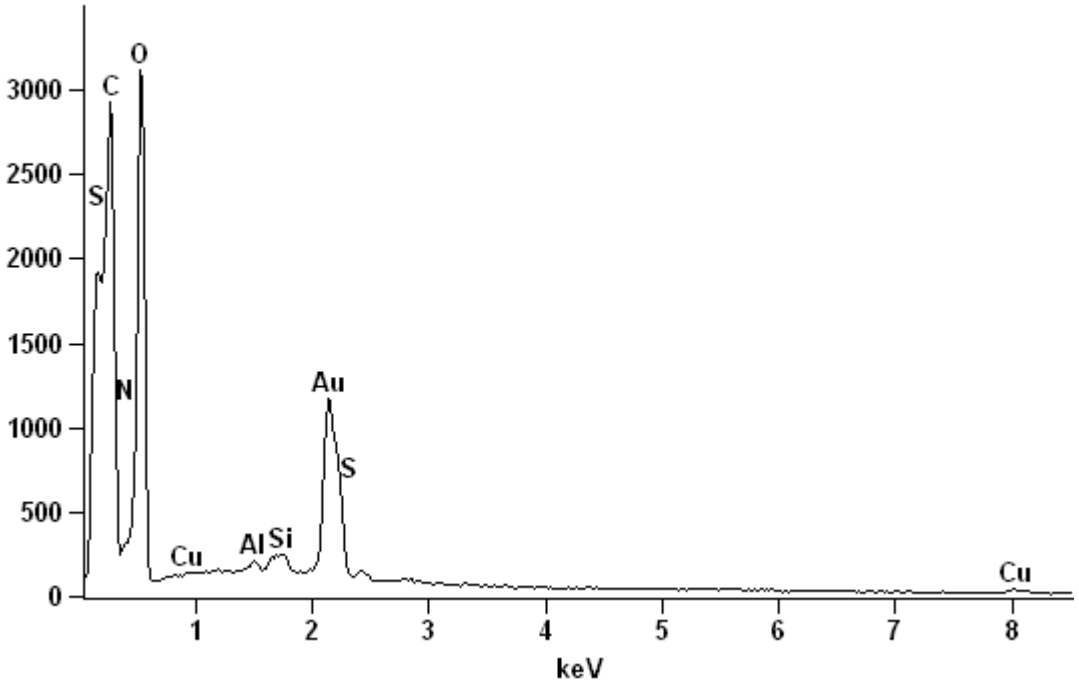


Image Name: D I (1)

Accelerating Voltage: 15.0 kV

Magnification: 500

Full scale counts: 3113 D I(1)_pt1



Thu Oct 04 11:07:12 2012
Filter Fit Chi-squared value: 2.123 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.7 deg

Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	20.13	+/-0.30	35.78	+/- 0.54	C	20.13
N K	7.60	+/-1.00	11.58	+/- 1.53	N	7.60
O K	35.56	+/-0.44	47.44	+/- 0.59	O	35.56
Al K	0.17	+/-0.03	0.14	+/- 0.02	Al	0.17
Si K	0.38	+/-0.03	0.29	+/- 0.02	Si	0.38
S K	0.00	+/-0.00	0.00	---	S	0.00
Cu K	1.86	+/-0.27	0.62	+/- 0.09	Cu	1.86
Zn K	2.01	+/-0.33	0.66	+/- 0.11	Zn	2.01
Au L	32.30	+/-4.51	3.50	+/- 0.49	Au	32.30
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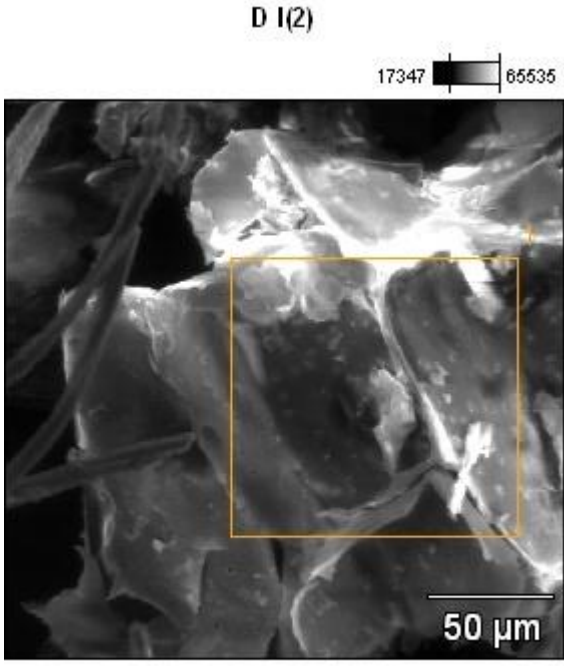
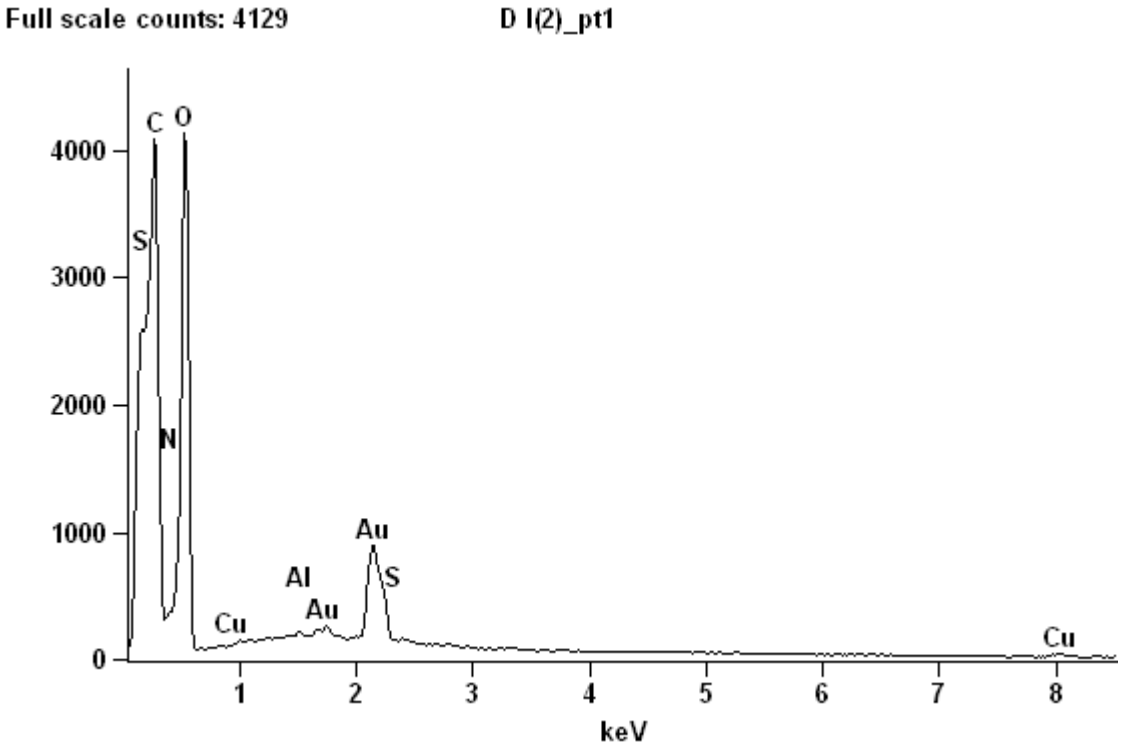


Image Name: D I (2)

Accelerating Voltage: 15.0 kV

Magnification: 500



Thu Oct 04 11:09:46 2012
Filter Fit Chi-squared value: 3.407 Errors:+/-1 Sigma
Correction Method: Proza (Phi-Rho-Z)
Acc.Voltage: 15.0 kV Take Off Angle: 35.7 deg

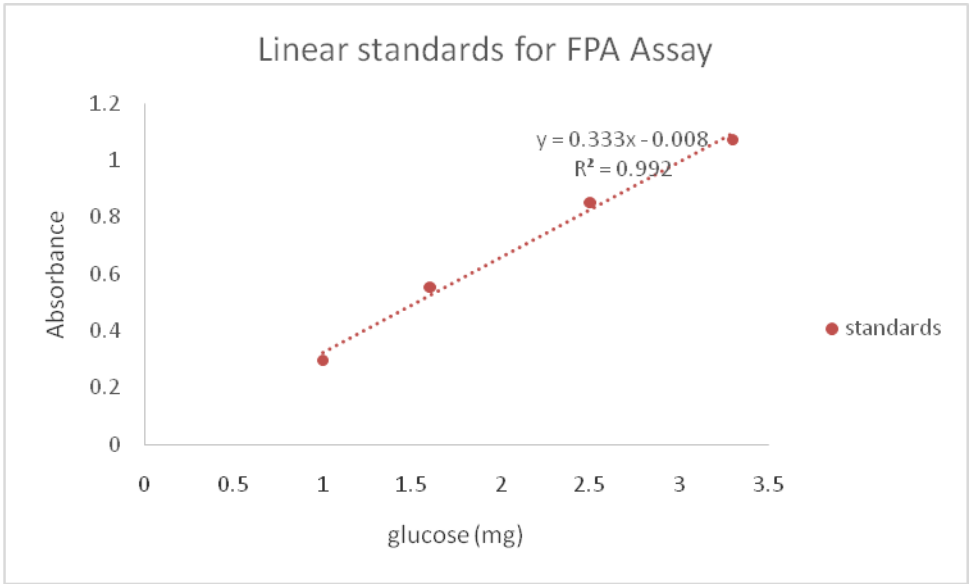
Element Line	Element Wt.%	Wt.% Error	Atom %	Atom % Error	Compnd Formula	Compnd Wt.%
C K	23.11	+/-0.29	35.91	+/- 0.45	C	23.11
N K	8.50	+/-1.05	11.32	+/- 1.40	N	8.50
O K	42.74	+/-0.45	49.85	+/- 0.53	O	42.74
Al K	0.18	+/-0.03	0.12	+/- 0.02	Al	0.18
S K	0.06	+/-0.06	0.03	+/- 0.03	S	0.06
Cu K	1.82	+/-0.26	0.53	+/- 0.08	Cu	1.82
Au L	23.60	+/-2.63	2.24	+/- 0.25	Au	23.60
	---		---			---
Total	100.00		100.00			100.00

Annexure C

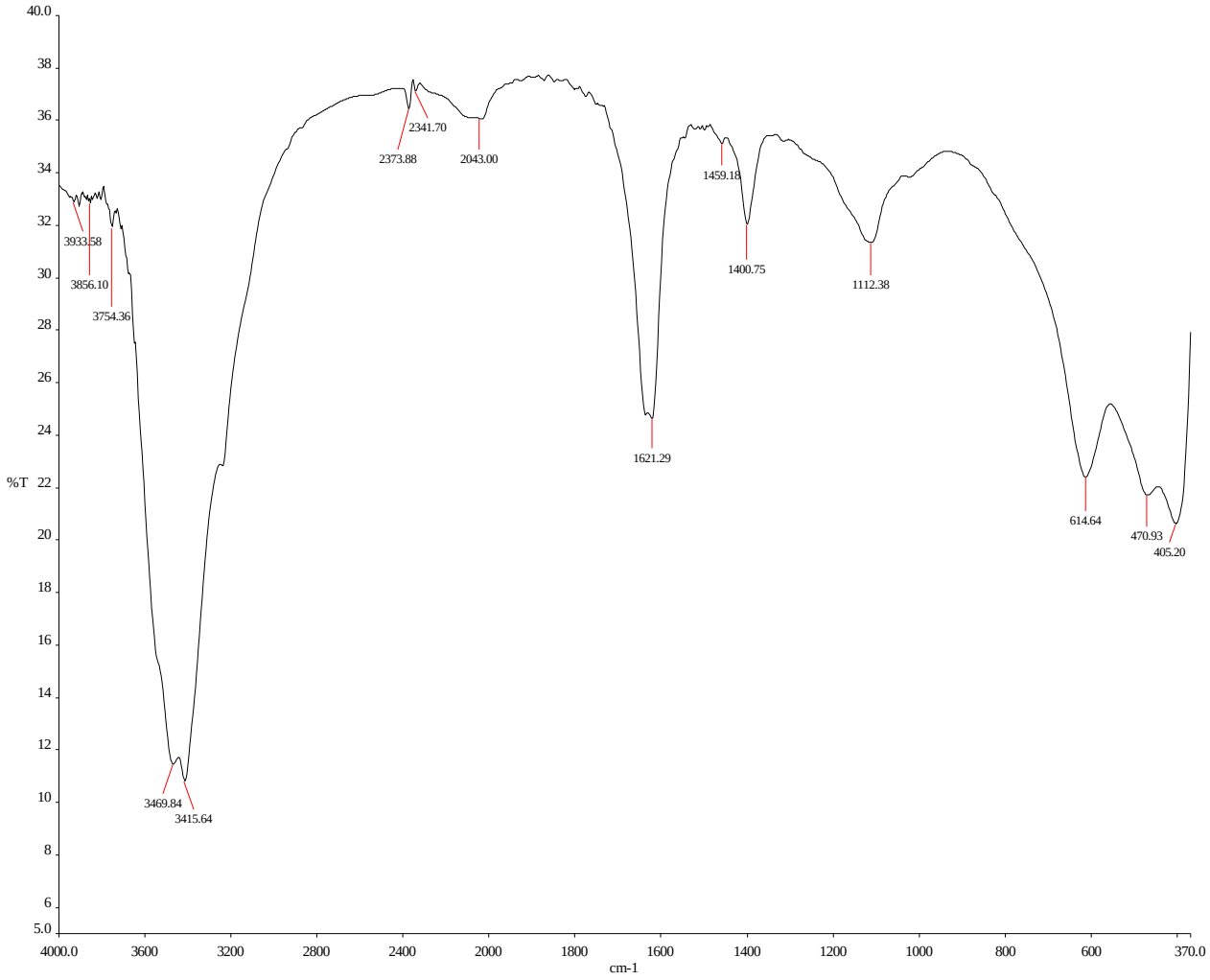
Crystallinity Indices

Material/treatment	**Crystallinity Index (%)**
Alkali Peroxide	
Raw	28.0
24 h	45.6
48 h	46.4
Conc Acid treatment	
30 % acid	31.7
72 % acid	26.0
Dilute acid treatment	
1.6 M acid	35.7
2 M acid	32.2

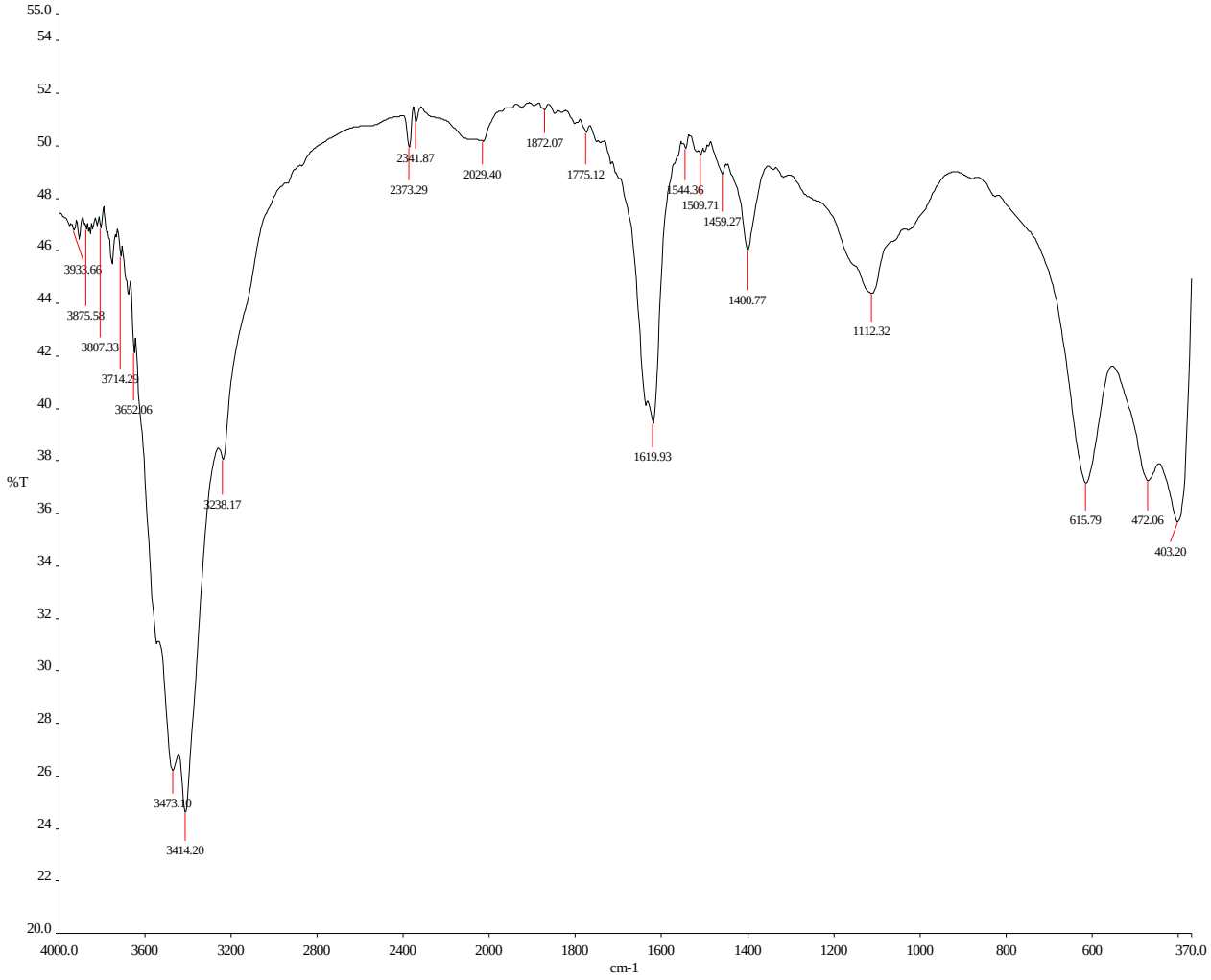
FPA assay graph



Annexure D: FTIR Results



72%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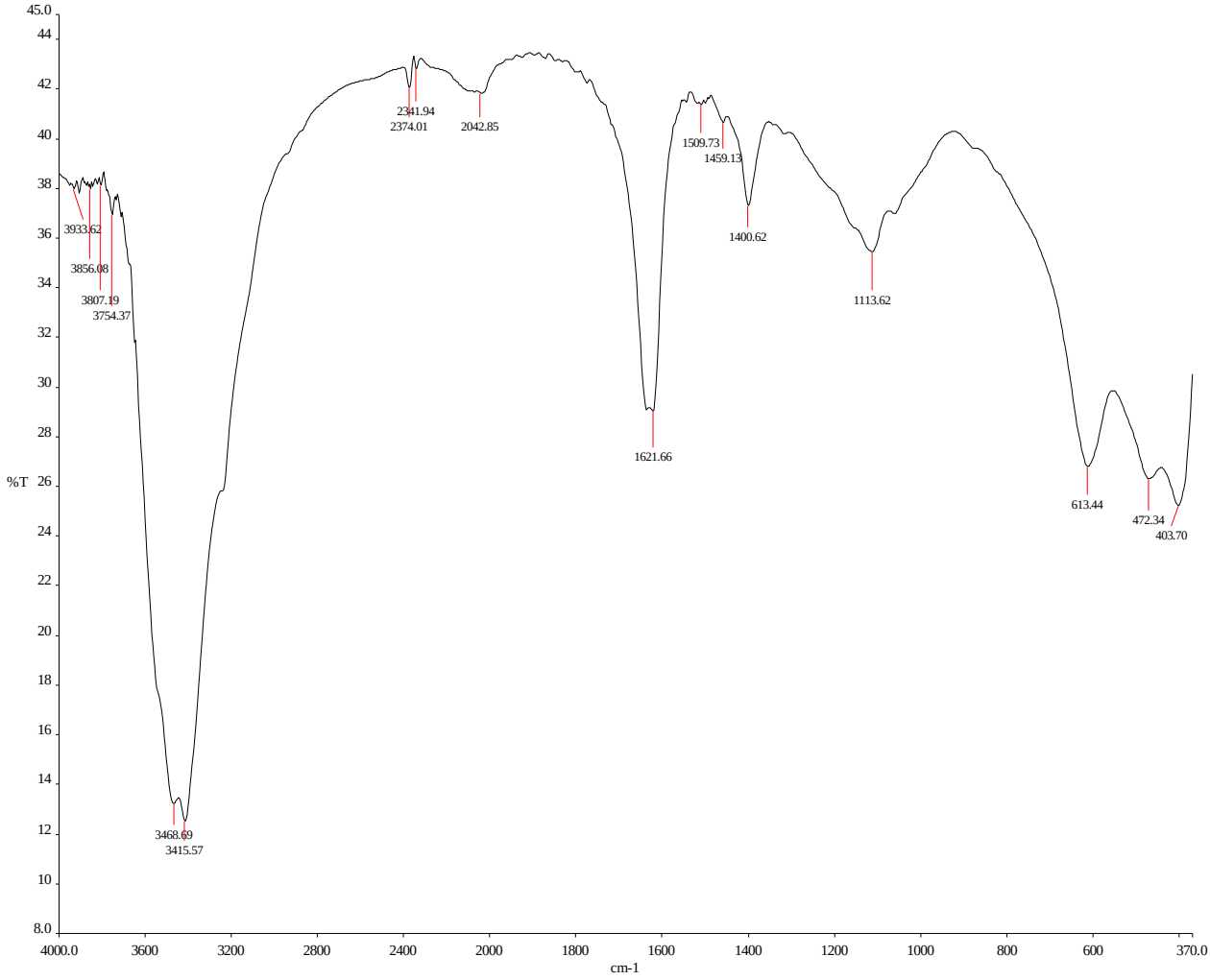
54
52
50
48
46
44
42
40
38
36
34
32
30
28
26
24
22
20.0
%T
4000.0
3600
3200
2800
2400
2000
1800
1600
1400
1200
1000
800
600
370.0
cm-1
3933.66
3875.58
3807.33
3714.29
3652.06
3473.10
3414.20
3238.17
2373.29
2341.87
2029.40
1872.07
1775.12
1619.93
1544.36
1509.71
1459.27
1400.77
1112.32
615.79
472.06
403.20

32%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3600
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1600
1400
1200
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800
600
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cm-1
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3856.08
3807.19
3754.37
3468.69
3415.57
2341.94
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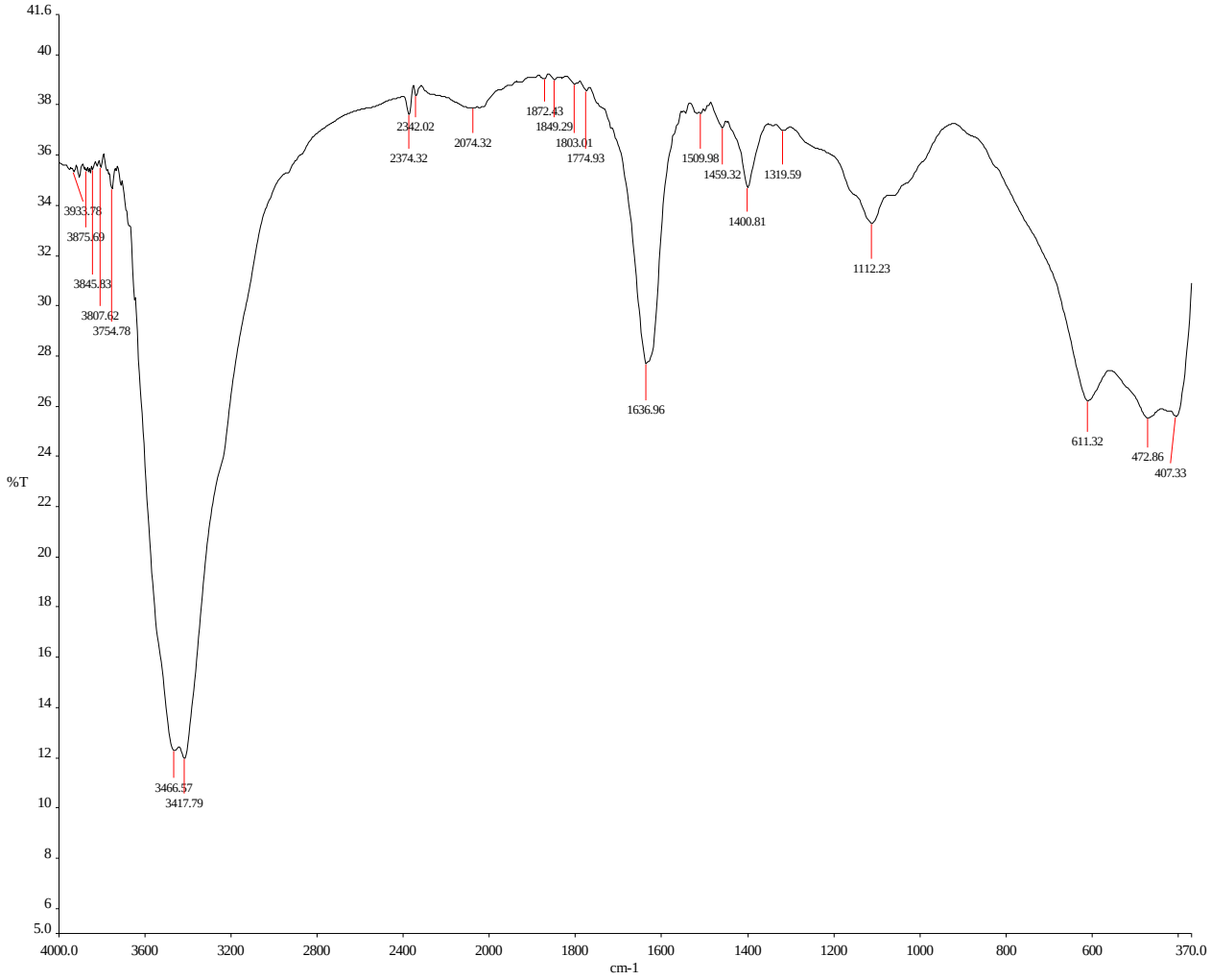
1113.62
613.44
472.34
403.70

1.6M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4000.0
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3875.69
3845.83
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2074.32
1872.43
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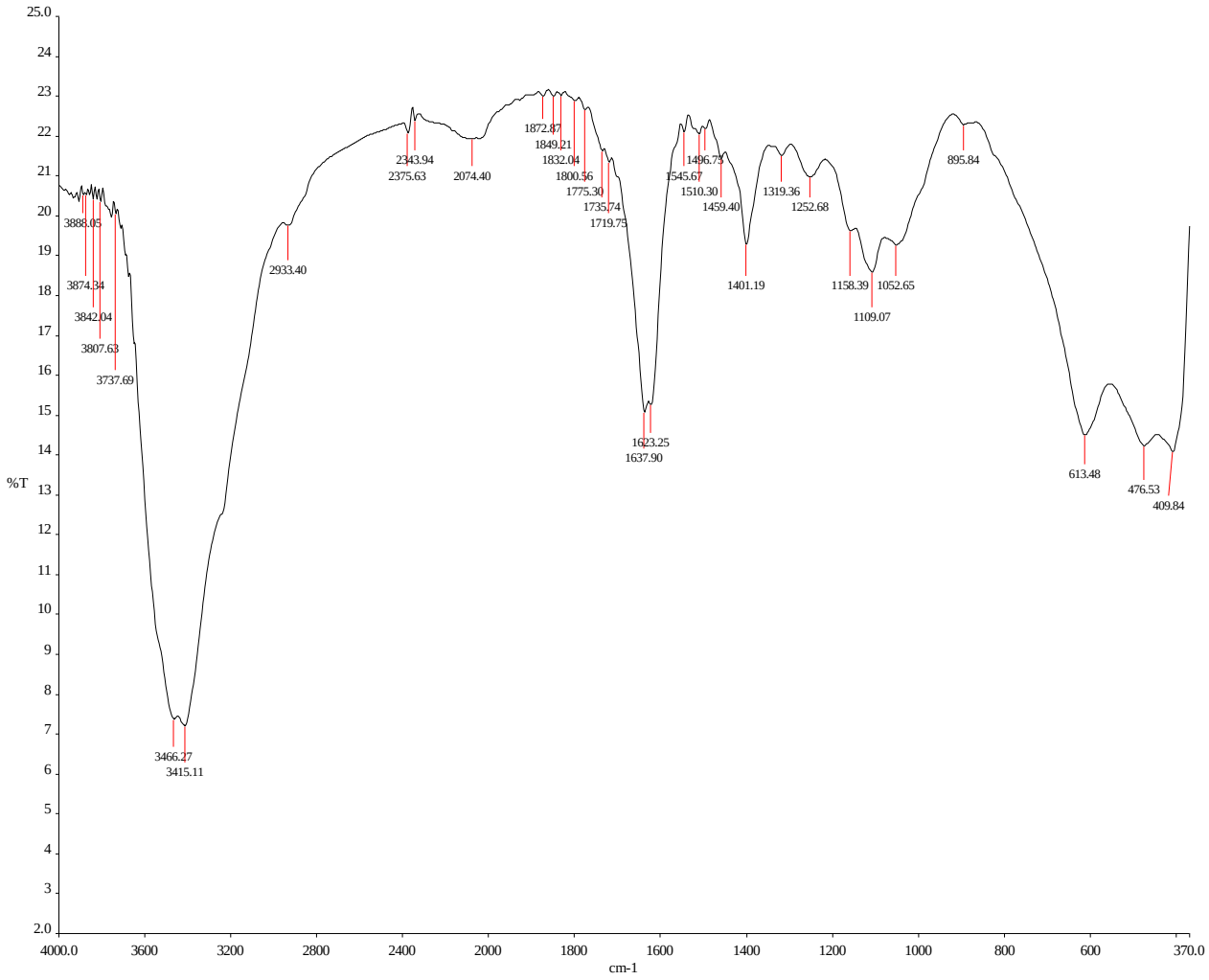
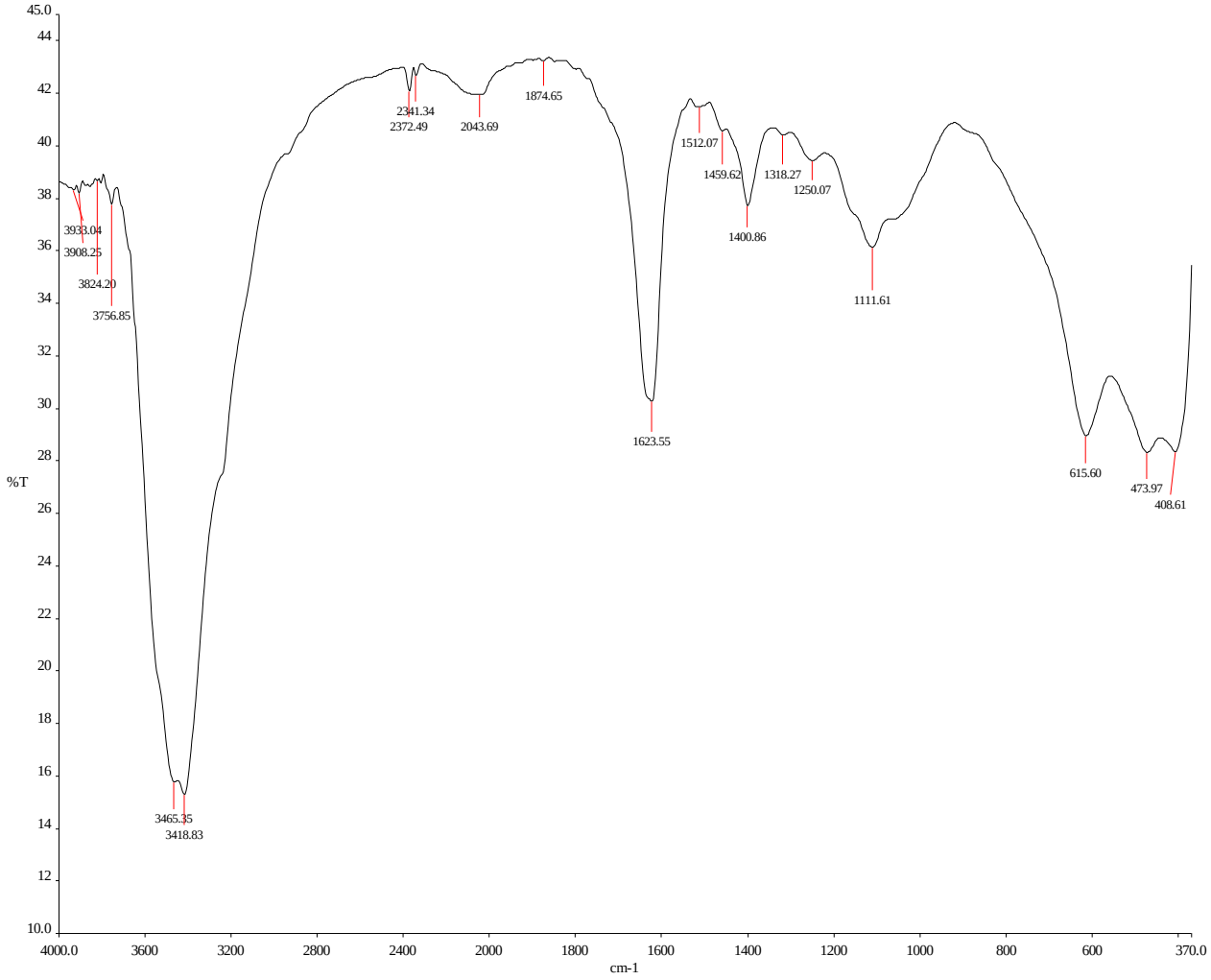
472.86
407.33

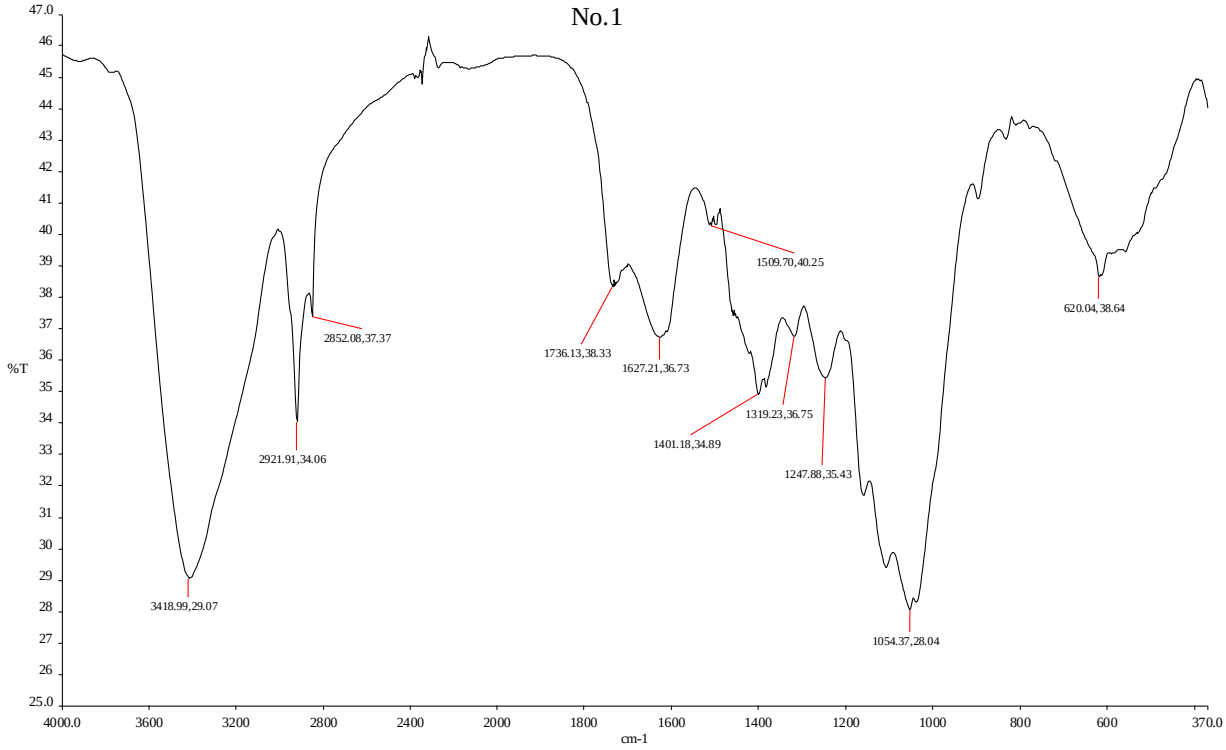
2M



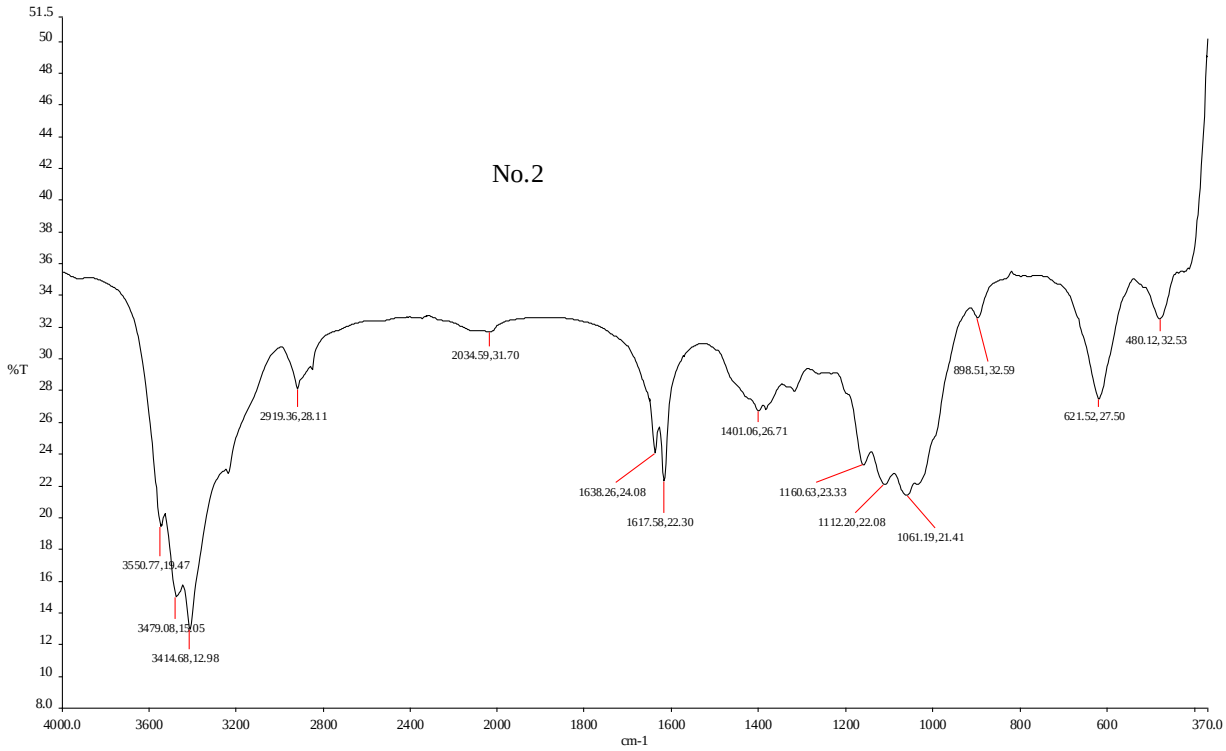
Deionised water



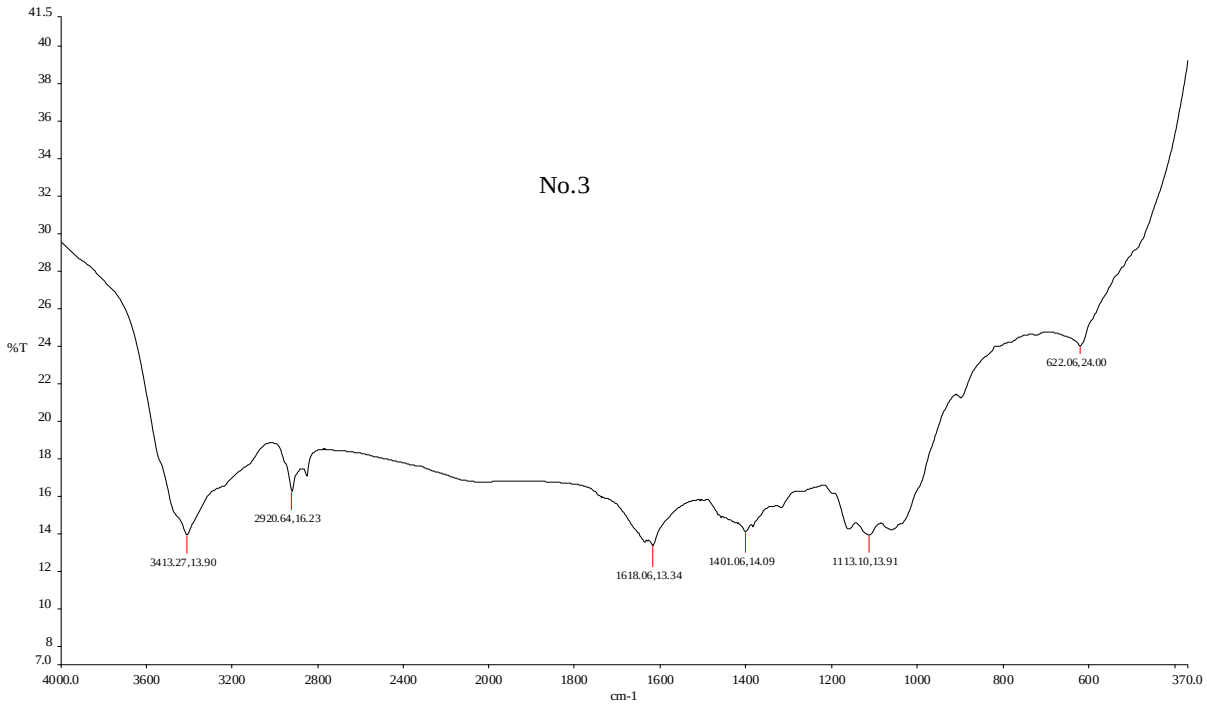
Untreated raw



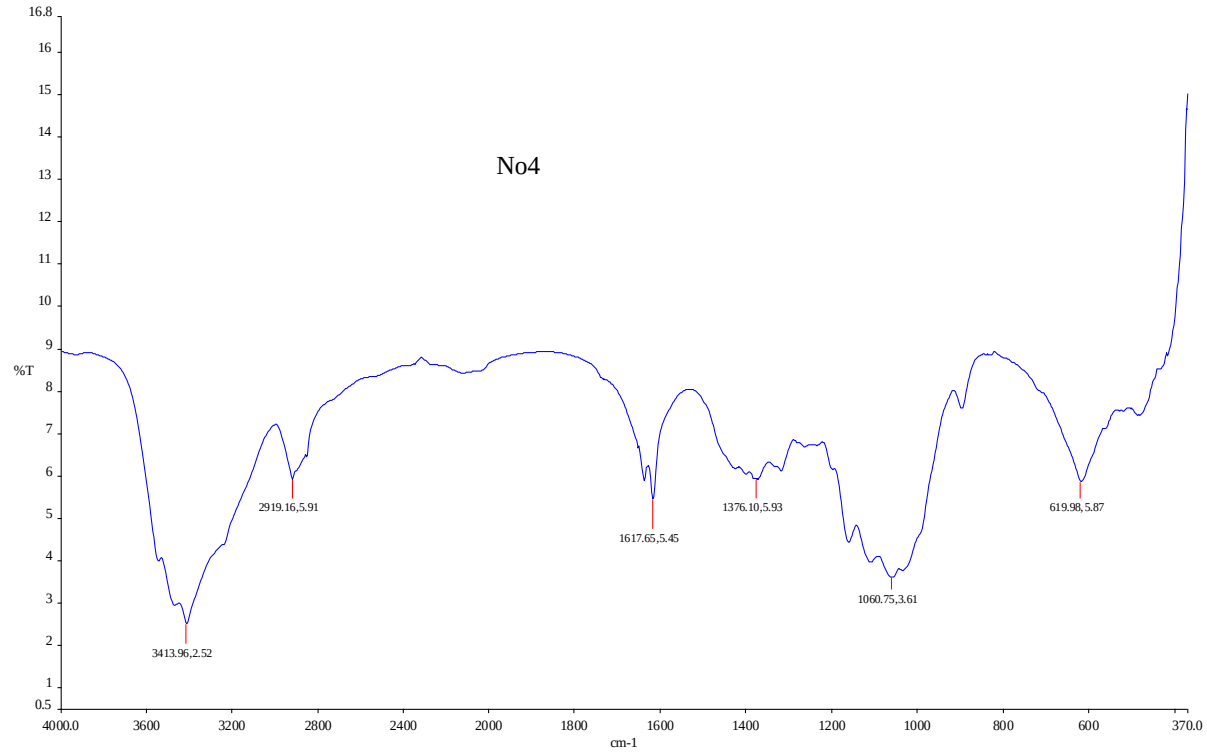
No 1: 0.5 % peroxide treated lignocellulose



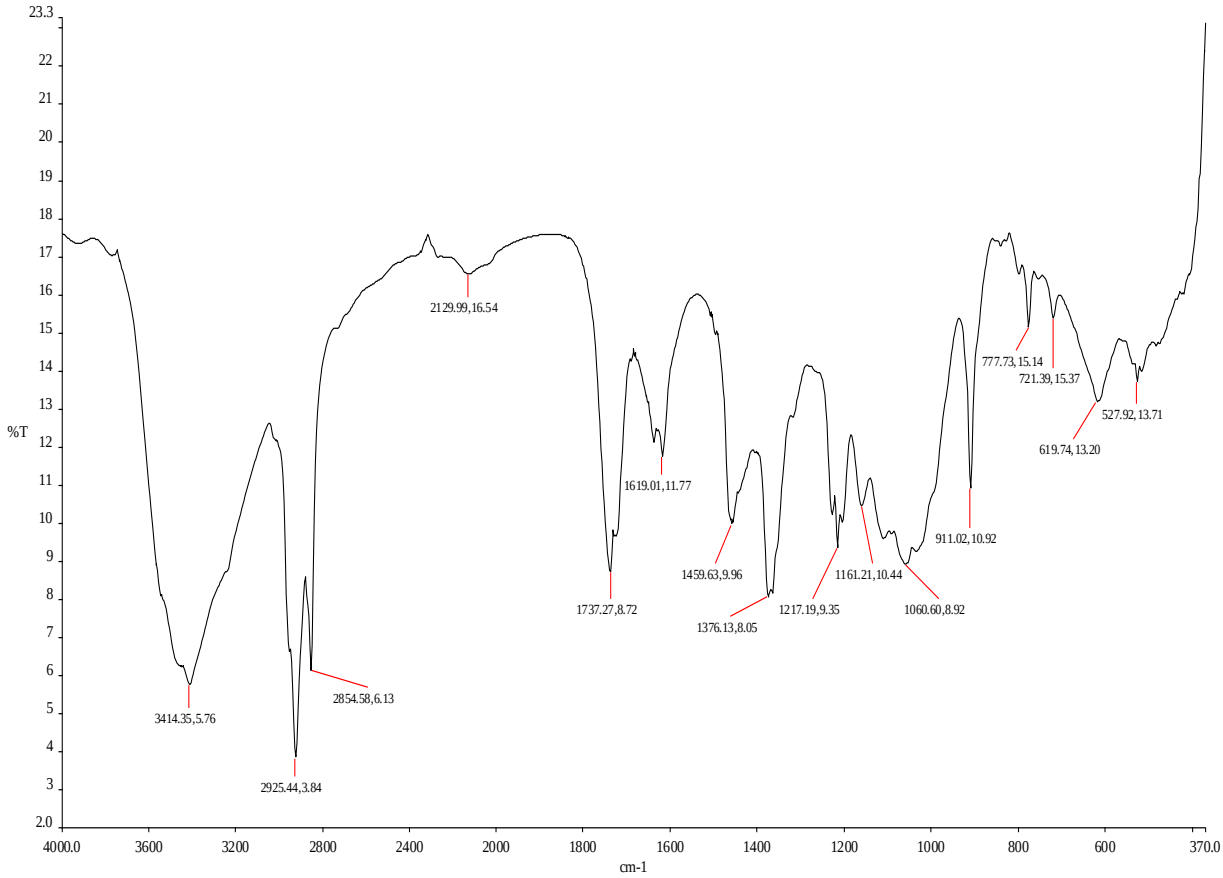
No 2: 3 % alkali peroxide treated lignocellulose



No 3 - 5% Peroxide treated lignocellulose



7 % peroxide treated lignocellulose



Raw untreated lignocellulosic material

The table below shows peak assignment (Silverstein et al., 1981, Adapa et al., 2011)

Wavenumbers (cm^{-1})	Peak assignment
3800 - 2800	3414 cm^{-1}O – H stretch, intermolecular hydrogen bonding. 2854 and 2925 - C – H stretch
1900 – 1500	1737 aromatic ring stretch (typical lignin) 1619 –very strong aromatic ring stretch, aromatic –C – O stretch. C = C, C = O, C = N , aromatic skeletal vibration
1500 – 1300	1459 – C – H deformation, 1376 –weak C – O stretching,
1300 – 1100	1161 – glycosydic linkage, C – O – C – Hemicellulose ring vibrational stretching, 1717 – cellulose– C – O stretch
1050 – 1200	1060 – β- polysaccharide, 1045- C – OH bending, 1035 – C – O in ether , C= C and C – C – O vibrational stretching

Annexure E Fermentation results

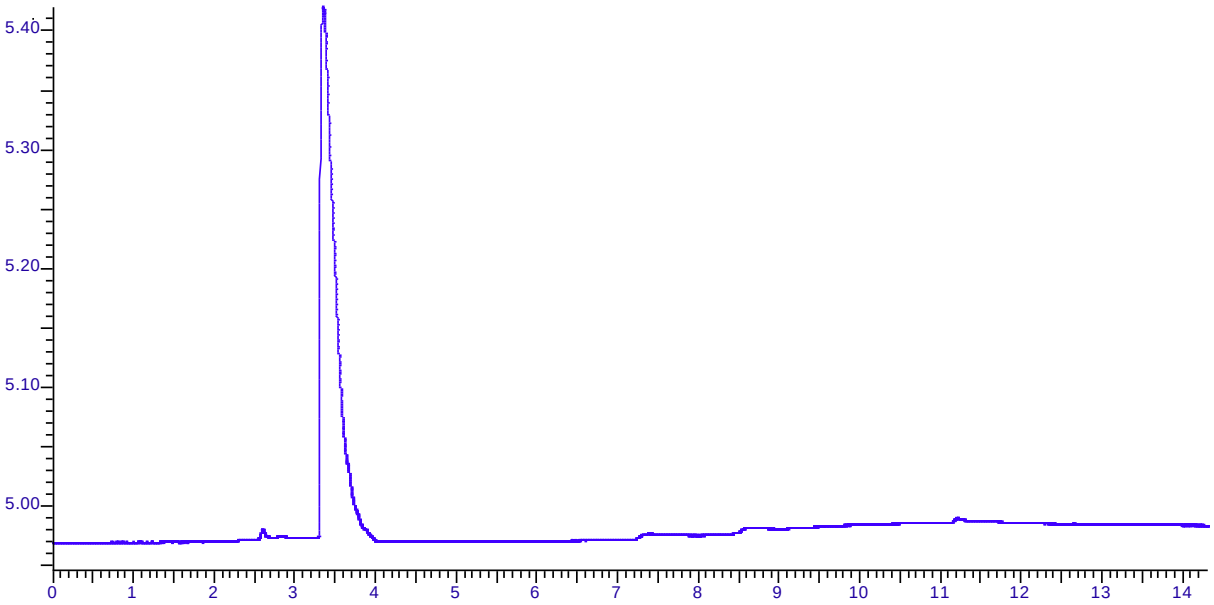


Fig 1: Bioethanol obtained from enzyme hydrolyzates after 24 h of fermentation

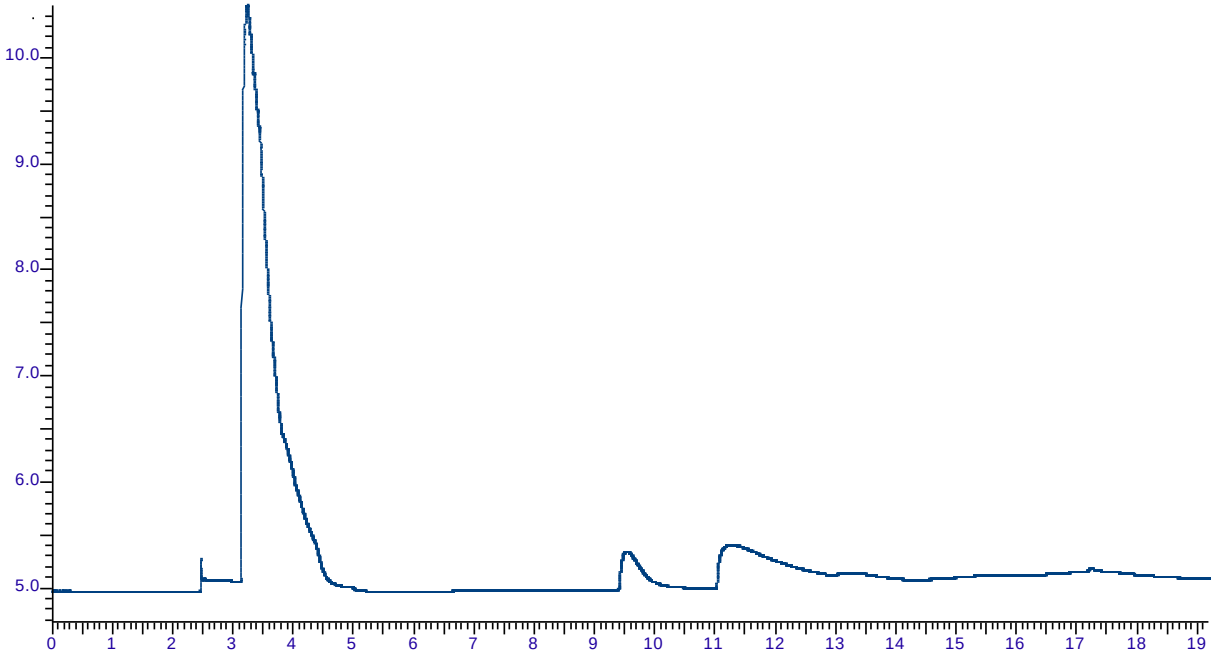


Fig 2: Enzyme treated –bioethanol content after 72h

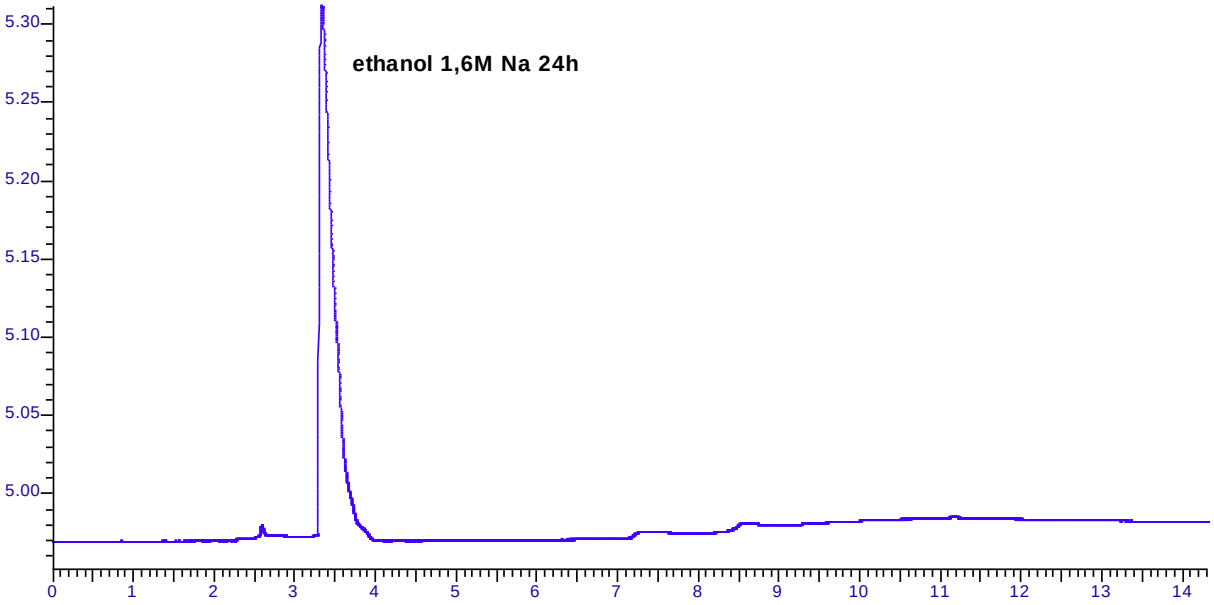


Fig 3: Bioethanol from 1.6 M sulphuric acid treated lignocellulose hydrolyzates after 24 h fermentation

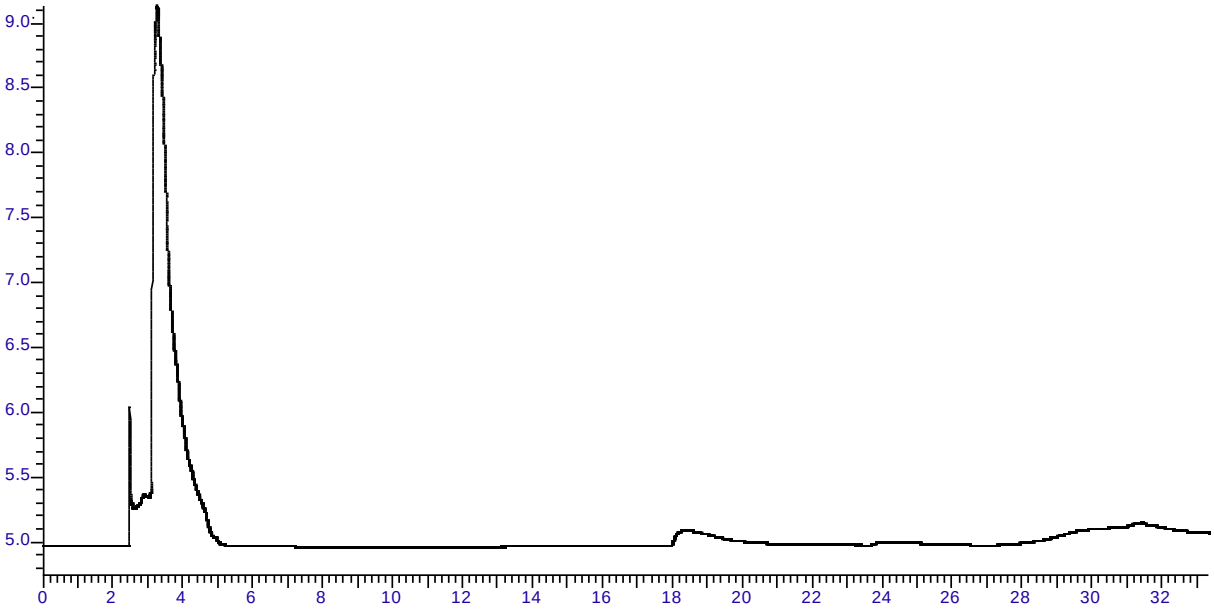


Fig 4: Bioethanol from 1.6 M treated hydrolyzates after 72 h fermentation

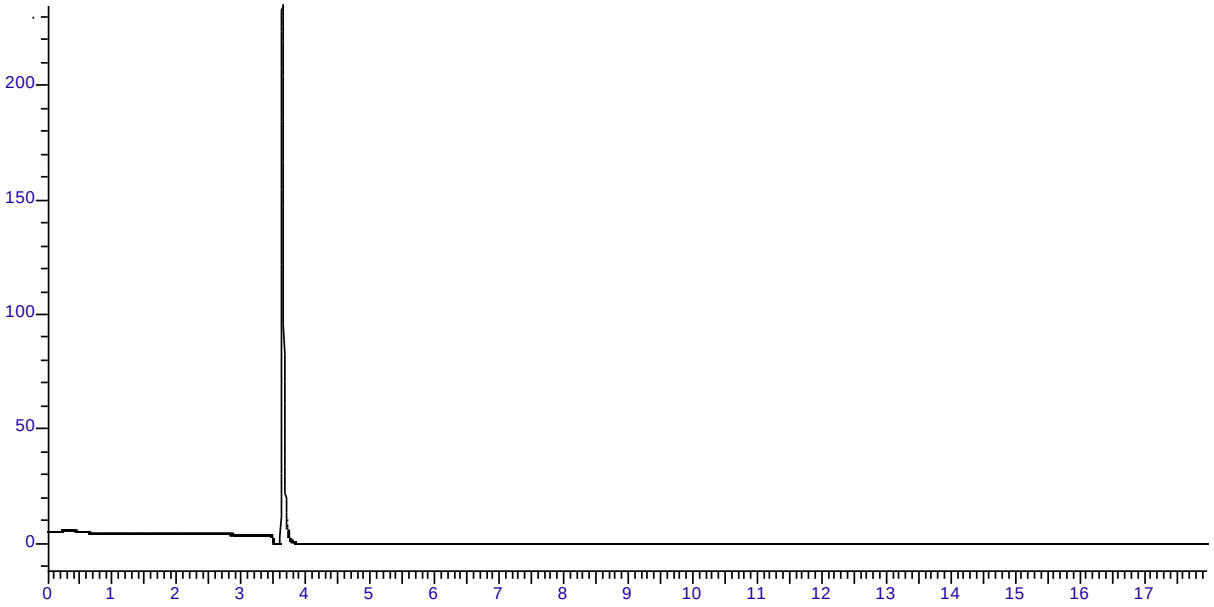


Fig 5: Pure ethanol control