Ultrasonic-assisted lignocellulose pretreatment of amaranth stem for bioethanol production

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It all starts here ™

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ABSTRACT

The depletion of fossil fuels, increasing energy demands in the world, and the effects associated with global warming has led to a search for alternative energy forms that are renewable such as bioethanol. Bioethanol produced from biomass such as a plant or organic waste could help reduce the production of carbon dioxide (CO_2). First generation ethanol which is derived from food crops can offer some CO_2 reduction benefits, but has the disadvantage of competing with food crops, which limits the production of first generation ethanol. Second generation ethanol offers the potential of providing novel biofuels. Second generation bioethanol is produced by breaking down the lignocellulose plant structure into fermentable sugars, which can further be fermented into ethanol. However, one major drawback in bioethanol production is lower ethanol yields due to ineffective pretreatment.

The aim of this study was to assess the effectiveness of ultrasonic pretreatment in combination with acid and alkali treatment to liberate fermentable sugars from amaranth lignocellulose for ethanol production. High Performance Liquid Chromatography (HPLC) was used to analyze and quantify the sugars and ethanol, and the solid residues were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). The effect of energy input (54 -423kJ.g^{-1}), sonication time (15-60minutes), calcium hydroxide concentration (10 - 50 g.kg^{-1} in $Ca(OH)_2$ in water) and sulphuric acid concentration (10 – 50g.kg^{-1} H_2SO_4 in water) and biomass loading (10 - 100g.kg^{-1} biomass) on sugar and ethanol yields were studied.

The highest sugar yield using ultrasonic-assisted dilute acid pretreatment (340g.kg^{-1} substrate) was obtained at 270 kJ.g^{-1} energy input for 30min in the presence of 30 g.kg^{-1} H_2SO_4 in water. The highest sugar yield using ultrasonic-assisted dilute alkali pretreatment (240 g.kg^{-1} substrate) was obtained at 270 kJ.g^{-1} energy input for 30 min in a solution of 30 g.kg^{-1} $Ca(OH)_2$ in water. The highest ethanol yield of 110 g.kg^{-1} of biomass was obtained after 24 hours of fermenting an alkaline pretreated hydrolysate, and 90 g.kg^{-1} ethanol yield was obtained from an acid pretreated hydrolysate. The combined pretreatment was shown to be effective in liberating fermentable sugars from amaranth stem with ultrasonic-assisted dilute acid pretreatment resulting in 92% conversion of total sugars. With reduced pretreatment time, and relatively low catalyst concentrations, assisted ultrasonic pretreatment is an economical attractive lignocellulose pretreatment step.

Keywords: Bioethanol; Amaranth, Pretreatment; Ultrasound, Enzymatic hydrolysis.

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DECLARATION

I, **AMANDA MBELWANA MDITSHWA** declare that the dissertation entitled ultrasonic assisted amaranth stem pretreatment for bio-ethanol production is my own work and it has not been submitted to any other university.

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Amanda Mbelwana Mditshwa

Potchefstroom

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ABBREVIATIONS

Acronyms	Definition
ARC	Agricultural Research Council
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
Min	minutes
MTBE	Methyl-tertiary butyl ether
SEM	Scanning Electron Microscopy
US	United State
W	Watts
Wt	weight
USA	United States of America
EU	European Union
Kg	kilogram
t/ha	tones per
kHz	kilohertz
s	seconds
°C	degrees Celsius
µm	micrometers
kW	kilowatts
FPU/g	Filter Paper Unit per gram
mg	milligram
g	grams
β	Beta
α	Alpha
v/v	volume per volume
w/v	weight per volume
L	Litre
wt%	weight percentage
rpm	rounds per minute
g/L	gram per litre
µL	microlitre
hrs	hours
psi	Pounds per square inch

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CHAPTER ONE: GENERAL INTRODUCTION

1.1. INTRODUCTION

The global demand for energy is increasing because of high fuel consumption and growing industrialization (Sunday, 2011). Currently, fossil based energy resources are the most used resources for energy and fuel production. Fossil based energy is one of the major contributors to environmental pollution (Gahukar, 2012) that has resulted in global warming. Global climate change and increased energy demand has driven researchers to find alternative energy resources that will overcome the limited energy supply, be affordable, sustainable and ecological friendly.

Ethanol (ethyl alcohol) has been used as an alternative source of fuel energy since the oil crisis in the 1970's (Shen *et al*., 2012). Ethanol, an excellent transportation fuel, can be used as blend with petrol and are currently used in the US and Brazil (Firth *et al*., 2014). Since it is an oxygenated fuel that contains 35% oxygen, it reduces particulate and NO**x** emission from combustion (Saratale & Eun Oh, 2010). Ethanol blending with petrol oxygenates the fuel and reduces the formation of carbon monoxide and ozone, which lowers greenhouse gas emission (Kumar *et al*., 2009). It is a renewable, cost effective and cleaner fuel than petrol (Chaudhary & Qazi, 2011).

Biofuels are fuels derived from biomass such as renewable organic materials from plants or animals (Uyigue & Archibong, 2010). Biomass feedstock is classified into: first, second and third generation feedstock. First generation feedstock are those crops that are used mostly as food and feed for humans and are rich in sugar, oil, and starch (Gasparatos *et al*., 2011). However, the use of first generation feedstock for biofuel production has raised arguments that fuels competes with food and is responsible for increases in food prices (Joshi *et al*., 2011). The latter has resulted in the shift towards using second generation feedstock. Second generation feedstock are dominated by lignocellulosic biomass such as agricultural residues (Naik *et al*., 2009) and also municipal and industrial waste (Joshi *et al*., 2011). Lignocellulose consists of cellulose and hemicellulose, which is converted to sugars through chemical pretreatment and biological processes and eventually fermented to bioethanol (Gahukar, 2012). However, second generation feedstock has raised concern over the land use requirements and land use change and as a result, researchers have directed their focus towards third generation fuels (Naik *et al*., 2009). Third generation biofuels produced from

microscopic organisms are considered to be viable alternative energy resources that do not possess the major drawbacks associated with first and second generation (Nigam & Signh, 2011). Third generation feedstock are also non-food crops such as microalgae (Gahukar, 2012) and microbes (Nigam & Sighn, 2011). Biofuel feedstock can be converted into solid, liquid, and gaseous form of biofuels. Amongst all the liquid biofuels, biodiesel and bioethanol are the most studied and promising as alternative fuels or used as blends with petroleum petrol (Payne, 2010). Amaranth is considered as potential plant for second generation feedstock ethanol production. Amaranth possesses characteristics such as fast growth rate through a C4 photosynthetic pathway, good tolerance to stress, high potential biomass yield, (De la Rosa *et al*., 2008) and the ability to absorb heavy metals from surrounding soil which makes it an excellent renewable energy source (Akond *et al*., 2013).

Various types of pretreatment methods for production of bioethanol such as physical, physico-chemical, chemical and biological methods and combinations thereof (Ninomiya *et al*., 2011) has been investigated. The application of ultrasound irradiation in pretreatment strategies has been found to be more effective in converting lignocellulose material into fermentable sugars (Nikolinic *et al*., 2011). Application of ultrasound-assisted pretreatment under selected conditions can increase the overall bioethanol yield rapidly through the increased porosity of cellulose fibre (Ninomiya *et al*., 2012) and the cleavage of glycosidic linkages in lignin (Sakthiselvan *et al*., 2012). It can also promote a decrease in mass transfer limitations and improve hydrolysis (Werle *et al*., 2013:129).

1.2. PROBLEM STATEMENT

Cellulosic ethanol is one of the most attractive alternative fuels in bioethanol production. There are three major steps involved in producing second generation ethanol from lignocellulose, i.e. pretreatment, enzymatic hydrolysis, and fermentation (Talebnia *et al*., 2009). Pretreatment is one of the major steps to reduce cellulose crystallinity and expose the polymers in lignocellulose for enzymatic attack (Kumar *et al*., 2009). Many types of pretreatment methods have been investigated to improve the ethanol production processes but existing technologies require high energy input in terms of heat (Saratale & Oh, 2012). So there is a need to seek newer methods that will require less energy, increase enzymatic hydrolysis rate and decrease the crystallinity of cellulose in order to increase product yield without being expensive (Balat *et al*., 2008).

Cao *et al*. (2012) suggested a synergistic approach to pretreatment where two or more methods are applied on a particular feedstock. Application of ultrasound irradiation in

combination with either acid or base-assisted pretreatment is considered to be promising to enhance enzymatic hydrolysis rate. In this study ultrasonic pretreatment will be utilized in combination with sulphuric acid or calcium hydroxide treatment to enhance the yield of bioethanol from amaranth stems

1.3. AIMS AND OBJECTIVES:

The aim of this study was to assess the effectiveness of ultrasonic pretreatment in combination with acid and alkali treatment to convert amaranth lignocellulose to ethanol. The aim of this study was met and through the following specific objectives:

- Evaluation of the effect of ultrasonic-assisted acid pretreatment on monomeric sugar yields from amaranth stems at varying sulphuric acid concentration.
- Evaluation of the effect of ultrasonic-assisted alkali pretreatment on monomeric sugar yields from amaranth stems at varying calcium hydroxide concentration.
- Determine the effect of pretreatment on the morphology and composition of amaranth stems.
- Evaluate the efficiency of the pretreatment on hydrolysis and fermentation of sugars to ethanol.

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CHAPTER TWO: LITERATURE REVIEW

2.1. INTRODUCTION

The need to respond to climate change, increasing energy consumption and the depletion of fossil fuel resources has led to a need for alternative energy sources (Subhedar & Gogate, 2013). The development of renewable energy resources such as biomass is believed to address the issue of energy reliability and sustainability globally because biomass feedstock is often locally available (Bussemaker & Zhang, 2013). Biomass energy can play an important role in reducing greenhouse gas emissions, mainly carbon dioxide and methane, since carbon dioxide from fuels produced from biomass is offset by the carbon dioxide used to grow the feedstock. Bio-based fuels can help reduce greenhouse gas emission from fuels-based fuels (Edgar & Zhang, 2009). Worldwide biofuel production has increased from 4.4 to 50.1 billion liters between 1980 and 2005 (Koh & Ghazoul, 2008).

Biofuel is energy derived directly or indirectly from biological sources (Uyigue & Archibong, 2010). Biofuels can be classified into; primary biofuels which are unprocessed fuels and are commonly used for cooking and heating and secondary biofuels that are processed fuels; in liquid, solid and gaseous form. The most attractive of these have been liquid biofuels which are used for transportation. Amongst these, bioethanol and biodiesel are the most used biofuels (Payne, 2010).

2.2. BIOETHANOL

Globally, the use of ethanol as fuels has been favorable, due to the high octane content. Ethanol (an alcohol produced through fermentation of carbohydrates) (Chaudhary & Qazi, 2011) has been used as a fuel oxygenate to enrich fuel with oxygen to increase compression ratios resulting in increased performance (Joshi *et al*., 2011). Bioethanol has numerous advantages over conventional fuels, when used as fuel. For example, it is cleaner and blends well with other fuels to reduce greenhouse gas emission and has a reduced carbon dioxide emission (Chaudhary & Qazi, 2011). Bioethanol production from plant biomass has gained considerable attention, with sugarcane and maize being the main raw material of choice. United States, Brazil, and China are the front-runners in global first generation bioethanol production. However, there are numerous debates to the use of non-food crops in the production of bioethanol and as a result there are other feedstock being investigated including second and third generation biomass (Kocar & Civa, 2013).

2.3. BIOETHANOL FEEDSTOCK

The availability of biomass feedstock for bioethanol production is of paramount importance as it constitutes approximately 80% of the total cost of bioethanol (Balat et al., 2008). Different types of biomass include woody plants, herbaceous plants, grasses, aquatic plants, agricultural crops and residues, municipal solid waste and manures (Agbor et al., 2011). Since the cost of biofuels production is determined by the cost of feedstock, geographic location and availability has to be carefully considered before a production plant is planned (Canilha et al., 2012). The commonly used types of feedstock for biofuel production in different countries are listed in the Table 2. 1 below:

Table 2.1: Production of liquid biofuels in different countries from different type of biomass (Kocar & Civas, 2013).

Countries	**Bioethanol Feedstock**	**2006 Production (million litres)**	**2007 Production (million litres)**	**2010 Production (million gallons)**
Brazil	Sugar cane, palm oil	16,998	18,798.2	6,577.89
Canada	Corn, wheat, straw	579	1000.0	290.59
China	Corn, wheat, cassava, sweet sorghum	3,849	1599.9	541.55
EU	Wheat, sugar beet, wine, alcohol and other grains		2302.8	1,039.52
India	Molasses, sugarcane	1,900	400.1	
Indonesia	Sugar cane, cassava		0	
Malaysia	None		0	
Thailand	Molasses, cassava, sugar cane	352	300.1	
United State	Primary corn		24597.6	13,230.00

2.4.BIOFUELS CLASSIFICATION

Depending on the feedstock for biofuel production, biofuels can be classified as first generation, second generation or third generation biofuels. The primary distinction is whether the feedstock is obtained from food crops, residues of food energy crops or algae. First

generation biofuels are generated from crops that are also used for human and animal consumption such as sugars (sugar cane, sweet sorghum), grains or seeds (Gasparatos et al., 2011; Joshi et al., 2011). First generation technologies are well developed and studies have shown that first generation ethanol production have a negative impact on the environment, biodiversity the climate, because of the large land and water requirements to grow first generation crops. The effect of first generation fuel production on food security and food prices in the long term has been under debate (Zhang & Bussemaker, 2013). The use of first generation biofuels is currently not competitive with existing fossil fuels, because the cost of biofuels per liter is more than that of petrol at current average crude oil prices (Gahukar et al., 2012; Joshi et al., 2011).

Second generation biofuels are obtained from lignocellulose biomass that is the inedible parts of plants or leftovers after harvest, which includes stem, leaves, and husks (Antizar-Ladislao & Turrion-Gomez, 2008). Lignocellulose biomass is composed of energy-rich cellulose, hemicellulose and lignin. These polymers make up the cell wall of plants and are comprised of polysaccharides. Second generation biofuels has become an attractive source of energy, because the lignocellulose material is abundantly available, is cheap and have a potential to offer novel biofuels. Moreover, these non-food feedstock avoid the food vs. fuel argument (Joshi et al., 2011; Gahukar et al., 2012). Examples of second generation feedstock include maize stover, maize fiber, woodchips, and cotton stalks (Payne, 2010). Typical agricultural residues include sugarcane bagasse, rice straw, alfa - alfa, rice hull, maize cob, oat hull and switch grass (Joshi et al., 2011).

Third generation biofuels are derived from water-based biomass such as algae and microbes (Gahukar et al., 2012). Third generation technologies are not well developed but current research has shifted to focus towards the third generation biofuels, because of the potential to negate the drawbacks associated with first and second generation feedstock (Nigam & Singh, 2010). For example, microalgae which is one commonly used third generation feedstock has been found to grow appropriately with minimal freshwater input and can utilize land that is not productive for plant crop (Kumar & Sarma, 2013). However even though algae can grow in wastewater, it still requires large amounts of water, nitrogen and phosphorus to grow. The production of fertilizers that meet these requirements would produce more greenhouse gas emission than saved by using algae as biofuel feedstock therefore third generation biofuels are not environmental friendly.

2.5. AMARANTH IN SOUTH AFRICA

Amaranth is an annual herbaceous plant species with significantly high dry matter yields as well as seed (Suchomel et al., 2009). It belongs to the Amaranthaceae family and there are numerous Amaranthus species known with approximately 60 wild types (Berneo & Aguirre, 2008). Amaranth originates from America and is recognized as one of the oldest food crops in the world. It exists in many ecosystems around the world such as India, China, Southeast Asia, Mexico, South America and United State (Department of Agriculture, Forestry and Fisheries, 2010).

2.6. COMPOSITION AND PROPERTIES OF AMARANTH PLANT

Amaranth is a carbon 4 (C4) photosynthetic plant, and has the ability to maintain high rates of carbon dioxide fixation, and is very efficient at converting carbon dioxide assimilation of minerals from the soil, energy of the sun, and water into plant tissue. Amaranth is capable of tolerating stressful environmental conditions such as temperature, drought, salinity, and alkalinity, acidic or poor soil (de la Rosa et al., 2009). Considering its cell wall biochemical composition, amaranth has an unusual potential as a low – lignin fermentation feedstock (Akond et al., 2010). The genetic and phenotypic variation of morphological traits that contributes biomass in amaranth is not yet understood. Also not much data has been collected on its biomass, composition of the cell wall and its economic viability is still uncertain (Akond et al., 2013). Amaranth grain has high potential a lower gluten nutritional source (Cai & Corke, 2004). Amaranth holds potential as a biofuel feedstock as it can absorb heavy metals from surrounding soil, it has higher yields under marginal soil conditions and has a short life cycle (Akond et al., 2013).

In South Africa, amaranth is known to grow naturally after the first rains. The level production of amaranth is not known, but amaranth is estimated to produce fresh leafy yields up to 40metric tonne per hectare. The yield of grain is highly variable with 1000kg.ha-1 considered a good yield. KwaZulu Natal, Limpompo, North West, and Mpumalanga are the main producing areas in South Africa (Department of Agriculture, Forestry and Fishiries, 2010). Further efforts on amaranth production can be beneficial for the South African biofuel industry. The main aspects to consider in amaranth production will therefore be directed in the selection of species cultivation methods and its harvesting techniques. As the focus on the production of liquid fuels from lignocellulose feedstock is the better alternative in the biofuels industry, amaranth hold an attractive potential as biomass for production of biofuels.

2.7. LIGNOCELLULOSE STRUCTURE

Lignocellulose biomass is composed of three major structural polymers, each having a different and intricate structure, i.e. cellulose, hemicellulose and lignin. Generally, lignocellulose is found to contain approximately 30 – 50% cellulose, 25 – 30% hemicellulose and 20 – 25% lignin. It is the largest renewable energy resource and the most abundant (Saratale & Oh, 2009).

2.7.1. CELLULOSE

Cellulose ($C_6H_{10}O_5)_n$) is an unbranched linear polymer of β–D–glucose unit joined together by β–(1-4) glycosidic bonds (Chandel *et al*., 2007) leading to long chains of approximately 7,000 to 15,000 glucose molecules per polymer. It is one of the main components of the plant cell wall and is also known to have a high degree of polymerization from 100 – 20,000 (Bussmaker & Zhang 2013). The multiple hydroxyl groups on the glucose residues from one chain of hydrogen bonds with side and resulting in order crystalline structure called the micro-fibrils which makes a recalcitrant compact structure. The micro-fibrils are group of individual cellulose chains and are packed to form fibrils and these fibrils are further packed to form cellulose fiber (Saratale & Oh, 2009). This tightly packed structure makes cellulose resistant to hydrolysis.

2.7.2. HEMICELLULOSE

Hemicellulose heteropolymer is made up of carbon six (hexose), carbon five (pentose) sugars and uronic acid (Bussmaker & Zhang, 2013). Hemicellulose has short chains of approximately 500 – 3000 sugar units and is branched and non-crystalline polymers (Khullar, 2012). The hexoses sugars in hemicellulose are D-glucose, D-galactose, and D–mannose, while the pentoses are D-xylose and L-arabinose. Acid sugars in the hemicellulose structure are acetic acid, D–gluccuronic acid and 4-O-methyl-D-glucuronic acids. These sugars are linked together by glycosidic linkages and can be affected by chemical and physical attack. Hemicelluloses are classified according to the oligomers that are present in the main polymer, i.e. xylan, glucomannan and galactan (Canilha *et al*., 2012). According to Saratale & Oh (2009), glucomannans and galactomannans are the major components in softwood whereas xylan is the main component in hardwood. Xylan and mannan protects cellulose from enzymatic attack (Saratale & Oh, 2009).

2.7.3. LIGNIN

Lignin is an amorphous polymer that is composed mainly of three aromatic alcohol monomers, namely p-coumaryl, coniferyl, and sinapyl alcohol. These aromatic alcohols have a molecular weight ranging from 100 to 1000 and have similar chemical properties (Saratale

& Oh, 2012). The alcohol monomers are bonded together by alkyl-aryl, alkyl-alkyl, and aryl-aryl ether linkages forming p-hydoxyphenyl, guaiacyl, and syringyl phenylpropanoid units within the lignin polymer (Khullar, 2012). Each monolignol differs according to its plant species, location in the cell wall, and plant tissue. Hardwoods contain mainly guaiacyl and syringyl, softwoods contain guaiacyl and grass contains both guaiacyl and syringyl with higher p-hydoxyphenyl units. (Saratale & Oh, 2012; van Dyk & Pletschke, 2012).

It is difficult for enzymes to access carbohydrate polymers found in lignocellulose material, because of the intricate lignocellulose structure. Therefore, the process to convert lignocellulosic material into bioethanol needs three steps, namely pretreatment, hydrolysis and fermentation. The pretreatment and hydrolysis are the major steps because this is where the polymer structure is broken down to monomeric sugars.

2.8. PRETREATMENT OF LIGNOCELLULOSE MATERIAL

During the pretreatment process, the biomass structure is disrupted and altered so that hemicellulose and cellulose can be easily accessed by enzymes in the hydrolysis process, thus resulting in increased reaction rate and greater yields (Balat *et al*., 2008). The choice of pretreatment method is crucial to ensure break down of the lignin and hemicellulose matrix that protects the cellulose, thus affording cellulase enzyme to hydrolyze cellulose into glucose (Shen *et al*., 2012). Furthermore, pretreatment must not only increase the porosity of the biomass but also be able to disrupt or loosen the crystalline structure of cellulose (Joshi *et al*., 2011). Overall, pretreatment process has to be cheap, must have little degradation or loss and as little formation of inhibitory substances as possible for effective ethanol production (Theerarattananoon, 2012). Bioconversion of lignocellulosic material to bioethanol is normally hindered by the structural and chemical complexity of biomass; therefore it is necessary to modify the native form of biomass by means of pretreatment. According to Sul'man *et al*., (2010) there are four classes of pretreatment methods i.e. physical, chemical, physico-chemical and biological pretreatment technologies. Every pretreatment method has its own specific way of disrupting the cell wall components based on the mechanistic application with the conditions applied (Canilha *et al*., 2012).

2.8.1. PHYSICAL PRETREATMENT

Physical pretreatment technique involves mechanical treatment, auto hydrolysis and irradiation (FitzPatrick *et al*., 2010), and is effective for breaking down cellulose crystallinity with no chemicals involved. This technique is effective in the reduction in size of biomass

materials to improve enzymatic hydrolysis and mass transfer characteristics. However, it has lower efficiency, has a large energy requirement, and is expensive (Saratale & Oh, 2012; FitzPatrick *et al*., 2010).

2.8.1.1. Milling

Mechanical pretreatment that reduces particle size includes grinding, milling, and chipping. Milling makes materials easy to handle and increases surface / volume ratio (Canilha *et al*., 2012). Milling is used so to alter the lignocellulose ultrastructure, the degree of crystallinity and to make cellulose more accessible to cellulase enzymes. The reduction in size caused by milling can improve susceptibility to enzymatic hydrolysis and the efficiency of downstream processing. Milling is usually carried out before enzymatic hydrolysis and also before other pretreatment methods such as dilute acid, steam or ammonia explosion on several lignocellulose materials (Kumar *et al*., 2009).

Colloid, fibrillator, and dissolver mills are suitable for wet materials whereas extruders, rollers, and, hammer mills are commonly used for dry material (Sul'man *et al*., 2010). Ball mills can be used for either wet or dry materials (because there are no chemicals used during this method) (Taherzadeh & Kamiri, 2008). It is an environmental friendly pretreatment method, and there is no formation of inhibitors (Talebnia *et al*., 2009). The desired particle size depend on the pretreatment method to be used, however the use of very small particles results in higher energy consumption in the milling stage and it also impact negatively on the pretreatment method used (Talebnia *et al*., 2010).

2.8.1.2. Steam Explosion (Auto hydrolysis)

Steam explosion also known as auto hydrolysis is the most widely used method for lignocellulosic biomass pretreatment. This method is a combination of mechanical forces, due to explosive decompression, and the chemical effects of the water protons and acetyl groups present in the hemicellulose (Talebnia et al., 2010). During this process, high pressure saturated steam is injected into a batch or it is continuously filled with reduced sized biomass. When the steam is injected, the temperature rises to temperatures of between 160°C and 220°C, the pressure is dropped suddenly and the biomass undergoes an explosive decomposition. As a result, hemicellulose gets degraded and the lignin matrix gets disrupted. The energy costs of this method are relatively moderate and it satisfies all the requirements of the pretreatment process (Tabelinia et al., 2010).

2.8.1.3. Ultrasonic Pretreatment

Ultrasound energy at frequency ranging from 16 – 100 kHz has been applied to lignocellulose biomass disruption through a sound transducer (horn) (Rehman *et al*., 2013). It has been

postulated that sonication causes cavitation resulting in the formation of micro-bubbles within the solution that contains biomass and as the bubbles collapse, energy is released in the form of a shock wave. Cavitation increases temperature and pressure and the formation of free radicals. Turbulence enhances mixing, and the accessibility of the biomass for subsequent processing and mass transfer at the solid liquid interface. It also increases the enzymatic hydrolysis rate (Bussmaker & Zhang, 2013).

Ultrasonic assisted pretreatment has been employed in numerous studies. Goshadrou *et al*. (2011) used ultrasonic assisted alkali pretreatment of sweet sorghum bagasse to observe the effects of ultrasonicaton on the bagasse. Ultrasonication was combined with sodium hydroxide to pretreated bagasse, at 28 ±1.4 kHz, and 50W for 3 hours. Under these conditions partially delignification, reduced cellulose crystallinity, distorted rigid structure increased external surface area and porosity was observed. The results showed 81% theoretical yield and ethanol yield 0.70g/l/h productivity could be obtained with *M. hiemalis* used as fermentation organism. It was concluded that sonoalkalization increased the hydrolysis rate. Also Sul'sum *et al*. (2010) used ultrasonic pretreatment with 368W/cm^2 for 15min and cellulose and lignin destruction of 18% and 11.4% were observed respectively.

Irradiation energy from ultrasound affects physical and chemical properties of lignocellulosic biomass and increases the saccharification of cellulose (Bussmaker & Zhang, 2013). It was observed that ultrasonication with lime pretreatment improved the quality and recovery of pretreated lignocellulosic biomass. Shi *et al*, (2012) suggested that ultrasonic pretreatment destroyed the physical structure of cellulose and caused significant swelling of cellulose in water. Ultrasonication increased the surface area of cellulose and reduced depolymerisation and crystallinity.

2.8.2. BIOLOGICAL PRETREATMENT

Biological pretreatment processes involve the use of cellulolytic microorganisms and cellulolytic enzymes in lignocellulose pretreatment (Saratale & Oh, 2012). Although little attention is paid to technique, many microorganisms are involved in waste material and delignification of lignocellulose biomass (Canilha *et al*., 2010) Biological pretreatment offers low energy input and require mild operational conditions also there are no chemicals required (FitzPatrck *et al*., 2010). However, the drawback of this method of pretreatment is the long treatment time, and loss of hydrocarbon (Canilha *et al*., 2010). In addition, the use of microorganisms always requires carefully controlled growth conditions such as pH and fermentation.

2.8.3. CHEMICAL PRETREATMENT

Chemical pretreatment processes include the use of alkali, acid, oxidizing agents, and solvents (Saratale & Oh, 2012). Hemicellulose is degraded and lignin is removed and biodegradability of biomass is prompted (Canilha *et al*., 2010). On the other hand, chemical pretreatment involves harsh reaction conditions (FitzPatrck et al., 2010) and may cause secondary pollution problems (Saratale & Oh, 2012).

2.8.3.1. Alkaline pretreatment

Alkaline pretreatment employs the use of alkaline solutions to remove lignin and part of the hemicellulose from biomass resulting in improved reactivity of the remaining polysaccharides in further enzymatic hydrolysis steps (Joshi *et al*., 2011). Commonly used alkalines include sodium hydroxide, potassium hydroxide, calcium hydroxide, and ammonia hydroxide (Canilha *et al*., 2010). The mechanism of alkaline pretreatment is believed to be degradation of the ester and glycosidic bonds crosslinking xylan hemicellulose and other components such as lignin. Alkaline pretreatment changes the structure of lignin, and partially decrystalizes and swells cellulose (Joshi *et al*., 2011). Alkaline pretreatment is often performed at ambient conditions but only requires longer time to achieve effective pretreatment. The only limitation of this approach is the formation of irrecoverable salts that are incorporated as salts into biomass pretreatment reactions (Suhedar & Gogate, 2013).

Traditionally, sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$) have been used in the pretreatment of lignocellulose (Joshi *et al*., 2011). When NaOH is used as alkaline for pretreatment, swelling in the fibers occurs which increase the internal surface area, and decreases the degree of polymerization and crystallinity of cellulose, separating the structure linkages that are between lignin and carbohydrates and disrupting the lignin structure making cellulose and hemicellulose available for enzymatic degradation (Canilha *et al*., 2012). Despite the advantages of NaOH, $Ca(OH)_2$ is preferred due to it is cheap, safe and can easily recovered from water as insoluble calcium carbonate by reaction with carbon dioxide (Mosier *et al*., 2005).

2.8.3.2. Acid pretreatment

Acid pretreatment normally solubilizes hemicellulose resulting in a relatively accessible surface area increase in cellulose. Acid treatment can be employed in concentrated or diluted acid conditions. Dilute acid pretreatment has been commercially used widely for lignocellulose material pretreatment (Saratale & Oh, 2012). Other common acids used include hydrochloric acid, nitric acid, and phosphoric acid. Depending on the type of feedstocks dilute acid pretreatment operates at medium temperature for shorter reaction

times, thus reducing the energy cost (Cannilha *et al.*, 2012). Although this technique is effective in lignocellulose pretreatment it is associated with the formation of by-product such as furfurals, hydroxy methyl furfural, phenolic acids, and acetate (Joshi *et al*., 2011). Dilute sulphuric acid is very corrosive and thus expensive equipment is required. The acid also needs to be neutralized before fermentation of the sugars and the neutralizing have to be salts removed (Mosier *et al*., 2004).

2.8.3.3. Wet Oxidation

During this pretreatment, lignocellulosic biomass is treated with water and high pressure oxygen or air at elevated temperature (above 120°C) and oxygen pressures between 120 and 3309.48 kPa (Talebnia *et al*., 2010). Temperature, reaction time and oxygen pressure are the main factors that influence sugar yield during wet oxidation. The main reaction of wet oxidation is the formation of acids from hydrolytic processes as well as oxidative reactions. This process is very effective in separating cellulose from lignin and hemicellulose, thus all three fractions of lignocellulosic are affected. During this process lignin undergoes both oxidation and cleavage while hemicellulose is extensively cleaved to monomeric sugars and cellulose gets partly degraded making it highly susceptible to enzymatic hydrolysis (Taherzadeh & Karimi, 2008).

2.8.4. PHYSICOCHEMICAL PRETREATMENT

Physicochemical pretreatment refers to the combination of both physical and chemical pretreatments and may include steam explosion, carbon dioxide explosion, and liquid hot water pretreatment. However, the methods are expensive (FitzPatrck *et al.*, 2010), consume large amounts of energy and may result in the formation of secondary pollutants (Saratale & Oh, 2012).

2.8.4.1. Carbon dioxide Explosion

Carbon dioxide (CO_2) explosion is similar to steam explosion except that CO_2 is used instead of steam. It is believed that CO_2 reacts with carbonic acid, thereby improving the hydrolysis rate (Canilha *et al*., 2012). The delignification with carbon dioxide at high temperatures can be improved by the use of co-solvents such as ethanol, water or acetic acid and can increase lignin removal. Carbon dioxide has been used as an extraction solvent because it is available at relatively low costs, its non-toxic, non-flammable, its recovered easily after extraction, is environmental acceptable and also shows gas – like mass transfer properties (Taherzadeh & Karimi, 2008).

2.8.4.2. Liquid Hot Water

This process is also known as hydrothermal, hydrothermolysis pretreatment, aqueous fractionation, sovolysis or aquasolv. Under high pressure, water can penetrate into the biomass to hydrate cellulose and remove hemicellulose and a part of the lignin. During this process, hot compressed water contacts with biomass for up to 15 minutes at high temperature ranging from 200°C to 230°C and 40% to 60% of total biomass is dissolved in the process with 4% to 22% of cellulose, 35% to 60% of the lignin, and all of the hemicellulose removed (Harmsen *et al*., 2010). The main advantages of this process are that there is no addition of chemicals necessary and there is no requirement of corrosion resistant materials for hydrolysis reactors (Taherzadeh & Karimi, 2008).

2.8.5. MECHANICAL/ CHEMICAL PRETREATMENT

2.8.5.1. Microwave assisted pretreatment

Microwave irradiation combined with chemical pretreatment has been used as an alternative to conventional heating. It has remarkable advantages such as faster reaction time; lower energy consumption and minimization of fermentation inhibitors (Canilha *et al*., 2012). Researchers have shown that microwave irradiation can alter the complex structure of cellulose by changing the ultrastructure of cellulose and degrade lignin and hemicellulose (Binod *et al*., 2010). The heated biomass and the applied electromagnetic waves interact with biomass, resulting in high heat zones that cause mechanical and thermal changes to the structure of the biomass. Polar bonds inside the biomass structure align with the microwave magnetic field which causes vibrations that disrupt the lignocellulose structure (Choudhary *et al*., 2012).

2.9. HYDROLYSIS OF CELLULOSE AND HEMICELLULOSE

Saccharification (also known as hydrolysis) is the process through which polysaccharides from lignocellulose biomass are converted into monomeric sugars. Cellulose and hemicellulose are hydrolyzed into simple sugars and lignin remains unaffected as a by-product (Taherzadeh & Karimi, 2007; Zheng *et al*., 2009). Saccharification can be done using acids or enzymes.

2.9.1. ACID HYDROLYSIS

Dilute acid is often preferred to convert lignocellulose into these simple sugars (Saxena *et al*., 2009). Acid hydrolysis of cellulose is done either at high acid concentrations at low temperatures or at dilute acid concentrations at high temperatures. Although acids are attractive for hydrolysis, it breaks down the cellulose and further degrades glucose into hydro

methyl furfural and other unwanted by-product if the process is not controlled carefully (Werle *et al*., 2007).

2.9.2. ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis is an eco-friendly way to hydrolyze cellulose. a mixture of cellulase enzymes, that are highly specific to degrade cellulose into reducing sugars (Cannilha *et al*.,2012). Cellulase enzyme from bacteria belonging to *Clostridium, Cellulomonas, Ruminococcas* and fungus *Trichoderma reesei*, are the most studied (Chandel *et al*., 2009). There are three main classes of cellulases i.e. endoglucanase, exoglucanase or cellobiohydrolase, and β-glucosidase. Endoglucanase enzyme creates free chain ends by attacking cellulose at the region of low crystallinity. Exoglucanase degrades the cellulose further by removing cellubiose units from free chain ends and β-glucosidase hydrolyzes cellubiose into glucose (Saxena *et al*, 2009).

Enzymatic hydrolysis processes are usually done under mild conditions of pH 4.5 to 5.0 and temperatures (45 to 50 °C) and with cellulose dosage of 10 to 30 (FPU/g cellulose) for 48 to 72 hours. Enzyme loading depends on the type of biomass and pretreatment technique used (Talebnia *et al*., 2009). Previous studies of enzymatic hydrolysis showed that parameters such as substrate type, cellulase activity, and reaction condition can influence monomeric sugar yields (Cannilha *et al*., 2012). Hemicellulose enzyme breaks down the β-1, 4–xylan chain into xylose monomers. Other hemicellulases that are known to be involved in hydrolysis are α–1–arabinofuranosidases, α–glucuronidases, acetyl xylan astarases, farulic acid astarases and α-galactosidases.

2.10. FERMENTATION

Lignocellulose hydrolysates can be converted to ethanol and carbon dioxide by variety of microorganisms (Lin & Tanaka, 2006). Hydrolysates from the pretreatment processes contain a number of different monosaccharides such as glucose, arabinose, xylose, and galactose. (Canilha *et al*., 2012). For this reason, microorganisms used for fermentation should be able to ferment all available sugars. Yeast is one of the most effective bioethanol producing microorganisms (Balat *et al*., 2008). To date, Z. *mobillis* and yeasts such as *Saccharomyces, Khiveryomyces,* and *Pichia* have been applied in the fermentation of sugar hydrolysate to ethanol (Binod *et al*., 2010). However, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are commonly used in industry (Sarkar *et al*., 2012).

2.11. CONCLUDING REMARKS

Although bioethanol production is attractive for alternative fuel or blended in fuel there are still challenges and these need to be investigated. Various pretreatment methods have been investigated, however most these application have not been optimized to make them suitable for commercialization. Combination of chemicals has a promising potential to increase the rate of enzymatic hydrolysis and the bioethanol yield. In this study ultrasonic assisted pretreatment will be investigated for enhanced hydrolysis rate and ethanol yields. It is envisaged that the integration of two or processes will reduce the process steps; energy input and makes the conversional process economical.

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CHAPTER THREE: MATERIALS AND METHODS

3.1. OVERVIEW

In this chapter the materials and experimental procedure used in this study are described in details. Section 3.1 outline the materials used and 3.2 describes the methods used in the production of bioethanol from amaranth stem.

3.2. MATERIALS AND CHEMICALS

3.2.1. CHEMICALS

All materials used in this study are shown and described in Table 3.1.

Table 3.1 Chemicals used in this study

Chemical name	Supplier	Purity (%)	Purpose
Sulphuric Acid	Associated Chemical Enterprises (ACE)	95 - 98%	Pretreatment
Calcium Hydroxide	Sigma-Aldrich	97%	Pretreatment
NS 22192	Novozymes	Not Applicable	Hydrolysis
Tween 80	ACE	Not Applicable	Hydrolysis
S.cerevisae	Local shop	Not Applicable	Fermentation
Amaranth stem	ARC	Not Applicable	Pretreatment
Citric acid	Sigma-Aldrich	99 – 102%	Buffer
Sodium citrate	Merck	99.0 – 100.5%	Enzyme assay
Sodium hydroxide	Sigma-Aldrich	98%	Neutralization

3.2.2. FEEDSTOCK

Amaranth plants were obtained from a local farm of the Department of Agriculture in Potchefstroom, South Africa (27°43'43 16" S - 27° 04' 47.71 °E). The roots were removed from the stem using a knife and the stems were sun dried to a moisture content of 10% and milled using a Hammer mill (Trapp-TRF 70). The milled samples were then stored in airtight bags at ambient conditions until used. The composition of the milled amaranth samples was analyzed by ARC Irene laboratories.

3.2.3. MICROORGANISMS AND ENZYMES

Baker's yeast was obtained from a local shop as commercial yeast (*Sacromyces cerevisae*) for use in fermentation. The yeast was revived before use, using small amounts of the fermentation broth.

3.2.4. MEDIA PREPARATION

The hydrolyzate and glucose (glucose as control) solution was supplemented with yeast extract, 5.0; $(NH_4)_2SO_4$, 7.5; $MgSO_4 \cdot 7H_2O$, 0.75; K_2HPO_4, and 3.5; $CaCl_2 \cdot 2H_2O$. The pH of the broth was adjusted to 5.5 using 2.5M sodium hydroxide buffer or 0.5 M sulphuric acid (Goshadrou *et al.*, 2011).

3.2.5. EXPERIMENTAL PROCEDURE

The main aim of pretreatment step is to disrupt the lignocellulose structure, making more cellulose accessible to the enzyme used in enzymatic hydrolysis (Joshi *et al*., 2011). In this study, indirect ultrasonication was combined with alkali and acid treatment to release monomeric sugars and to break down the crystallinity of cellulose. The experimental procedure is followed in Figure 3.1.

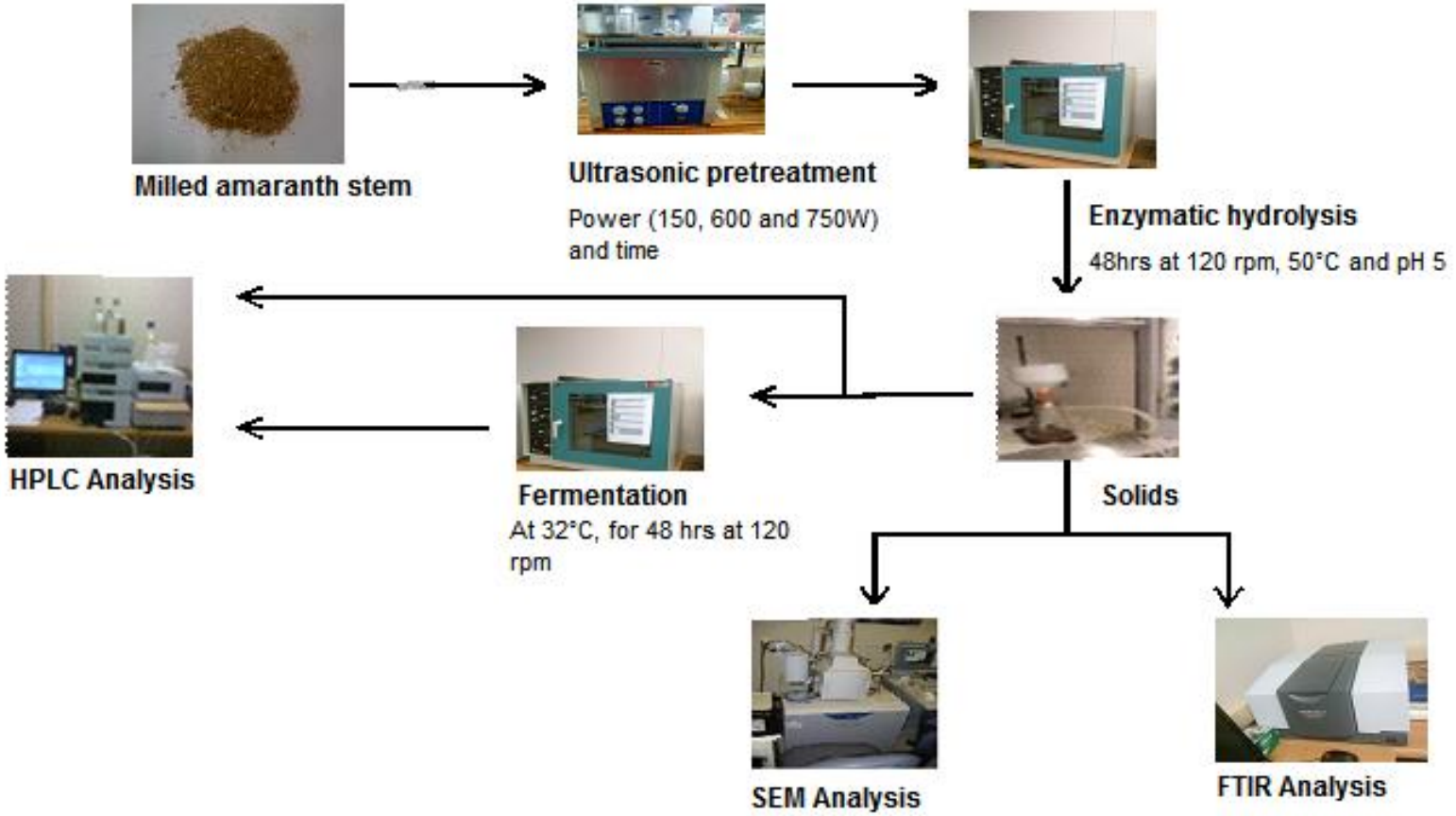

Figure 3.1 Experimental procedure followed in converting amaranth stem to ethanol.

3.2.6. PRETREATMENT

A milled amaranth loading of 50 $g.kg^{-1}$ was used in either acid (H_2SO_4) or alkali ($Ca(OH)_2$) solutions (10 or 30 $g.kg^{-1}$ in water). An ultrasonic bath (see Figure 3.1) was used to supply indirect ultrasonic irradiation to samples. The biomass in acid or alkali was kept at a constant ultrasonic bath temperature of 40°C for up to 300 minutes at varying power settings (75, 150, 375, 600, and 750W) at a frequency of 35 kHz. All experiments were repeated 3 times.

Figure 3.2 Ultrasonic water bath (Elma Transonic)

3.2.7. HYDROLYSIS

The pretreated sample was hydrolyzed with a commercial cocktail of cellulase enzyme (NS 22192) from Novozymes, (Denmark). The filter paper unit of the enzyme was determined according to the standard procedure recommended by IUPAC as described by Ghose *et al.*, (1987). The enzyme activity was found to be 47.4 FPU. A loading of 15 and 30 FPU per gram of substrate was used with 1.25$g.L^{-1}$ Tween 80 at a pH of 5.0. Tween 80 was added as a surfactant to limit the absorption of cellulase to lignin in the broth. The pH was adjusted to 5.0 using either $Ca(OH)_2$ or H_2SO_4 prior to the addition of the enzymes. The solution was then incubated in 250mL GL 45 laboratory glass bottles at 50°C for 48 hours at 150 rpm shaking speed in a rotatory shaker (see Figure 3.2). The hydrolysate was then filtered and analyzed for sugars using high performance liquid chromatography (HPLC). All experiments were repeated 3 times.

Figure 3.3 Rotatory Shaker Incubator (Labex Platform Shaker)

3.2.8. FERMENTATION

The hydrolysates obtained after hydrolysis were filtered using vacuum filtration with whatman no. 246 filter paper. The solid residue was analyzed using Fourier Transmittance Infrared spectroscopy and Scanning Electron Microscopy and the filtrate was used for fermentation. *S. cerevisae* was activated from the dry dormant state using small amounts of the hydrolysis filtrate. An *S. cerevisae* loading of 50 $g.kg^{-1}$ was aseptically inoculated into the hydrolysate broth. The broth was incubated at 32° C for 48 hours at a shaking speed of 120 rpm under anaerobic conditions. The fermentation products i.e. residual sugars, ethanol and by-products were analyzed at set intervals using HPLC (see Figure 3.4). All experiments were repeated three times.

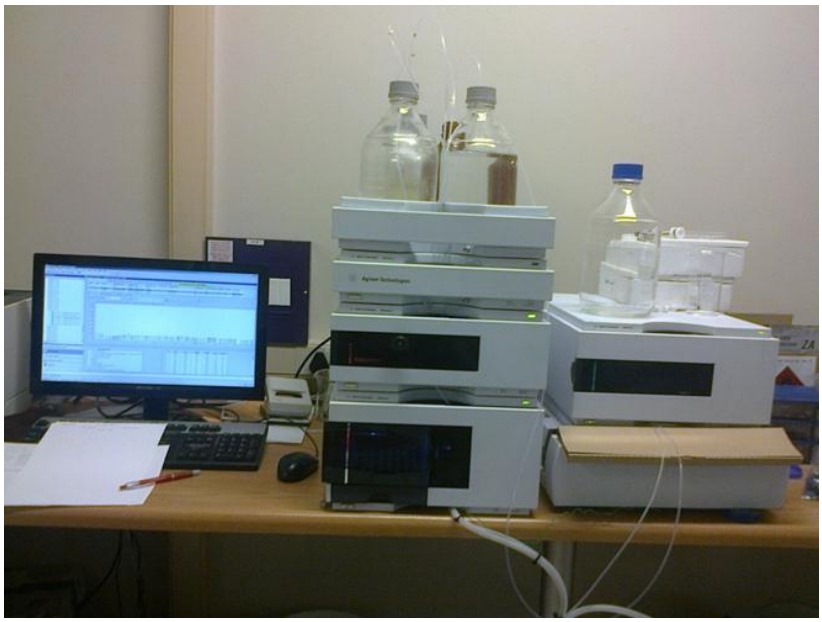

Figure 3.4 HPLC used in this study

3.3. ANALYTICAL METHODS

3.3.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The pH of sample was adjusted to 7.0. A volume of 2.0 ml of the sample was transferred to a clean polytop and the sample was diluted by adding 2.0 ml of distilled water. The sample was filtered into a clean HPLC vial using a 0.45µm and 0.2 µm syringe filter consecutively. Analysis was done using an Aligent series 1200 HPCL instrument fitted with a Shodex column for sugars and ethanol and an Aminex column for analysis of organic acids, HMF, and furfural. The instrument was fitted with an RI detector and water was used as mobile phase for the Shodex column and sulphuric acid for the Aminex column. The instrument condition for analysis are given in Table 3.2. The concentration of sugars and ethanol are presented in g/g. The concentration is calculated using the equation stated below.

Sugar concentration (g/L) = (Peak area / k-value)* dilution factor

Table 3.2:The instrument conditions for analysis with Shodex and Aminex column

Column conditions	Shodex	Aminex
Mobile phase	Water	5mM H_2SO_4
Flow rate	1.0 $ml.min^{-1}$	0.7$ml.min^{-1}$
Injection volume	2 µL	2µL
Column temperature	80°C	65°C
RID	59°C	59 °C

3.3.2. FOURIER TRANSFORM INFRA-RED (FTIR) – SHIMADZU FTIR 2000

Untreated and pretreated amaranth stem samples were analyzed using FTIR (see Figure 3.5) at 4 cm^{-1} and 40 scans per sample. The samples were prepared by mixing 3 mg of amaranth stem to 300 mg of potassium bromide (KBr) and the samples were then analyzed using the transmittance measure mode within the range of 400 – 4000cm^{-1}. This was conducted in order to see the effects of different pretreatments on the structure of amaranth stem.

Figure 3.5 Fourier Transform Infra-Red (FTIR)

3.3.3. SCANNING ELECTRON MICROSCOPY (SEM)

Samples of untreated and pretreated amaranth stem were analyzed in high vacuum mode to see morphological changes on the biomass. A Quanta 250 FEG instrument was used.

3.3.4. ULTRA-VIOLET SPECTROPHOTOMETER (UV)

Cell growth during fermentation was measured using a UV spectrophotometer (see Figure 3.5) at 600nm absorbance.

Figure 3.6 UV spectrophotometer

3.4. REFFERENCES

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Harmsen, P.F.H., Huijgen, W.J.J., Lopez, L.M.B. & Bakker, R.R.C. 2010. Literature review of physical and chemical pretreatment process for lignocellulose biomass. *Energy research centre of the Netherlands*, ECN-E --10-013

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. INTRODUCTION

In this chapter, the results obtained during ultrasonic-assisted pretreatment are presented and discussed in details. The main goal of the pretreatment step is to alter the structural and compositional elements of the biomass, thereby releasing fermentable sugars (Balat *et al*., 2008). The effect of parameters such as the energy input, catalyst concentration, sonication time and biomass loading on total sugar yield were investigated. The chapter is divided into three sections, i.e. in section 4.1 compositional analysis of amaranth stem are shown, in section 4.2 the results of ultrasonic assisted pretreatment with dilute acid/ and alkaline are given and in section 4.3 the fermentation results is shown.

4.2. COMPOSITION OF AMARANTH STEM

Amaranth stem was obtained from Agricultural Research Council (26°41'36"S, 27°05'35"E), Potchefstroom and was compositionally analyzed by ARC, Irene laboratories. The compositional analysis showed that there is 26.06 wt% cellulose, 12.07 wt% hemicellulose and 8.01 wt% lignin in amaranth stem used in this study. The results indicate that the maximum total sugar yields obtainable from amaranth stem used is 38.13%.

Table 4.1 Chemical composition of amaranth stem used in this study

Analysis	Composition (wt %)	Method Number
Dry matter	93.81	ASM013
Moisture	6.19	ASM013
Ash	16.38	ASM048
*Protein	9.41	Not SANAS accredited
Fat (ether extraction)	0.85	ASM044
Neutral detergent fibre (NDF)	46.24	ASM060
Acid detergent Fibre (ADF)	34.07	Not SANAS accredited
Acid detergent lignin (ADL)	8.01	Not SANAS accredited
HHV ($MJ.kg^{-1}$)	13.39	ASM053
Cellulose[a]	26.06	Calculated
Hemicellulose[b]	12.17	Calculated

*For the conversion of nitrogen to protein content, a factor of 5.25 was used.

[a]Cellulose content = ADF-ADL

[b]Hemicellulose content = NDF-ADF

4.3. PRETREATMENT RESULTS

4.3.1. ULTRASONIC ASSISTED DILUTE ACID PRETREATMENT

4.3.1.1. Comparison of ultrasonic assisted and dilute acid pretreatment

The effect of sonication time on the total sugar yield from amaranth stem was investigated with 30g.kg^{-1} and 10g.kg^{-1} of H_2SO_4 in water at a fixed ultrasonic power of 750W and varying treatment times (15 – 60 minutes)

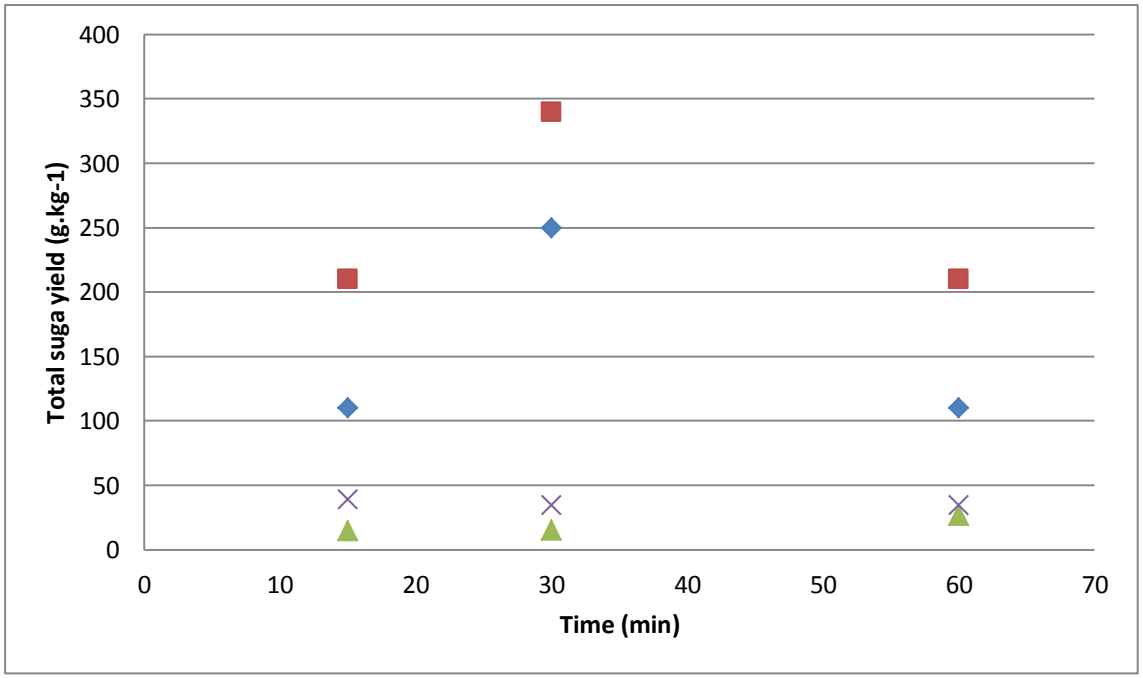

Figure 4.1 Effect of treatment with and without ultrasonication on total sugar yield at a sonication power of 750W (♦ -with sonication in 10g.kg H_2SO_4, ■ –with sonication in 30g.kg H_2SO_4, ▲- without sonication in 10g.kg H_2SO_4, × - without sonication in 30g.kgH_2SO_4).

From Figure 4.1 it can be seen that there is almost no increase in total sugar yield as pretreatment time increases for pretreatment without ultrasound. For ultrasonic assisted pretreatment, an increase in total sugar yields is observed from 0 to 30 min and a decrease with time is then observed after 30 min. The total sugar yields without ultrasound assistance are comparatively low compared to the ultrasonic assisted pretreatment. The significant increase in total sugar yields when ultrasound is used indicates its effectiveness in enhancing dilute acid pretreatment of amaranth stems. This improvement is associated with acoustic cavitation of ultrasound which is known to disrupt the bonds within polymers (Rehman *et al.*, 2013) and the breaking down of heterocyclic ether bond between monomers found in the

polymeric chain caused by dilute acid pretreatment (Velmurugan & Muthukumar, 2011). High amounts of total sugar yields (340g.kg^{-1}) were obtained after 30min of sonication pretreatment in 30 g.kg^{-1} sulphuric acid solution in water, demonstrating that amaranth stem requires short sonication time. After 30min, the amount of total sugar yields are seen to gradually decrease showing that prolonging sonication treatment beyond this point has no benefits in terms of sugars released rather resulting in degradation of sugars.

4.3.1.2. The effect of dilute H_2SO_4 concentration

The influence of sulphuric acid loading (10, 30, and 50 g.kg^{-1}) on total sugar yields at different power densities (54, 216, and 270 kJ.g^{-1}) (different wattages in the same treatment time of 30 minute) is shown in Figure 4.3.

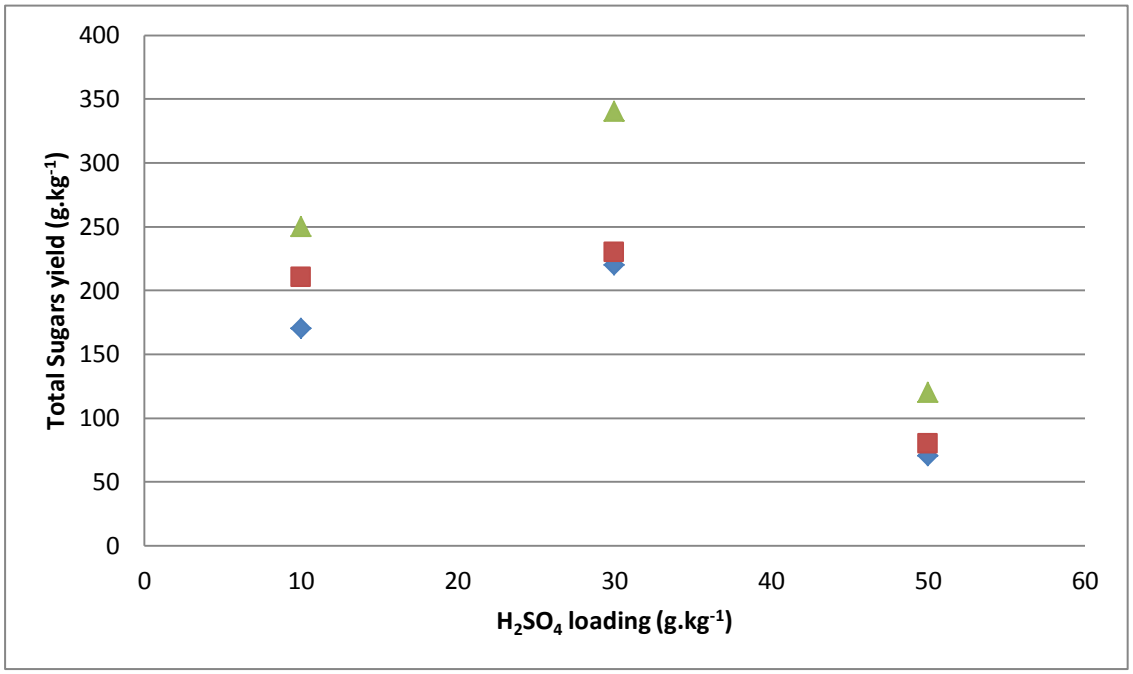

Figure 4.2: The effect of H2SO4 loading on the total sugar yield at a fixed treatment time of 30 minutes and different power setting, (♦ - energy input of 54kJ.g^{-1}, ■ – energy input of 216kJ.g^{-1}, ▲ – energy input of 270kJ.g^{-1}).

From Figure 4.2 it is seen that total sugar yield increases with an increase in sulphuric acid concentration up to a certain point, where the total sugars starts decreasing. It was observed that at the same pretreatment time and power, pretreatment with 50g.kg^{-1} H_2SO_4 in water resulted in significantly lower total sugar yield than 10 and 30g.kg^{-1} H_2SO_4 in water. Maximum total sugar yield (340g.kg^{-1}) was observed with 30g.kg^{-1} H_2SO_4 in water at an energy input of 270kJ.g^{-1}. The increase in total sugars yields may be attributed to the fact that dilute sulphuric acid hydrolyzes and removes most of hemicellulose as dissolved sugars

(xylose) and glucose yield from cellulose increase with hemicellulose removal (Kumar *et al*., 2009). Also with increase in H_2SO_4 concentrations the hydrolysis of hemicellulose increases, hence the increase in sugars with the increase in sulphuric acid concentrations. The decrease observed in total sugars at 50g.kg^{-1} H_2SO_4 in water concentrations is associated with degradation of sugars at high acid concentrations. It is also observed that at lower acid concentrations total sugar yields gradually increase however into smaller amounts whereas at moderate concentration (30 and 50g.kg^{-1}) there is a significant increase observed. This sudden increase is attributed to the improved contact between sulphuric acid and biomass due to ultrasonic at higher levels of energy input.

4.3.1.3. Effect of biomass loading on total sugars yields

The effect of biomass loading on total sugar yields were investigated at biomass loadings of 10, 50, and 100 g.kg^{-1} in 30 g.kg^{-1} sulphuric acid in water. The energy input was fixed at 270kJ.g^{-1}. Figure 4.3 represents the total sugar yields obtained at different biomass loading.

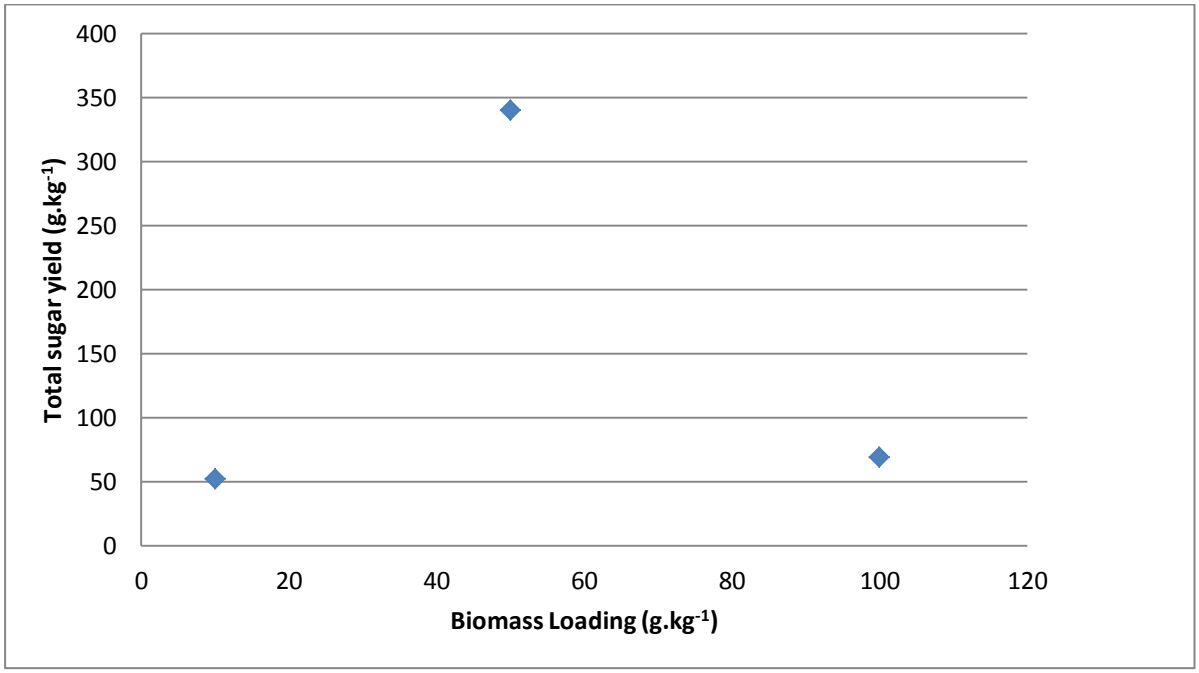

Figure 4.3: The graph demonstrating the effect of biomass loading on total sugar yields and other by-products formed (♦ -total sugar yield g.kg^{-1}).

From Figure 4.3 it can be seen that there is a sharp increase observed with an increase in biomass loading from 10 to 50g.kg^{-1} biomass loading, followed by a significant decrease. At a biomass loading of 10g.kg^{-1} biomass, comparatively low total sugar yield are observed. A maximum total sugar yield (340g.kg^{-1}) was observes with a 50g.kg^{-1} biomass loading. At

increased biomass loadings (100g.kg^{-1}) a significant decrease is observed in total sugars yield and it is also observed that at lower biomass concentrations, total sugars yield are not increasing. This is attributed to reduced physical and chemical ultrasonic effect caused by too much biomass in the medium. Since physicochemical properties of the medium such as viscosity and surface tension influence the propagation of sound waves, cavitation and the generation of radicals (Rehman *et al.*, 2012).

4.3.1.4. Effect of energy input on sugar yield after hydrolysis

To determine the effect of energy input on sugar yields, 50 g.kg^{-1} of amaranth stem was pretreated with 10 and 30 g.kg^{-1} sulphuric acid (H_2SO_4) at a constant treatment time of 30 minutes and varying wattage settings (54, 216, 270, 432 and 540 kJ.g^{-1}) for the ultrasonic bath, results shown in Figure 4.4.

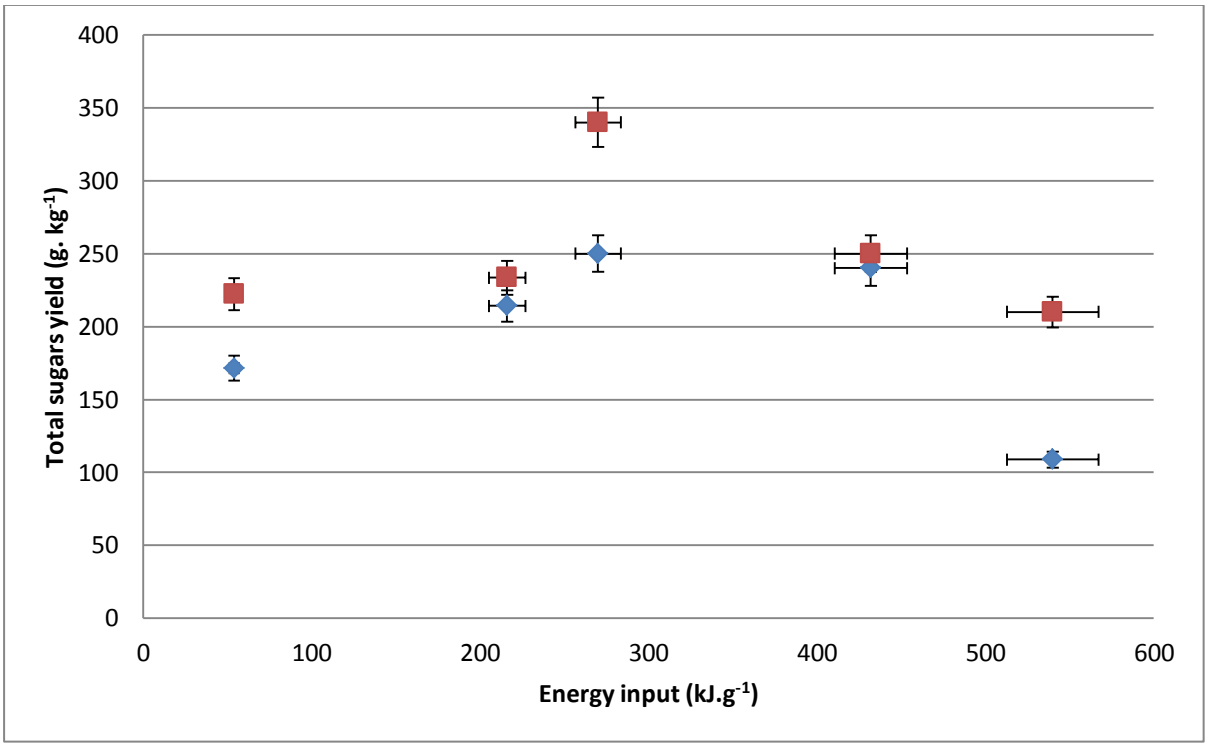

Figure 4.4: **Effect of energy input on sugars yield at different sulphuric acid loadings (♦ -10g.kg^{-1} sulphuric acid, ■ -30g.kg^{-1} sulphuric acid).**

From Figure 4.4 it is observed that the total sugars yield initially increased with an increase in energy input and acid concentration. As energy input increases, high pressure localized waves produced by the ultrasonic vibrations causes increased shear forces that break down the lignocellulose structure thereby releasing monomeric sugars. The highest total sugars yield (350g.kg^{-1}) biomass was obtained using 30g.kg^{-1} H_2SO_4 in water as a solvent and at energy input of 270 kJ.g^{-1} biomass. The conversion efficiency was 90% of the theoretical sugar yield. At lower energy input, the interactions between solvent and biomass are inadequate

hence the lower amounts of sugar yields are obtained between 54 to 216kJ.g^{-1}. It is observed that an increase in energy input beyond 270 kJ.g^{-1} causes a decrease in total sugar yields due to sugar degradation at higher energy inputs. The maximum total sugars yield obtained at 270 kJ.g^{-1} energy inputs indicate that moderate energy input are enough to disruption the amaranth stem structure sufficiently to liberate monomeric sugars.

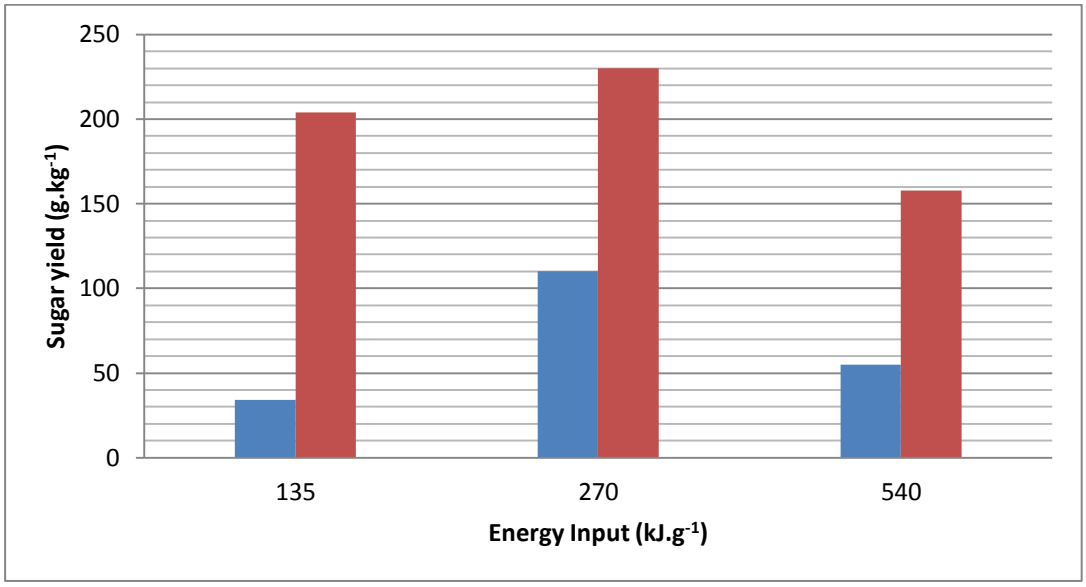

Figure 4.5: Effect of sulphuric acid pretreatment of pentose and hexose sugar liberated (■ –hexose sugar yield g.kg^{-1}, ■ – pentose sugar yield g.kg^{-1}).

From Figure 4.5 it is seen that the amount of hexose sugars liberated were higher than pentose sugars liberated. A maximum yield of both pentose and hexose sugar were liberated at an energy input of 270 kJ.g^{-1}. The major components of lignocellulose are hemicellulose which contains both hexose and pentose sugars and cellulose that only contains hexose sugars. Dilute acid pretreatment removes all hemicelluloses by hydrolysing it into monomeric sugars. As hemicellulose is removed digestibility of cellulose increases thereby increasing the total yield of hexose sugars. Ultrasonic cavitations interact with sulphuric acid and improve the mass transfer thus enhancing the liberation of sugars. There are lower pentose sugars yield compared to hexose total sugars yield, this is attributed to degradation hydrolysed hemicellulose.

4.3.1.5. Effect of ultrasonic-assisted dilute H_2SO_4 pretreatment on amaranth stem structure

4.3.1.5.1. Scanning Electron Microscopy analysis

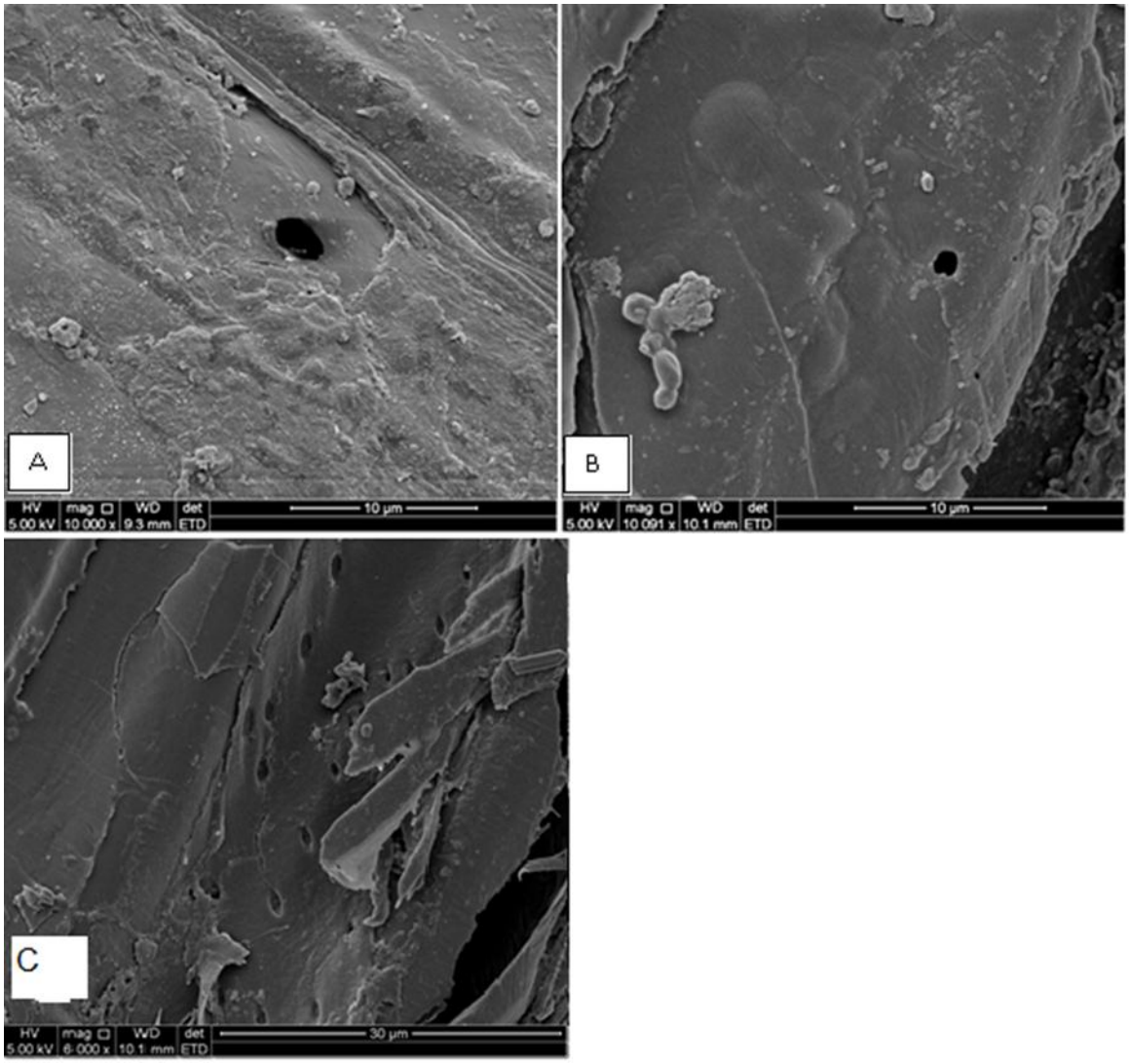

Figure 4.6 Scanning electron microscope images of unpretreated amaranth stem (A), H_2SO_4 pretreated amaranth stem (B) and ultrasound-assisted H_2SO_4 pretreated amaranth stem (C).

Scanning electron microscopy (SEM) analysis was used to assess the morphological changes to the plant material during pretreatment. Figure 4.6 show the SEM micrographs of milled amaranth stem before (4.6a), after pretreatment with H_2SO_4 (4.6b) and after ultrasonic-assisted H_2SO_4 pretreatment (4.6c). Ultrasonic-assisted pretreatment was done in $30g.kg^{-1}$ sulphuric acid in water for 30 minutes at 750W power setting. It is observed from the SEM images that the untreated amaranth stem cell wall is intact and smooth with minor fragmentation as a result of milling. The amaranth stem that was pretreated with dilute acid

without sonication showed no significant morphological changes except for minor damages on the outer plant wall. The plant cell wall surface of the ultrasound-assisted acid pretreated sample was disrupted indicating that the ultrasound-assisted dilute acid was able to open up the cell wall. The results confirms what was observed on the results above that ultrasonic assisted pretreatment was more effective in disrupting the lignocellulose material and thereby liberating more sugars than dilute acid pretreatment alone.

4.3.1.5.2. Fourier Transform InfraRed (FTIR) FTIR Analysis

The Fourier Transform Infrared (FTIR) analysis was used to characterize structural changes that occur in the milled amaranth stem after ultrasound-assisted dilute acid pretreatment. The FTIR spectra for untreated amaranth stem (A) and ultrasound-assisted acid pretreated amaranth stem (B) shown in Figure 4.7. The spectra show significant structural changes as a result of pretreatment.

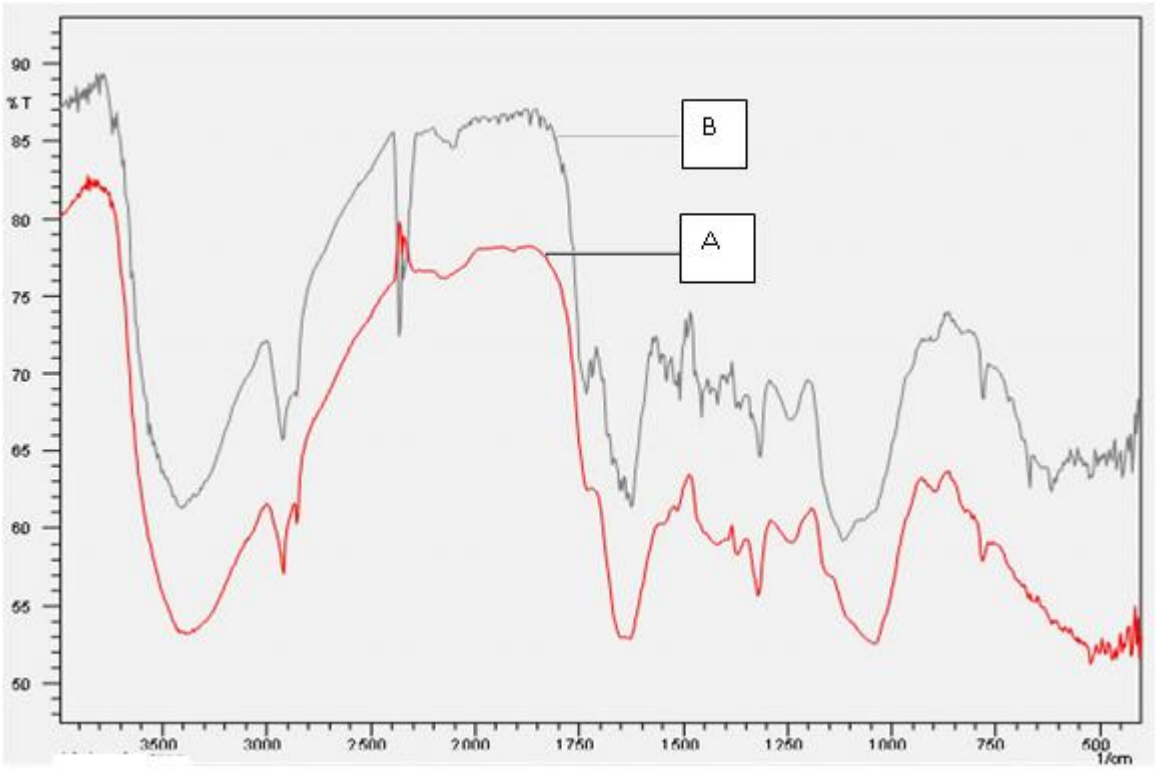

Figure 4.7: The FTIR spectra of (A) unpretreated amaranth and (B) ultrasonic assisted amaranth stem treated with 30g.kg^{-1} H_2SO_4 in water at 750W for 30 minutes.

Table 4.2 shows the summarized vibrational frequencies of different functional groups in the infrared spectrum of the biomass.

Table 4.2: The assignment of the band position in the FTIR spectra (Singh *et al*., 2014)

Band Position	Assignment
3000-3800	O-H stretching (related to the rupture of cellulose hydrogen bonds)
2900	C-H stretching (related to rupture of methyl/methylene group of cellulose)
2850	Associated with cellulose
1745	Carbonyl bonds (related to lignin side chain removal)
1738	C=O stretching due to carbohydrate linked with lignin
1700	Carboxylic acids or ester groups
1595	Aromatic ring stretch and aromatic skeletal vibrations(related to removal of lignin)
1508	Aromatic ring and skeletal vibration (related to removal of lignin)
1458	Aromatic ring vibration (related to removal of lignin) and asymmetric C-H deformations
1428	Band of cellulose and aromatic skeletal vibration
1378	Symmetric C-H bending
1260	Ester absorbance (related to removal of uronic acids)
1245	C=O absorption (resulting from acetyl groups cleavage)
1238	Hemicellulose- lignin linkage
1059	C=O stretching due to carbohydrate-lignin linkage
1050 - 1250	-C-O-C and C-OH bending in lignin and hemicelluloses

A broad peak was observed on the untreated sample at 3500-3400 cm^{-1} which represents the stretching vibrations of hydroxyl group and hydrogen bonding that is associated with the presence of cellulose. For the ultrasound-assisted treated sample the peak lessens confirming reduction of cellulose crystallinity. There were also two peaks observed at 2900 and 2850 cm^{-1}, these peaks corresponds with the C-H vibrations in the methyl and methylene groups. The peak at 2900 cm^{-1} decreases and the one at 2850 cm^{-1} completely disappears also indicating the removal of lignin from the amaranth stem after pretreatment. There is a peak observed at 2350 cm^{-1} on the untreated amaranth stem which completely disappears after pretreatment, this is attributed to the decreased crystallinity of cellulose. There is also a significant reduction observed on the pretreated sample at 1600-1650 cm^{-1} this band is attributed to the

presence of C=O bonds in non-conjugated and conjugated ketones which confirms that lignin has been broken down. This is evidence and confirmation that the ultrasound-assisted acid was able to disrupt the lignocellulose structure of the amaranth stem. However the bands at 1050 cm^{-1} and between 900 – 800cm^{-1} demonstrated a slight change, indicating not all of the cellulose sugars were liberated as the ultrasound-assisted acid pretreatment displayed a selective degradation pattern characterized by the preferential removal of lignin during pretreatment.

4.3.2. ULTRASONIC ASSISTED ALKALI PRETREATMENT

Ultrasonic parameters such as energy input and time were investigated so to see their effect on the total sugar yields and also other parameters such biomass loading and different concentrations of the $Ca(OH)_2$ were evaluated.

4.3.2.1. Comparison of ultrasonic assisted and dilute alkali pretreatment

The effect of treatment time on the total sugars yield from amaranth stem were investigated at a constant power setting of 750W in 10g.kg^{-1} or 30g.kg^{-1} $Ca(OH)_2$ in water solution. The results are presented in Figure 4.8.

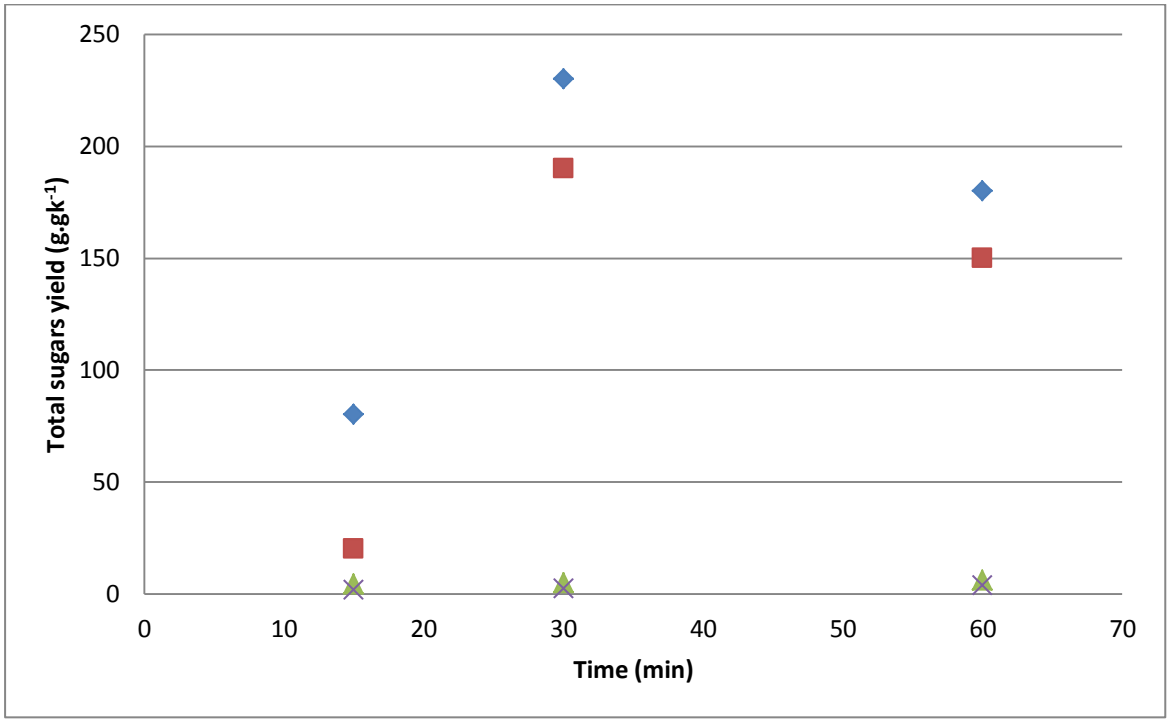

Figure 4.8: Effect of sonication time on total sugars yield at a sonication power of 750W (♦ -with sonication in 30g.kg^{-1} $Ca(OH)_2$, ■ –with sonication in 10g.kg^{-1} $Ca(OH)_2$, ▲- without sonication in 30g.kg^{-1} $Ca(OH)_2$, × - without sonication in 10g.kg^{-1} $Ca(OH)_2$).

From Figure 4.8 there is a sharp increase observed on the total sugars yield with increase in treatment time up to a certain point, where an increase in time gradually decreases total

sugars yield. The total sugar yields obtained when dilute alkaline was used without ultrasound are very low, and the total sugar yields obtained when ultrasonic assisted dilute alkali pretreatment is used are significantly higher. The results observed indicates that ultrasound does enhance the liberation of total sugars, this improved performance is linked to the hydrodynamic shear forces produced by ultrasound promoting the breakage of chain polymers and to the alkaline catalyzed reaction which break the aryl ether linkages found in lignin thereby increasing enzyme accessibility, resulting in increased total sugar yield. The maximum total sugar yield ($230 g.kg^{-1}$) was obtained after 30 minutes of pretreating with $30 g.kg^{-1}$ $Ca(OH)_2$ in water, with the conversion efficiency of 61%. Ultrasonic-assisted alkaline treatment increased efficiency conversion of total sugar from 5.3 to 61% as compared to non-sonicated alkaline treatment amaranth stem. The results demonstrate that amaranth stem structure is not too reluctant since it requires shorter sonication time. However prolonging the sonication time further than 30 minutes does not improve the liberation sugars rather damage of the sugars occur due to degradation of sugars, as observed at the decrease of total sugars at 60 minutes.

4.3.2.2. The effect of $Ca(OH)_2$ concentrations on total sugars yield after hydrolysis

The effect of $Ca(OH)_2$ concentration on total sugars yield, from amaranth stem was investigated at different ultrasonic energy input (54, 216, and 270 $kJ.g^{-1}$) at a fixed treatment time of 30 minutes. Results presented in Figure 4.9.

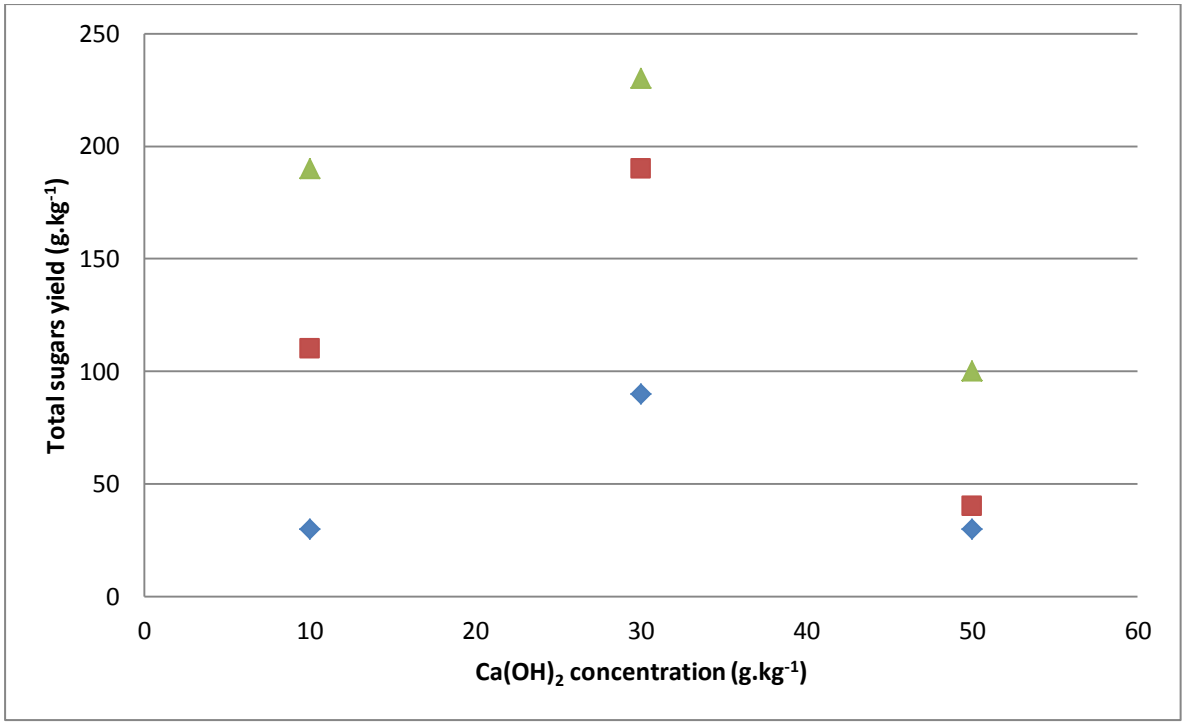

Figure 4.9: The effect of $Ca(OH)_2$ loading on the total sugars yield at a fixed treatment time of 30 minutes and different power setting, (♦ - energy input of 54kJ.g^{-1}, ■ –energy input of 216kJ.g^{-1}, ▲ –energy input of 270kJ.g^{-1}).

From Figure 4.9 it is observed that with increase in $Ca(OH)_2$ concentrations (10g.kg^{-1} to 30 g.kg^{-1} $Ca(OH)_2$ in water), total sugars yield also increase with $Ca(OH)_2$ concentrations, and a decrease in total sugars is observed with 50g.kg^{-1} $Ca(OH)_2$ in water. The increase of total sugars yield is attributed to the cavitation effect caused by ultrasound and alkaline reaction. Alkaline pretreatment mechanism is to be saponification of intermolecular ester bonds crosslinking xylan and hemicelluloses and lignin, thereby causing swelling to the lignocellulose structure, and separating the linkages between lignin and carbohydrates resulting in disruption of the lignocellulose structure (Signh *et al*., 2014). The maximum total sugar yield (230g.kg^{-1}) is observed with treatment of 30g.kg^{-1} $Ca(OH)_2$ in water at 270kJ.g^{-1} energy input. Lower amounts of total sugars yield are observed at higher concentrations of $Ca(OH)_2$ (50g.kg^{-1}) since alkaline pretreatment form irrecoverable salts or is incorporated as salt in the biomass thereby inhibiting the liberation of sugars. Therefore, indicating that lower concentrations of calcium hydroxide are more favourable than higher concentration to release sugars from amaranth stem.

4.3.2.3. The effect of biomass loading on total sugars yield

The effect of biomass loading on total sugar yields was evaluated at different biomass loading (1, 5, 10 wt%) was subject under 750W power for 30 min in 30 g.kg^{-1} $Ca(OH)_2$ solution in water.

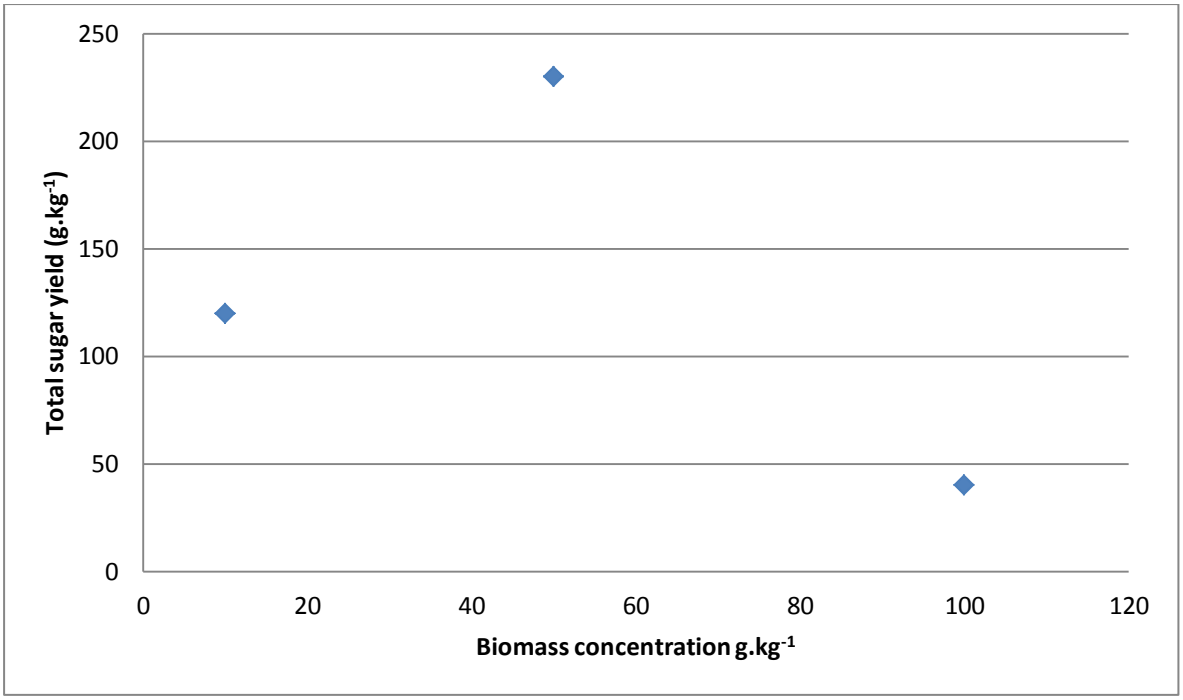

Figure 4.10: Graph showing the effect of biomass loading on total sugar yields (♦ -total sugar yield g.kg^{-1})

From Figure 4.10 it is observed increase in biomass loading (10g.kg^{-1} to 50g.kg^{-1}) also increased with total sugar yields from 53g.kg^{-1} to 340g.kg^{-1} up to a certain level, because when the biomass loading was furthered to 100g.kg^{-1} the total sugar yields decreased. The maximum total sugar yields 340g.kg^{-1} biomass were observed with 50g.kg^{-1} biomass loading, indicating that lower to moderate biomass loading are suitable for amaranth stems. The favourable loading conditions depend on the type of biomass as well as its reluctance (Subhedar & Gogate, 2014). The low amounts of total sugar yield observed at increased biomass loading may be attributed to the increased concentration of solids and ultrasound physical effect, since at increased biomass loadings the viscosity increases within the sample thus reducing mixing effect of the ultrasound.

4.3.2.4. The effect of energy input on total sugars yield

Amaranth stems treated with ultrasound and different concentrations of $Ca(OH)_2$ (10g.kg^{-1} and 30g.kg^{-1} $Ca(OH)_2$ in water), and different power settings (150, 600 and 750 W) to investigate the effect of energy input on the total sugars yield.

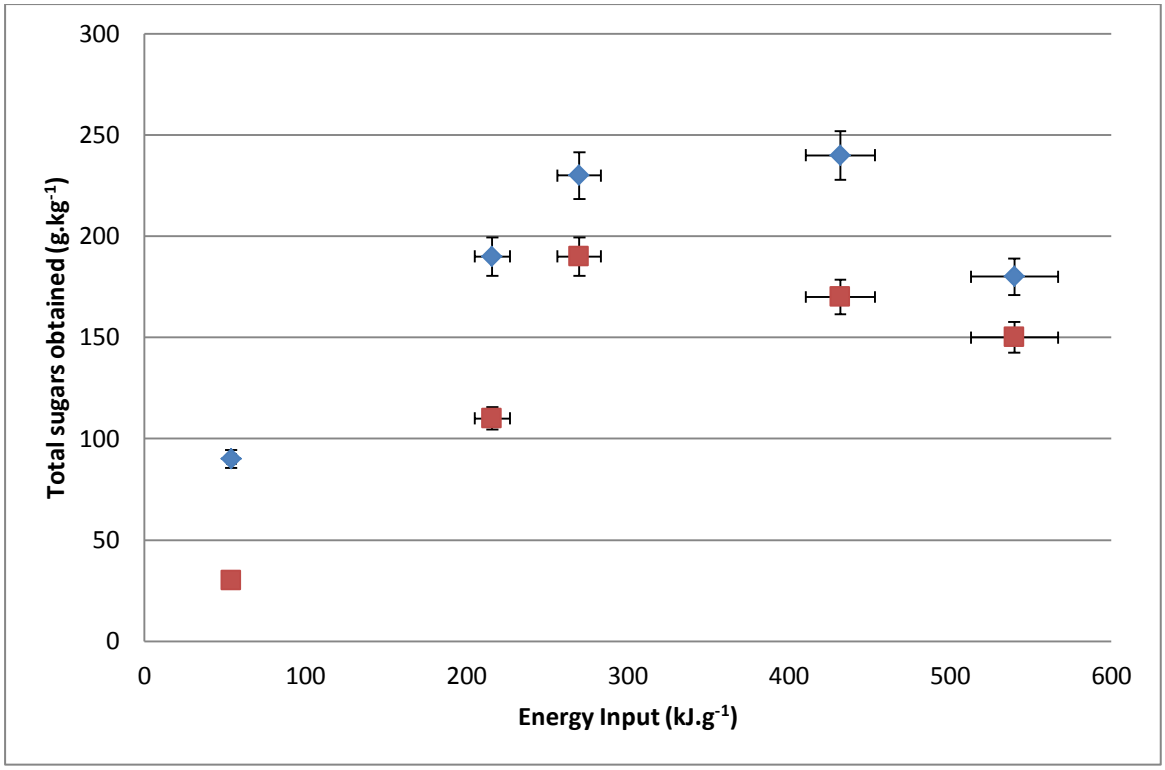

Figure 4.11: Effect of energy input on total sugars yield at different sulphuric acid loadings (♦ -30g.kg^{-1} $Ca(OH)_2$, ■ -10g.kg^{-1} $Ca(OH)_2$).

From Figure 4.11 it is observed that total sugars yield increase with the increase in energy input up to a certain level. The maximum yield for 10g.kg^{-1} and 30g.kg^{-1} is observed up to 270kJ.g^{-1} and 432 g.kg^{-1} energy input, respectively and a gradually decrease in total sugar yield is observed after. The increase in total sugars yield is associated with ultrasonic cavitation process in which, the asymmetric bubble collapses closer to the solid- liquid ratio interface, thereby generating liquid jets that fragment particles and increase their surface area promoting the alkaline pretreatment (Kim *et al*., 2010). Alkaline treatment break down aryl ether linkages found in lignin therefore casing disruption to the biomass thus an increase in total sugar yields. The maximum total sugar yield 240g.kg^{-1} was observed at 432kJ.g^{-1} when treated with 30g.kg^{-1} $Ca(OH)_2$ in water. However at higher energy input, sugars are damaged and formation of by-products thus a decrease in total sugars beyond 270 and 432 kJ.g^{-1} for 10 and 30g.kg^{-1}, respectively.

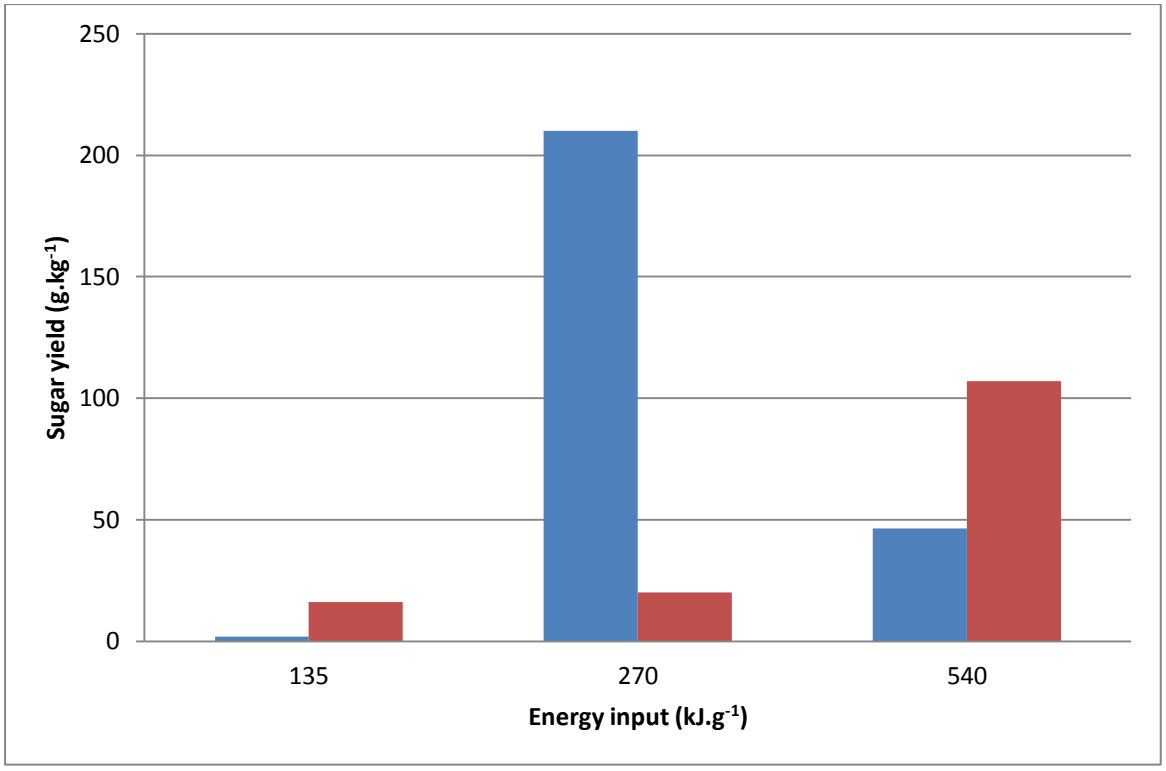

Figure 4.12: Effect of calcium hydroxide pretreatment on pentose and hexose sugars liberated (■ –hexose sugar yield g.kg^{-1}, ■ – pentose sugar yield g.kg^{-1}).[1]

From Figure 4.12 it is observed that hexose sugars were mostly released as compared to pentose sugars. However these results confirm that the calcium hydroxide combined with ultrasound was able to breakdown the complex lignocellulose structure, thereby liberating monomeric fermentable sugars. Lignocellulose material is composed mostly of cellulose and hemicellulose, therefore efficiency of a pretreatment technique is determined by its ability to break down either cellulose or hemicellulose. Hemicellulose is composed of both pentose and hexose sugars and cellulose is composed of only hexose sugars. At 270kJ.g^{-1} energy input highest cellulose sugars were obtained and a decrease at 540kJ.g^{-1} is observed which may be attributed to the degradation of sugars. The big spike observed of hemicellulosic sugars obtained at 270kJ.g^{-1} may be associated with the mechanism of alkaline pretreatment, which is known to selectively remove or solubilize lignin and hemicellulose but not affecting cellulose.

4.3.2.5. Effect of ultrasonic-assisted Ca(OH)2 pretreatment on amaranth stems structure

4.3.2.5.1. Scanning Electron Microscopy analysis

Scanning electron microscopy was used to study the morphological features and surface characteristics of the material before and after pretreatment with ultrasonic-assisted dilute alkaline solutions.

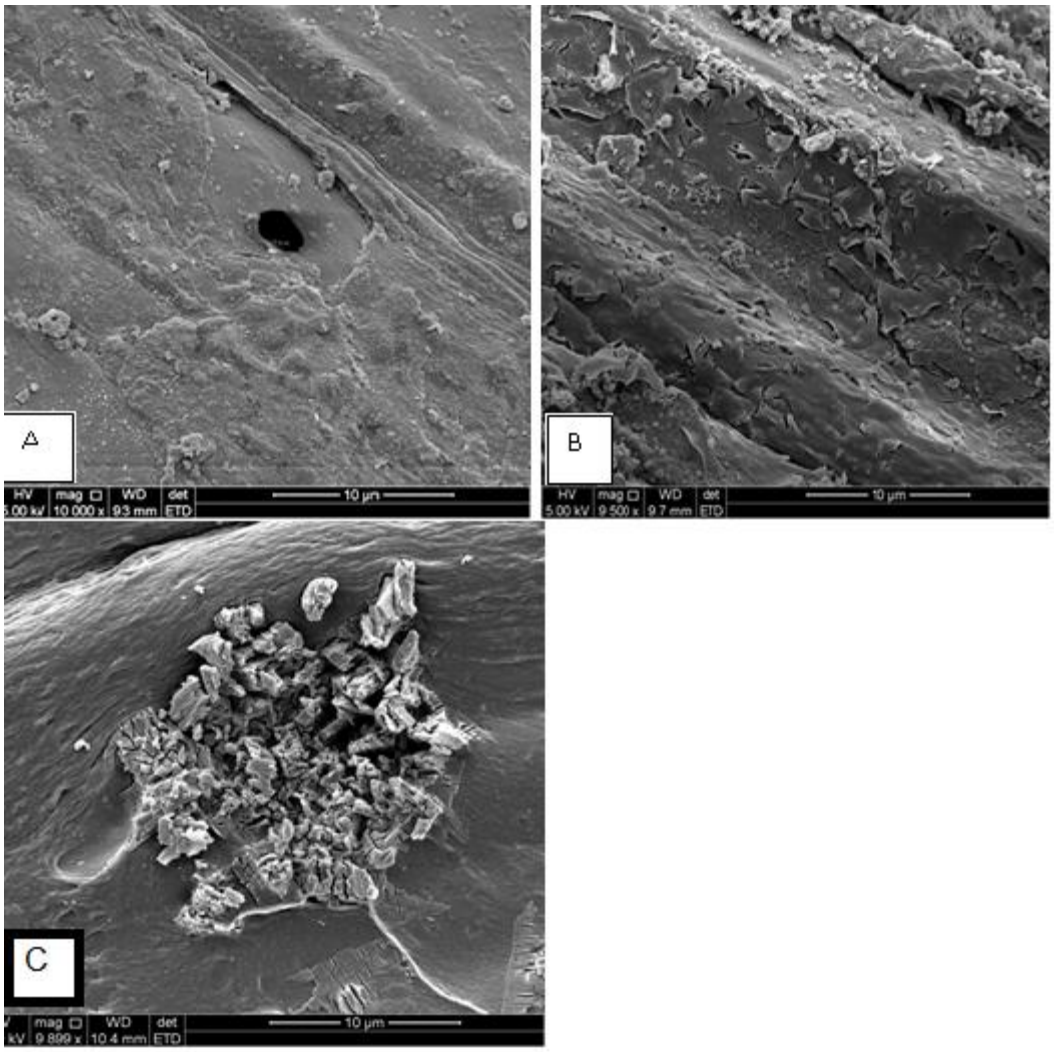

Figure 4.13: effect of calcium hydroxide pretreatment on pentose and hexose sugars liberated (■ –hexose sugar yield g.kg^{-1}, ■ – pentose sugar yield g.kg^{-1}).

Amaranth stems was pretreated with 30g.kg^{-1} $Ca(OH)_2$ in water for 30 minutes in an ultrasonic bath at 750W. From Figure 4.13, it is observed that the cell wall of the untreated sample (4.13a) is compact and hard with insignificant disintegration caused by milling. There was no significant difference in morphology observed after pretreatmet in alkaline solution without ultrasonication (4.13b) and the untreated sample. Confirming that the ultrasonic-assisted dilute alkali pretreated amaranth stem (4.13c) showed significant morphological

changes, the outer surface of the plant wall was not smooth it had a rough surface with cracks and openings, and complexes embedded on the surface. This is a very significant effect which confirms that ultrasonic assisted alkali was able to disrupt the amaranth stem structure as disruptions are observed on the Figure (4.13c).

4.3.2.5.2. FTIR Analysis

The FTIR analysis was used to monitor the changes that occur in the structure of amaranth stem after ultrasound-assisted alkali pretreatment. There was significant change in the pretreated spectra when compared to the untreated spectra as shown in Figure 4.14.

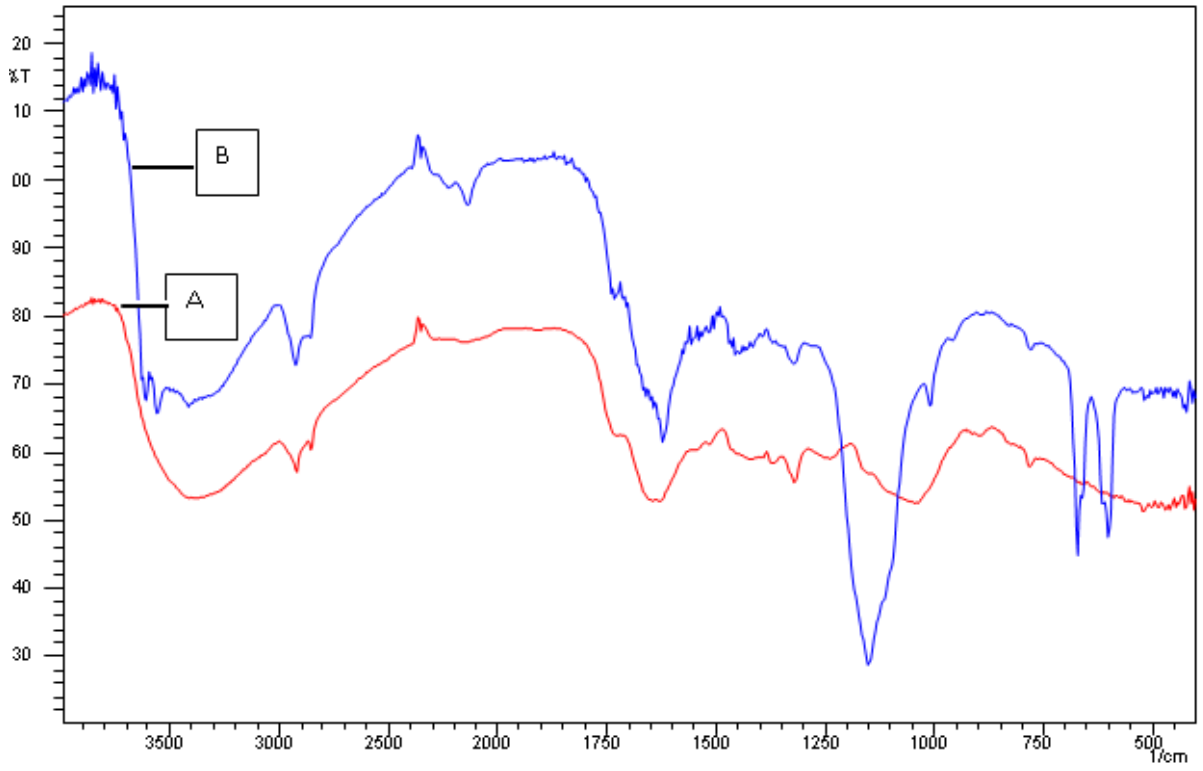

Figure 4.14: The FTIR spectra of (A) unpretreated amaranth and (B) ultrasound-assisted amaranth stem with 30g.kg^{-1} $Ca(OH)_2$ at 750W for 30min.

Table 4.2 shows the summarized vibrational frequencies of different functional groups in the infrared spectrum of the biomass.

Table 4.3: The assignment of the band position in the FTIR spectra (Singh *et al.*, 2014)

Band Position	Assignment
3000-3800	O-H stretching (related to the rupture of cellulose hydrogen bonds)
2900	C-H stretching (related to rupture of methyl/methylene group of cellulose)

2850	Associated with cellulose
1745	Carbonyl bonds (related to lignin side chain removal)
1738	C=O stretching due to carbohydrate linked with lignin
1720	Carboxylic acids or ester groups
1595	Aromatic ring stretch and aromatic skeletal vibrations(related to removal of lignin)
1508	Aromatic ring and skeletal vibration (related to removal of lignin)
1458	Aromatic ring vibration (related to removal of lignin) and asymmetric C-H deformations
1428	Band of cellulose and aromatic skeletal vibration
1378	Symmetric C-H bending
1260	Ester absorbance (related to removal of uronic acids)
1245	C=O absorption (resulting from acetyl groups cleavage)
1238	Hemicellulose- lignin linkage
1059	C=O stretching due to carbohydrate-lignin linkage
1050 - 1250	-C-O-C and C-OH bending in lignin and hemicellulose

From Figure 4.14 there is a prominent absorption band at 3500 – 3000 cm^{-1} seen on the untreated sample which is associated with the stretching vibrations of O-H bonding which is associates with cellulose, on the pretreated sample spectra this band narrows to three peaks and another band observed at 2350cm^{-1} which are attributed to reduced crystallinity of cellulose. Also the band at 2900cm^{-1} and 2800cm^{-1} which attribute to C-H vibration in the methyl and methylene groups indicating that some of the lignin was broken down and at 650 cm^{-1} and 700 cm^{-1} there are absorption bands that are demonstrate that there was some lignin remaining even after pretreatment. There is peak observed at 1600 cm^{-1} on the untreated spectra becomes sharper and bigger on the treated sample spectra is also associated with removal of lignin. There is also a very large and prominent peak observed at 1300 cm^{-1} which is associated with the removal of hemicellulose – lignin linkage. The FTIR analysis of the pretreated sample displayed significant differences in its functional group compare to the untreated sample confirming that the ultrasound-assisted alkali pretreatment did have an effect on the amaranth stem.

4.4. CONCLUDING REMARKS

In this chapter the pretreatment results were discussed and from the results it is clear that the ultrasound assisted with $Ca(OH)_2$ and H_2SO_4 was able to disrupt the amaranth stem structure liberating monomeric sugars. The effect of parameters such as energy input, catalyst concentration, energy input (time and power), and biomass loading on total sugar yield was evaluated and the results confirmed that the pretreatment was effective. The FTIR characterization also demonstrated a significant change in the functional groups found in the biomass after pretreatment and SEM analysis also validated the results with morphological effects of disrupted plant cell wall. The pretreatment has proven to be viable for liberating sugars from amaranth stem biomass.

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CHAPTER FIVE: FERMENTATION RESULTS

5.1. INTRODUCTION

In this chapter ethanol yields and sugar uptake curves obtained during fermentation are given and discussed. The results obtained after fermenting the hydrolysate obtained after using ultrasonic-assisted dilute acid pretreatment and after using ultrasonic-assisted dilute alkali pretreatment are shown in detail in sections5.2.1 and 5.2.2, respectively. Comparison between alkali and acid assisted treatment is discussed in section 5.2.3.

5.2. FERMENTATION RESULTS

Amaranth stems were treated with either 30g.kg^{-1} $Ca(OH)_2$ or 30g.kg^{-1} H_2SO_4 in water at 750W for 30 minutes, and the samples were subsequently hydrolyzed for 48hrs. After hydrolysis the samples were fermented with *S. cerevisae* at 32°C in a rotatory incubator for 48hrs. Samples were taken at set intervals for analysis of sugars and ethanol content.

5.2.1. FERMENTATION OF ULTRASONIC-ASSISTED ACID PRETREATED HYDROLYSATES.

Hydrolysates obtained after hydrolysis of ultrasonic-assisted pretreated amaranth stems were fermented using *S. cerevisae* at 32°C for 48 hours in a rotatory incubator. The fermentation results for hydrolysates obtained from dilute acid ultrasonic-assisted pretreatment with 30g.kg^{-1} H_2SO_4 in water is shown in Figure 5.1

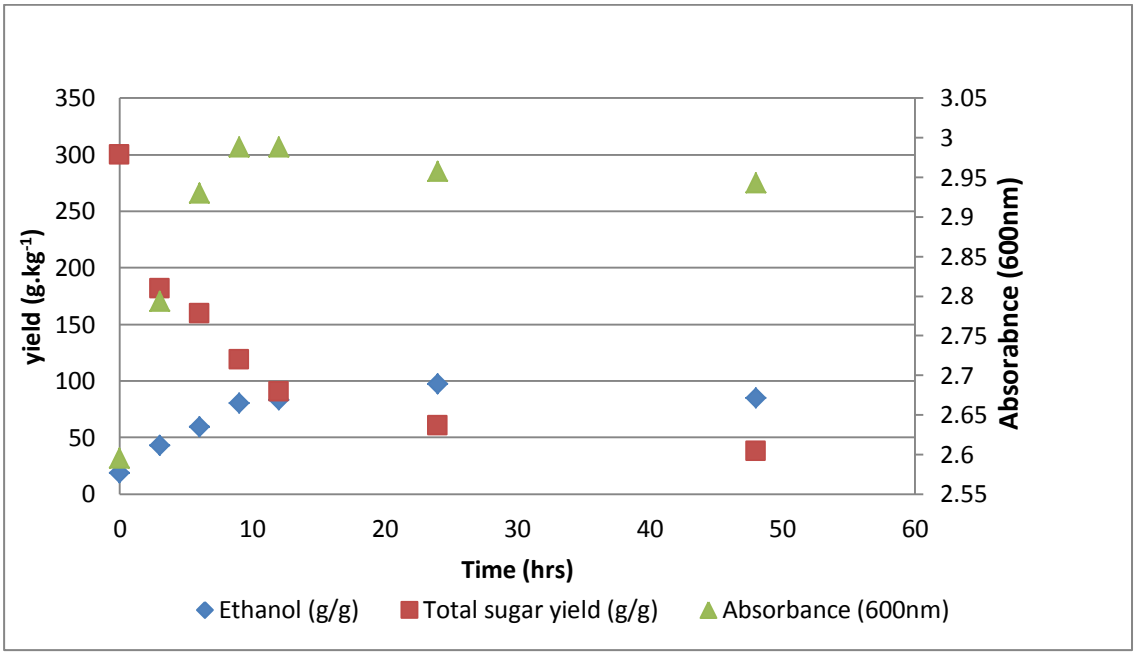

Figure 5.1: Sugar uptake curve and ethanol production during fermentation of hydrolysis broth with *S. cerevisae* (■ – total sugar yield g.kg^{-1}, ▲ – absorbance 600mn, ♦ - ethanol yield g.kg^{-1}).

From Figure 5.1 it can be seen that total sugar yield decreased with time as ethanol and absorbance increased gradually during fermentation. Ethanol was immediately produced which is probably the result of activating the yeast cells with the fermentation broth. The sugars decreased gradually with time as the cell density increased for the first 9 hours indicating growth and division of the cells. A stationary phase is observed on the cell density between 9 – 12 hours which illustrates that the resources for the cell are limited and cells stop dividing and started dying after 24 hours as a decrease on cell density is observed. *S. cerevisae* ferments the hexoses liberated from the lignocellulose biomass in ethanol but not the pentoses (Cardona *et al*., 2010). From Figure 5.1 it is observed that ethanol increases with the cell density and a decrease in total sugar content. The highest ethanol yield of 90 $g.kg^{-1}$ of biomass is obtained after 24 hours with a productivity of $0.31g.L^{-1}.h^{-1}$. The low yields of ethanol are due to the formation of inhibitory compounds that are formed as byproducts during dilute acid pretreatment. The inhibitory compounds are known to diffuse through the cell membrane and decrease the intracellular pH, thus causing adverse effects to the metabolism of the microorganism (Velmurugan & Muthukumar, 2011).

5.2.2. FERMENTATION OF ULTRASONIC-ASSISTED ALKALI PRETREATED HYDROLYSATES

Hydrolysate was filtered and fermented with $5g.L^{-1}$ of *S. cerevisae* for 48 hours in rotary incubator at 32°C for 48hrs. To observe the cell growth, sugar uptake, and ethanol production, the fermented hydrolysate was sampled out in three hour time intervals. The results are presented in Figure 5.2.

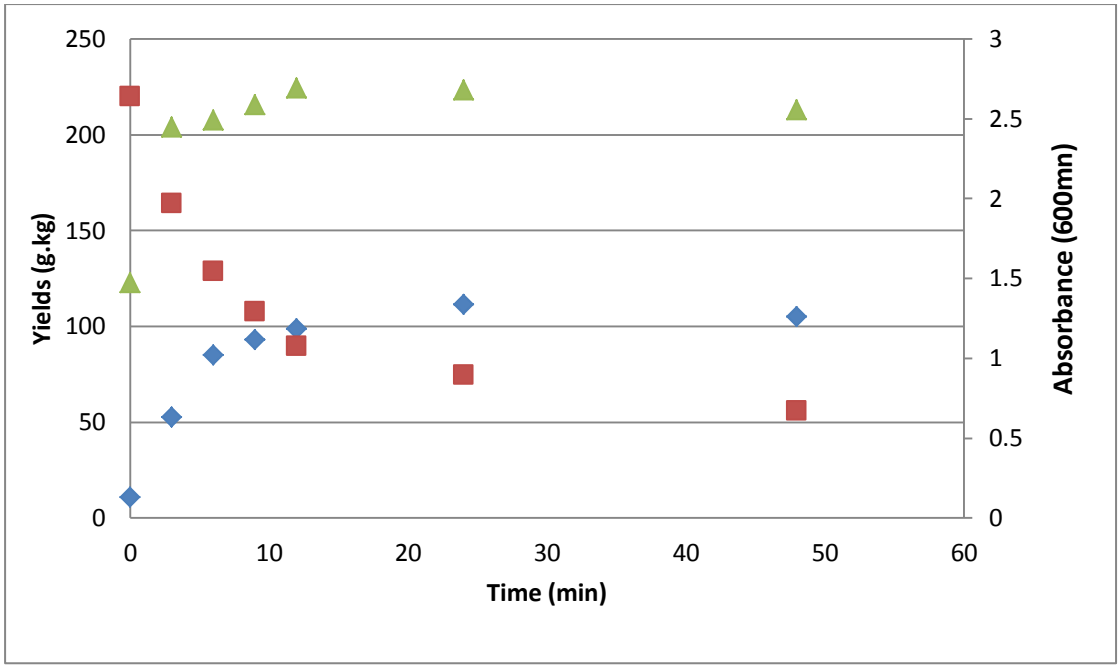

Figure 5.2: The effect of *S. cerevisae* on total sugar yield and ethanol production after ultrasonic-assisted alkali pretreatment (■ – total sugar yield g.kg^{-1}, ▲ – absorbance 600mn, ♦ - ethanol yield g.kg^{-1}).

From Figure 5.2, it is observed that with increase in ethanol yield and absorbance total sugar yield decreases gradually. Results shown in Figure 5.2 indicate that *S. cerevisae* started to ferment the sugars immediately due to the fact that the yeast cells were activated for 15min in 10ml of the hydrolysate prior to fermentation to reduce the lag phase. *S. cerevisae* was able to grow in the hydrolysate, assimilating glucose and producing ethanol as a major product. The highest ethanol yield (111 g.kg^{-1}) was obtained after 24 hours of fermentation. After 24 hours there was no significant effect on ethanol as the cell growth has reached stationary phase and the hexose sugars in the media have been depleted. Velmurugan & Muthukumar, (2011) reported an ethanol production of 0.43 g.g^{-1} of glucose when sugarcane bagasse hydrolysate was fermented using *S. cerevisae*. In this study ethanol production of 0.11g.g^{-1} was observed. Although lower ethanol yields were however this results show a successful fermentation.

5.2.3. COMPARISON BETWEEN DILUTE ALKALI AND ACID PRETREATMENT

Table 5.1: Total sugar yield and ethanol concentration obtained on the different pretreatments

Pretreatment	Total sugar yield (g.kg^{-1})	Total sugar conversion (%)	Ethanol concentration (g.kg^{-1})
$Ca(OH)_2$	250	92	112

H_2SO_4	350	66	90

As seen in Table 4.3, H_2SO_4 gave the highest sugar yield of 350 $g.kg^{-1}$ with 92% conversion as compared to $Ca(OH)_2$ which gave 250 $g.kg^{-1}$ with 66% conversion. These results indicate that H_2SO_4 is better able to disrupt the lignocellulose structure than $Ca(OH)_2$. Subhedar & Gogate, (2014) reported a 2-fold increase in delignification achieved when ultrasonic-assisted alkaline pretreatment compared to alkaline pretreatment. Also Velmurugan & Muthkumar (2011) found that the hydrolysate obtained during sono-assisted acid hydrolysis is more susceptible for bioethanol production. However, although H_2SO_4 is better at breaking down the lignocellulose structure, the by-products that are formed during pretreatment significantly inhibit the production of bioethanol. As a result, the highest ethanol yield was obtained on the hydrolysate that was obtained when pretreated with $Ca(OH)_2$ (111 $g.kg^{-1}$ of biomass).

5.2.4. CONCLUDING REMARKS

In this chapter the results obtained during fermentation were presented and discussed in details. Fermenting the hydrolysate with *S. cerevisae* was successful since ethanol was produced and the highest ethanol yield (111 $g.kg^{-1}$) was obtained when amaranth stem was pretreatment using 30 $g.kg^{-1}$ $Ca(OH)_2$ at 750W for 30 minutes. However lower yields were obtained on the hydrolysate pretreated with 30 $g.kg^{-1}$ H_2SO_4.

4. REFERENCES

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CHAPTER SIX: CONCLUSSION AND RECOMMENDATIONS

6.1. INTRODUCTION

In this chapter all the results obtained in this study will be summarized and necessary recommendations will be outlined. Pretreatment is the major step in bioethanol production process since it alters the lignocellulose structure enabling either enzymatic or chemical conversion of the biomass, thereby improving the enzymatic hydrolysis rate and increasing the fermentable sugar yields. The main objective of this study was to evaluate the effect of energy input, catalyst concentration, sonication time and, biomass loading on total sugar yield and the ethanol production.

6.2. CONCLUSION

Bioethanol has gained lots of attraction because of its properties when used as alternative fuel or blended in fuel. Challenges such as biomass resources, and cheaper and effective technologies to convert biomass to bioethanol still remains. The study was done to examine the feasibility of ultrasonic-assisted pretreatment on amaranth stems. Results obtained indicated that amaranth stems pretreated with ultrasound-assisted alkaline / acid pretreatment showed better delignification as compared to only alkaline / acid pretreatment. About 92% acid and 72 % alkaline increases in delignification were achieved by ultrasound-assisted pretreatment as compared to just alkaline or acid pretreatment. Overall, the significant decrease in the pretreatment time, energy input and alkali /acid requirement with enhanced effectiveness are the most attractive features of ultrasound-assisted pretreatment. The ultrasonic assisted alkali obtained hydrolysate was found to be more susceptible for bioethanol production compared to hydrolysate obtained after ultrasonic assisted acid pretreatment. The results prove that ultrasound can be used as effective pretreatment method and can be an economically attractive lignocellulose pretreatment method, also amaranth stems have shown a potential that it can be used as feedstock for bioethanol production.

RECOMMENDATIONS

A suitable fermentation strategy for both hexose and pentose sugar is necessary such as employing an engineered microorganisms that will convert both hexose and pentose sugars. Lastly, although laboratory results are promising, application of ultrasonic assisted lignocellulose pretreatment on pilot scale needs to be investigated to demonstrate scalability.

APPENDIX A

A.1 FILTER PAPER ASSAY FOR SACCHARIFYING CELLULASE ENZYME

A.1 INTRODUCTION

This section describes the method used to measure cellulase activity, the aim of this procedure is to measure cellulase activity in terms of "filter paper units" (FPU) per milliliter of undiluted enzyme solution. FPU is only defined based filter paper conversion, the value of 2.0 mg of reducing sugar as glucose from 50 mg filter paper (4% conversion) in 60 minutes is designed as the intercept for calculating filter paper units. This means therefore finding the dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4 % conversion in 60 minutes and then calculate the activity of the original stock in (FPU/mL) of the required dilution (Adney & Baker, 1996:1). Determination of cellulase enzyme was done first by preparing standards and constructing standard curve. The preparation of standards is outline on Table 1.1, citrate buffer was used at pH 4.8 and glucose stock solution was 10 mg/mL and absorbance was read at 540nm.

Table A.1:

Table A.1: Glucose standard dilutions

Glucose (ml)	Buffer (ml)	Abs @ 540nm	Glucose conc. mg/0.5ml
1	0.5	0.499	3.35
1	1.0	0.366	2.5
1	2.0	0.219	1.65
1	4.0	0.118	1

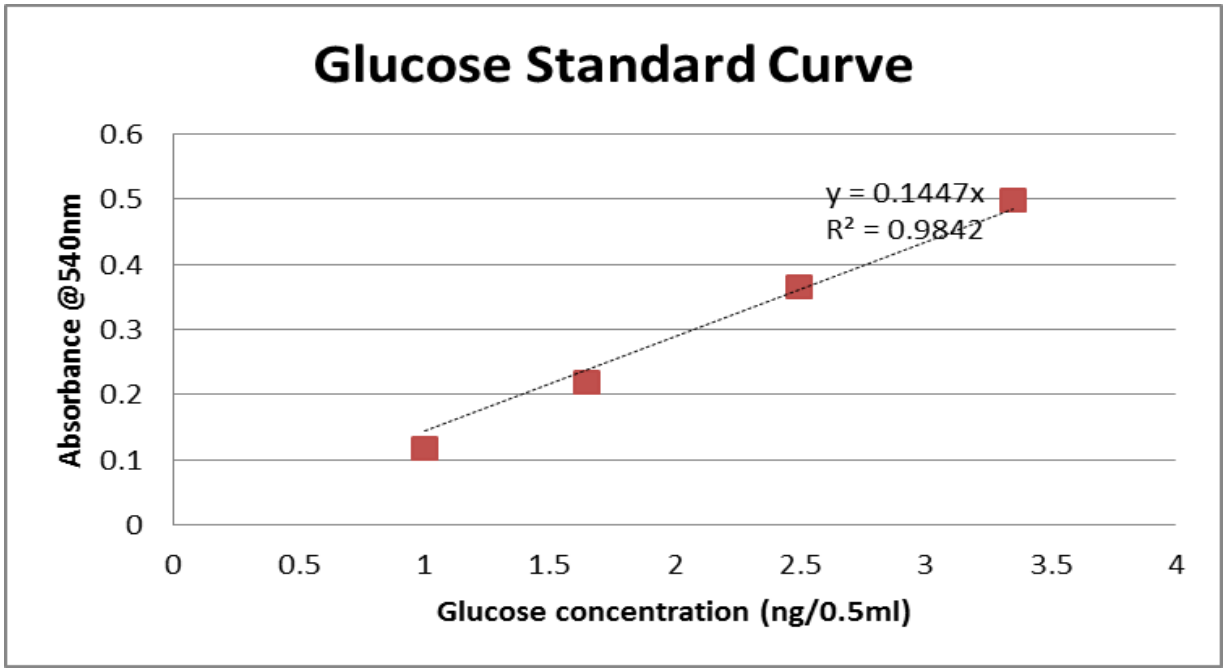

Figure A.1: : Graph showing glucose standard curve

The standard curve was then used to calculate the glucose concentrations in the diluted enzyme mixtures after hydrolysis. Meaning, the different samples containing 50 mg filter paper were hydrolyzed with the different enzyme dilutions and then the resulting glucose concentrations were measured using a spectrophotometer at 540 nm. The enzyme dilutions were done as presented in Table 1.2 below:

Table A.2: Enzyme dilutions, absorbance and glucose concentration of samples as determined from standard curve

Enzyme (ml)	Buffer (ml)	Final Volume	Dilution 2	Abs @ 540nm	Glucose released	Concentration
0.1	1.4	1.5	0.33	0.771	5.004	0.022
0.1	1.7	1.8	0.33	0.7105	4.634	0.0183
0.1	1.9	2	0.33	0.6195	4.077	0.0165
0.1	2.4	2.5	0.33	0.5465	3.63	0.0132
0.1	2.9	3	0.33	0.3865	2.651	0.011
0.1	3.9	4	0.33	0.371	2.556	0.0083
1	49	50	0.33	0.21	1.371	0.0066

* Concentration represents the proportion of the original enzyme solution present in the dilution added to the assay mixture.

Basically the main focus of this method is to find a specific enzyme concentration that will release 2.0 mg of glucose, so to estimate the activity of the undiluted enzyme solution. To find this required enzyme concentration, a graph of glucose liberated against logarithm of the enzyme concentration must be constructed as shown in Figure 1.2. Then take two data points that are very close to 2.0 mg and draw a straight line between them, this line is then used to

interpolate between the two points so to find the enzyme dilution that will produce exactly 2.0 mg glucose equivalents of reducing sugars.

Note: "enzyme concentration" refers to the proportion of the undiluted enzyme solution present in each enzyme dilution (thus, the number of mL of the original solution present in each mL of the dilution).

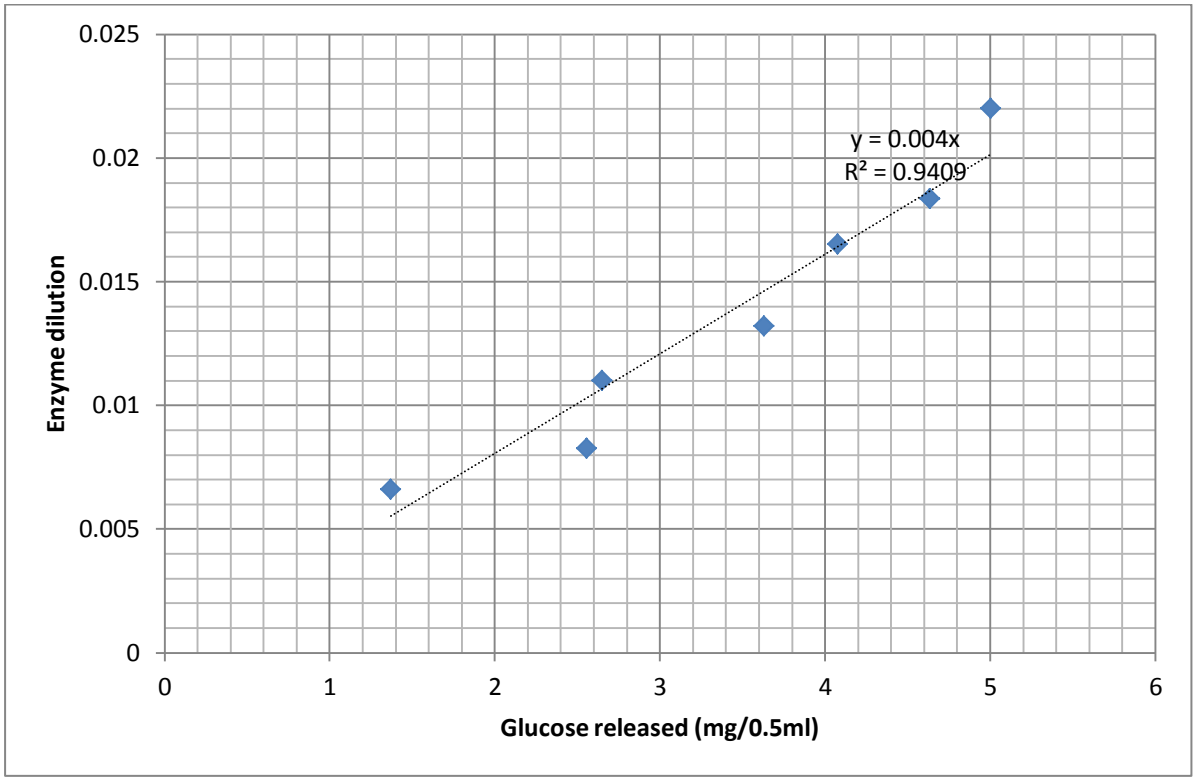

Figure A.2: Enzyme dilution vs. glucose concentration

Calculation of FPU:

Filter Paper Activity = [0.37 / (enzyme) releasing 2.0 mg glucose] units/ ml

Therefore from the graph above:

0.37/0.0078 = 47.4 FPU/ml

The numerator (0.37) in the equation is derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay to mmoles of glucose (2.0 ÷ 0.18016), from the volume of the enzyme being tested that is used in the assay (0.5 mL), and from the incubation time (60 minutes) required for generation of the reducing equivalents.

Thus, the enzyme activity of enzyme NS-22192 is 47.4 FPU/ml.

APPENDIX B

B.1 CALIBRATION CURVES

B.1.1 INTRODUCTION

The calibration curves that were used to analyze and quantify the sugars and ethanol using HPLC during this study are presented in this chapter. The concentration of the sugars, peak areas and calibration curves are shown in this section.

B.1 HPLC SUGARS ANALYSIS

The pure sugars (glucose, xylose, sucrose, arabinose, galactose, mannose, cellubiose, and xylan) and ethanol were dissolved in deionized water and each sugar was serial diluted to obtain the decreasing concentration of the sugar. The concentration was analyzed on the HPLC and a standard curve was obstructed for each sugar. From the standard curve, a straight line was plotted and the slope (k-value) was used to calculate the sugars. Table B.2 and B.3 show the concentration and peak area of the sugars and ethanol.

Table B. 1: Standardized sugars and their k-values

Sugars	K-value
Glucose	14288
Xylose	13297
Sucrose	15897
Galactose	14955
Cellubiose	17108
Xylan	12249
Arabinose	11275
Mannose	14811
Ethanol	6941.8

Table B.2: The concentration of diluted sugars and their peak areas.

Concentration (g/L)	Glucose	Xylose	Cellubiose	Xylan
10.01	1.53E+05	1.35E+05	1.51E+05	1.25E+05
	1.55E+05	1.39E+05	1.59E+05	1.27E+05
	1.68E+05	1.34E+05	1.55E+05	1.26E+05
5.005	4.18E+04	6.50E+04	7.25E+04	5.56E+04
	4.34E+04	6.17E+04	7.68E+04	6.13E+04
	3.67E+04	6.17E+04	7.65E+04	6.16E+04
2.5025	3.49E+04	3.16E+04	3.56E+04	2.29E+04
	3.51E+04	3.21E+04	3.92E+04	2.55E+04
	3.57E+04	3.04E+04	3.49E+04	2.42E+04
1.25125	1.57E+04	1.38E+04	1.46E+04	1.11E+04
	1.80E+04	1.53E+04	3.92E+04	9.62E+03
	1.64E+04	1.42E+04	3.49E+04	1.24E+04
0.625625	7.75E+03	7.21E+03	6.66E+03	3.15E+03
	8.61E+03	8.33E+03	6.43E+03	6.05E+03

	8.04E+03	6.72E+03	9.18E+03	4.60E+03

Table B.3: Concentration of sugars and ethanol and their peaks areas

Concentration (g/L)	Arabinose	Galactose	Mannose	Sucrose	Ethanol
10.01	1.18E+05	1.48E+05	1.39E+05	1.74E+05	7.03E+04
	1.15E+05	1.43E+05	1.55E+05	1.69E+05	7.13E+04
	1.03E+05	1.51E+05	1.50E+05	1.57E+05	7.02E+04
5.005	5.00E+04	7.65E+04	7.07E+04	6.64E+04	3.39E+04
	5.43E+04	7.88E+04	6.86E+04	8.55E+04	3.42E+04
	5.65E+04	9.16E+04	8.06E+04	5.54E+04	3.06E+04
2.5025	3.56E+04	3.13E+04	3.22E+04	6.88E+04	1.44E+04
	3.60E+04	3.71E+04	3.99E+04	1.29E+04	1.36E+04
	3.08E+04	3.21E+04	4.46E+04	2.22E+04	1.44E+04
1.25125	1.69E+04	1.63E+04	1.46E+04	4926.196	1.08E+04
	1.71E+04	1.71E+04	1.70E+04	6.08E+03	1.49E+04
	1.96E+04	1.96E+04	2.36E+04	5501.876	1.21E+04
0.625625	1.02E+04	8.07E+03	7.12E+03		3.02E+03
	1.01E+04	5.60E+03	1.07E+04		6.13E+03
	9.05E+03	6.84E+03	1.04E+04		4.63E+03

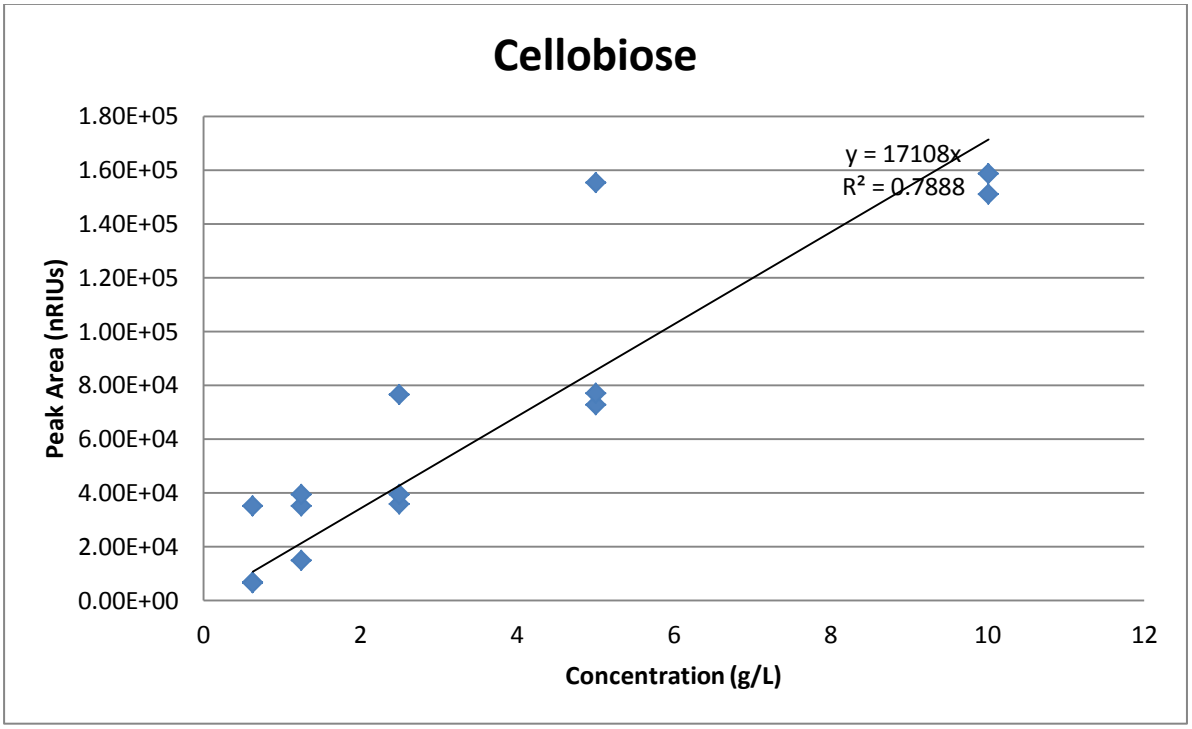

Figure B.1: Cellobiose calibration curve

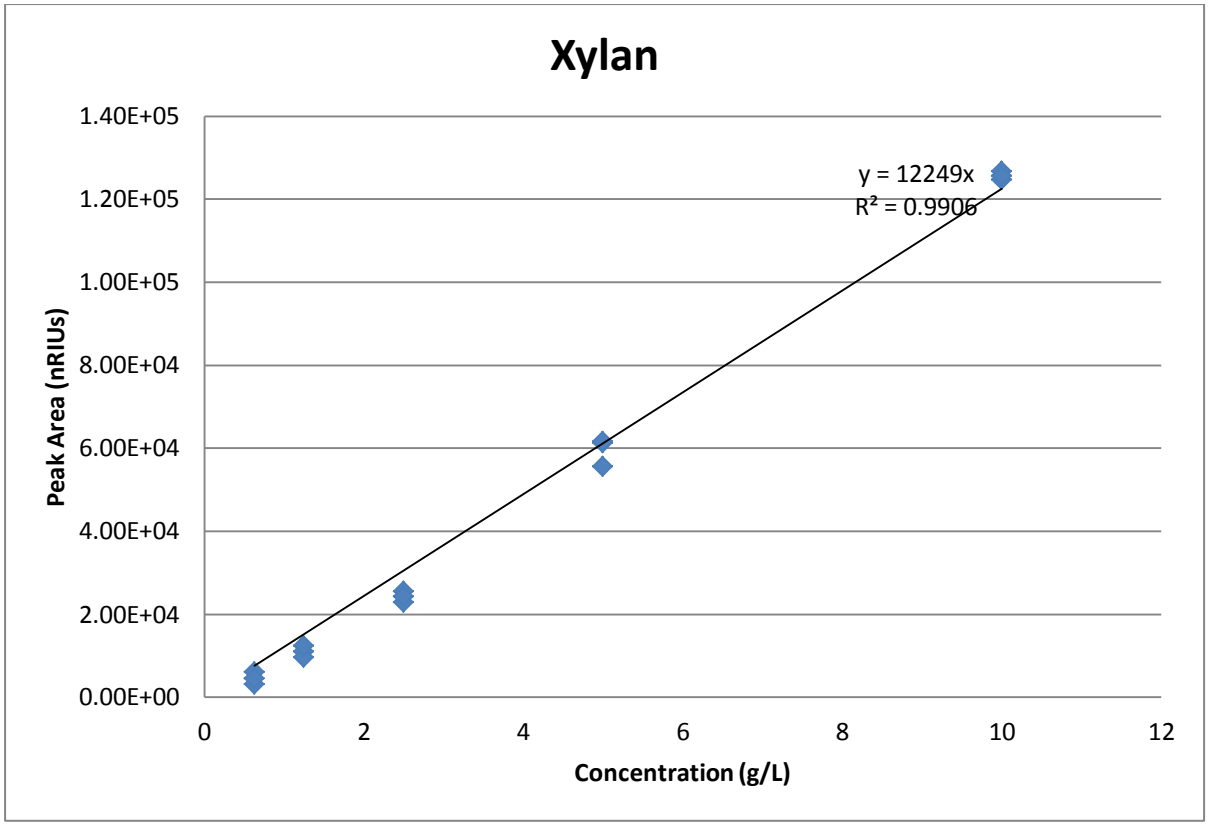

Figure B. 2: Xylan calibration curve

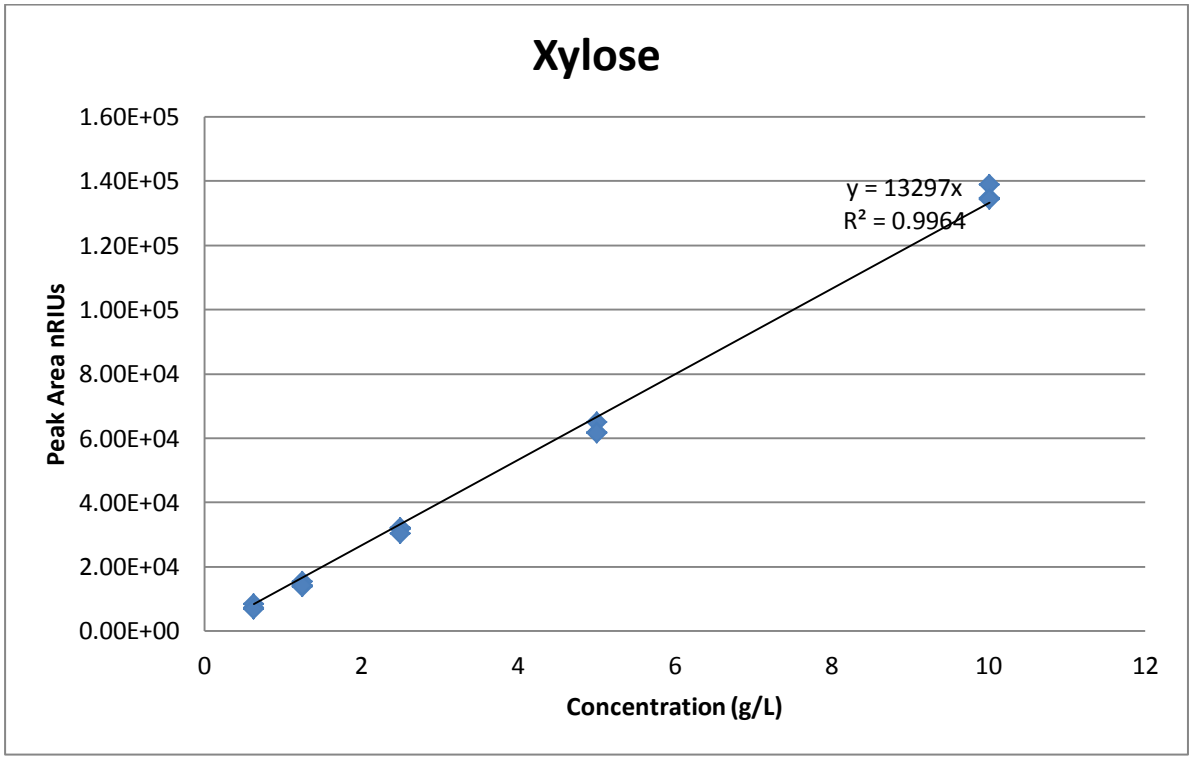

Figure B.3: Xylose calibration curve

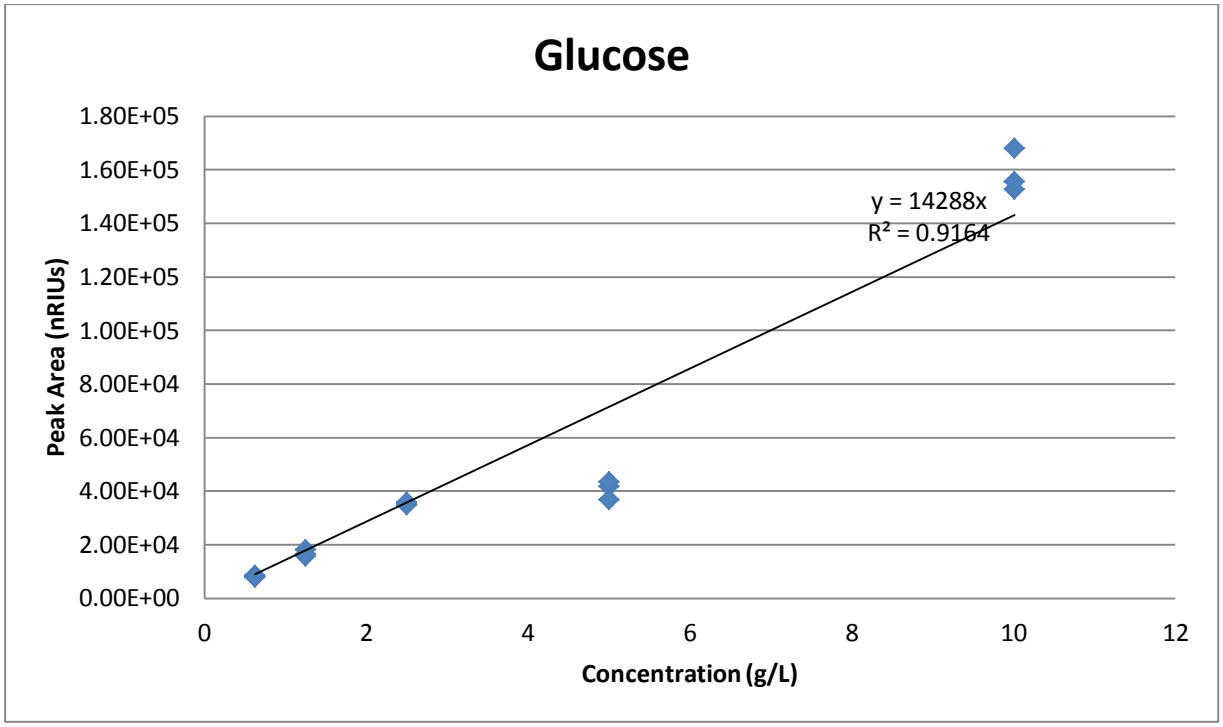

Figure B.4: Glucose calibration curve

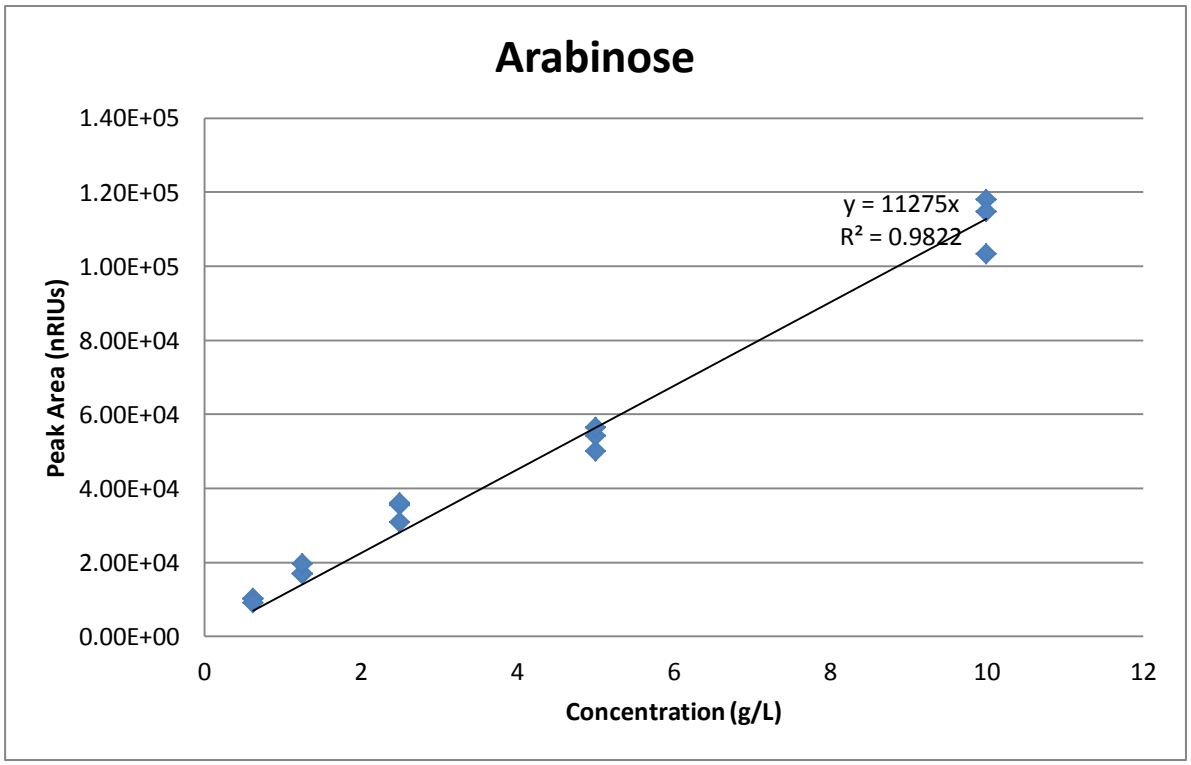

Figure B: 5 Arabinose calibration curve

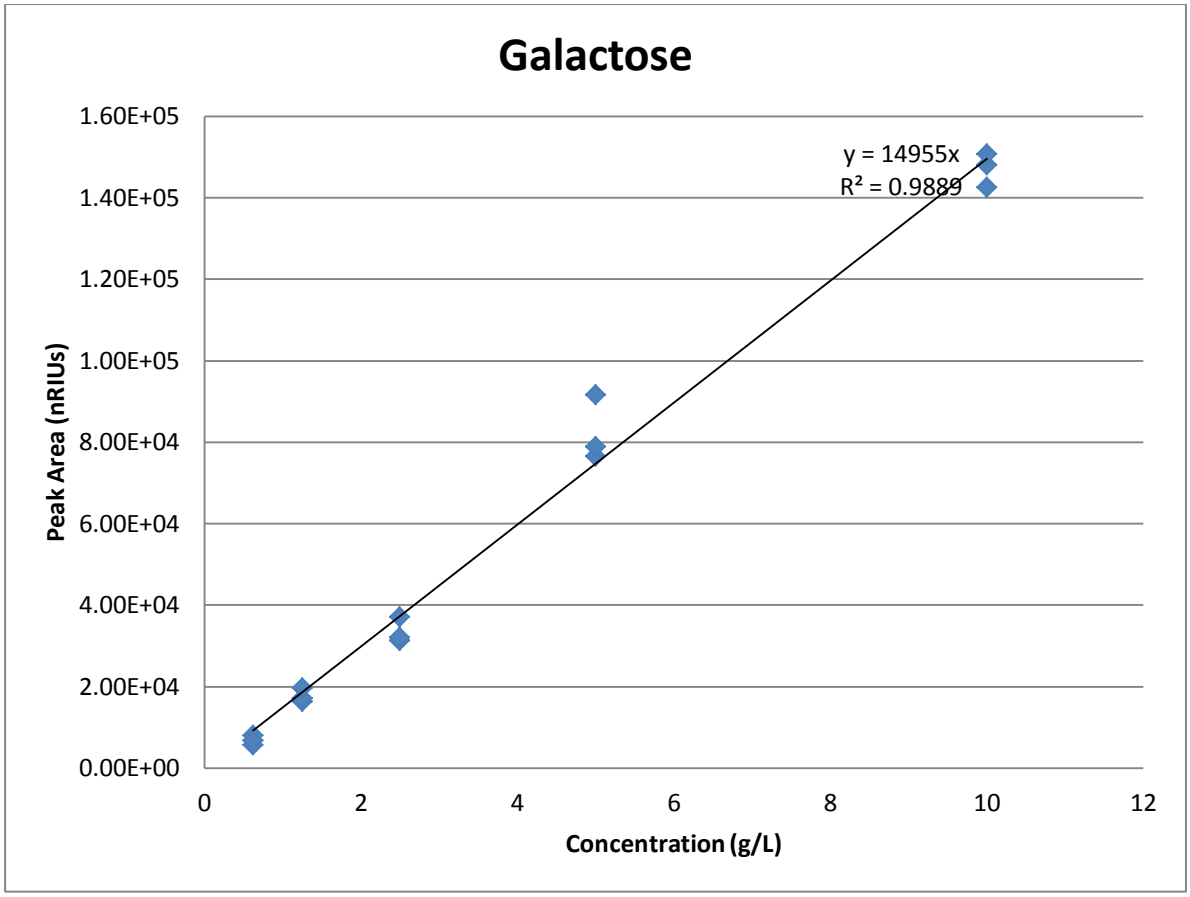

Figure B.6: Galactose calibration curve

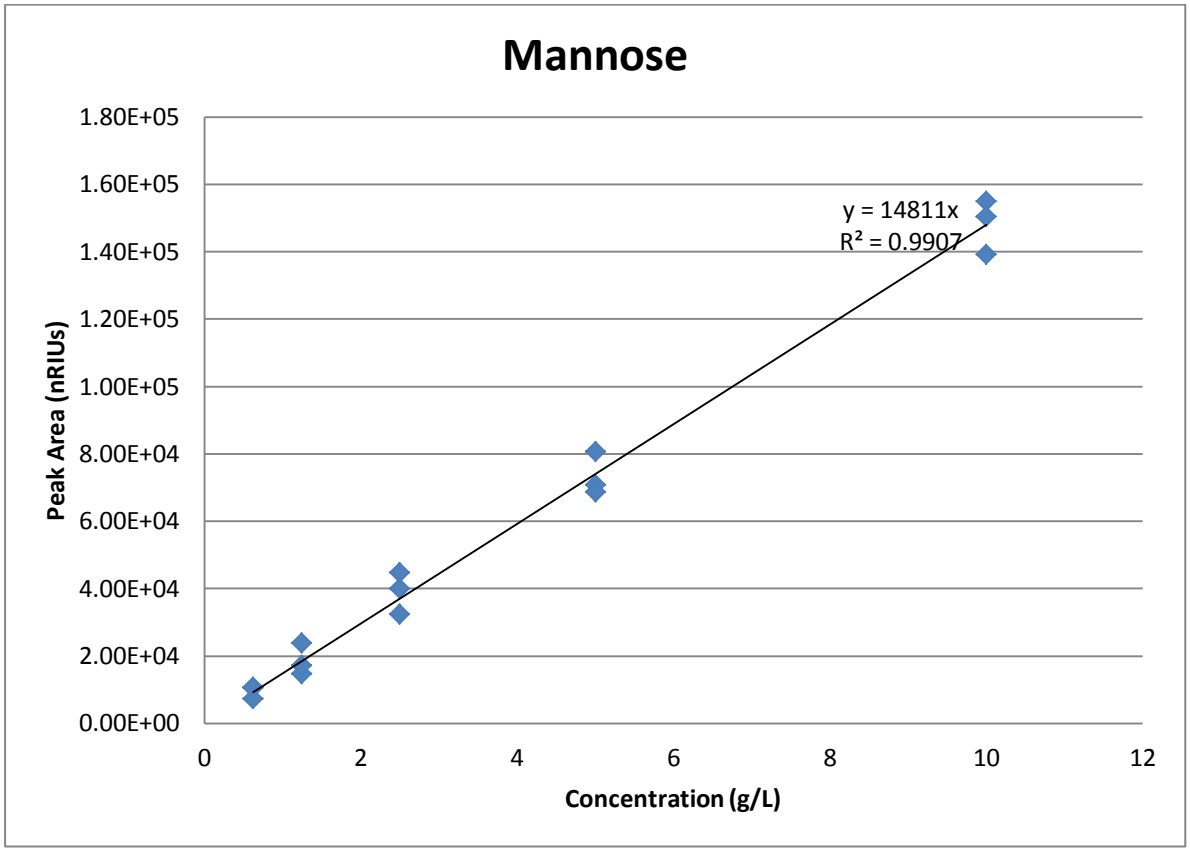

Figure B.7: Mannose calibration curve

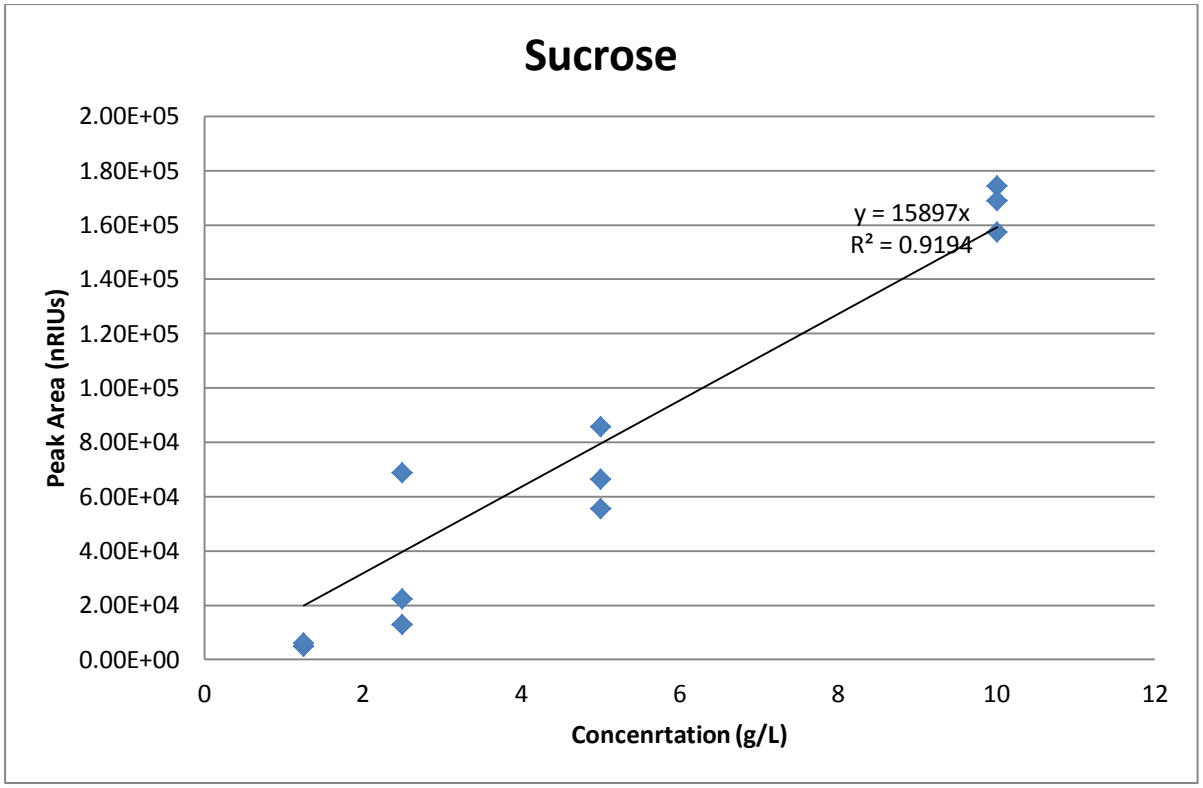

Figure B.8: Sucrose calibration curve

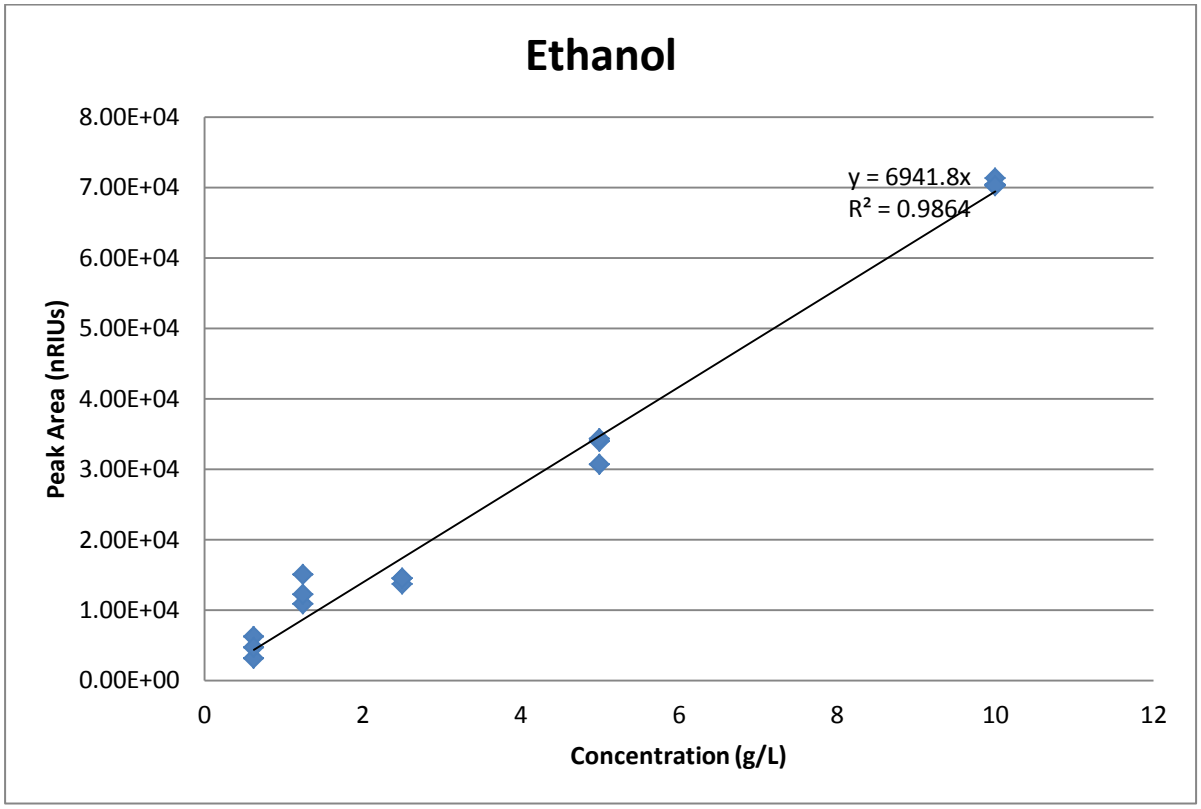

Figure B.9: Ethanol calibration curve

APPENDIX C

C.1 INTRODUCTION

This section shows the calculations that were used in this study, the calculation for quantifying sugars and ethanol, energy input and the experimental errors for this study.

C.2 CALCULATIONS

C.1.1 SUGAR CONCENTRATION CALCULATION

Sugar concentration (g/L) = (Peak area / k-value)* dilution factor

Sugars concentration (g/g) = (sugar concentration (g/L)* final volume)/ mass of biomass

C.1.2 ETHANOL CONCENTRATION CALCULATION

Ethanol concentration (g/L) = (Peak area / k-value)* dilution factor

Ethanol concentration (g/g) = (ethanol concentration (g/L)* final volume)/ mass of biomass

C.1.3 ENERGY INPUT CALCULATION

Energy Input (kJ/g) = [(Power (J/s)* time (s))/1000] / mass of biomass (g)

C.1 ERROR CALCULATIONS

Standard deviation Error = $\overline{x} \pm \frac{\sigma}{\sqrt{n}}$

$\delta = \sqrt{\frac{\sum(\bar{x}-x)^2}{n-1}}$

with *n* being a number of runs,

$\bar{x}$ = average, and

x = sample amount.

Table C1: The calculated experimental errors on the effect of energy input on total sugar yields after 30 minutes pretreatment.

Conditions	Run 1	Run 2	Run 3	Average	Standard deviation	Standard deviation error
1% H_2SO_4	0.23	0.25	0.26	0.25	0.02	0.01±0.25
3% H_2SO_4	0.32	0.36	0.34	0.34	0.02	0.01±0.34
1%$Ca(OH)_2$	0.19	0.15	0.18	0.17	0.02	0.01±0.17
3% $Ca(OH)_2$	0.24	0.22	0.18	0.21	0.03	0.02±0.21

APPENDIX D

D.1 EXPERIMENTAL CALCULATIONS

Table D1: Area, concentration and total sugars yield after 15 min pretreatment with 1% H_2SO_4 at different power (W).

Power (W)	Type of sugar	Concentration (g/L)	Sugar (g/g)	Total sugar yield (g/g)
	Pentose	9.461	0.024	0.03
	Cellubiose	0.098	0.002	
600	Pentose	0.098	0.002	0.002
750	Pentose	11.5745	0.104	0.11
	Cellubiose	0.095052	0.01	

Table D2: Area, concentration and total sugars yield after 30 min pretreatment with 1% H_2SO_4 at different power (W).

Power (W)	Type of sugar	Concentration (g/L)	Sugar (g/g)	Total sugars yield (g/g)
150	Hexose	5.69	0.09	0.17
	Xylose	5.56	0.07	
	Cellubiose	0.18	0.00	
600	Hexose	5.32	0.11	0.21
	Pentose	3.41	0.07	
	Cellubiose	1.68	0.04	
750	Hexose	6.72	0.14	0.25
	Pentose	4.45	0.13	

Table D3: Area, concentration and total sugars yield after 60 min pretreatment with 1% H_2SO_4 at different power (W)

Power(W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugars yield (g/g)
600	Hexose	5.70	0.13	0.24
	Pentose	5.40	0.08	
750	Hexose	3.42	0.08	0.11
	Pentose	0.12	0.03	

Table D4: Area, concentration and total sugars yield after 15 min pretreatment with 3% H_2SO_4 at different power (W).

Power (W)	Type of sugar	Concentration (g/L)	Concentration (g/g)	Total sugar yield (g/g)
150	Sucrose	0.025	0.00	
	Pentose	0.86	0.02	0.04
	Cellubiose	0.87	0.02	
600	Pentose	3.24	0.06	0.08

	Cellubiose	0.99	0.02	
750	Pentose	0.15	0.00	0.21
	Cellubiose	9.51	0.21	

Table D5: Area, concentration and total sugars yield after 30 min pretreatment with 3% H_2SO_4 at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugar yield (g/g)
150	Hexose	3.74	0.11	0.22
	Pentose	3.67	0.11	
600	Hexose	4.31	0.11	0.23
	Pentose	3.55	0.09	
	Cellubiose	1.48	0.04	
750	Hexose	10.33	0.22	0.34
	Pentose	5.16	0.11	

Table D6: Area, concentration and total sugars yield after 60 min pretreatment with 3% H_2SO_4 at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugars yield (g/g)
600	Hexose	5.71	0.13	0.25
	Pentose	5.15	0.12	
750	Hexose	5.66	0.16	0.21
	Pentose	1.96	0.06	

Table D7: Area, concentration and total sugars yield after 15 min pretreatment with 1% $Ca(OH)_2$ at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Concentration (g/g)	Total sugar yield (g/g)
150	Pentose	3.77	0.64	0.10
	Cellubiose	1.91	0.03	
750	Cellubiose	0.85	0.02	0.02

Table D8: Area, concentration and total sugars yield after 30 min pretreatment with 1% $Ca(OH)_2$ at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugars yield (g/g)
150	Pentose	4.07	0.09	0.09
600	Pentose	0.43	0.01	
	Hexose	6.50	0.18	0.19
750	Hexose	6.32	0.23	0.23

Table D9: Area, concentration and total sugars yield after 60 min pretreatment with 1% $Ca(OH)_2$ at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugar yield (g/g)
600	Pentose	3.92	0.08	
	Hexose	3.97	0.08	0.17
750	Pentose	2.73	0.06	0.18
	Hexose	5.40	0.12	

Table D10: Area, concentration and total sugars yield after 15 min pretreatment with 3% $Ca(OH)_2$ at different power (W).

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugar yield (g/g)
150	Hexose	9.33	0.12	0.12
600	Hexose	0.68	0.008	0.01
	Cellubiose	0.38	0.005	
750	Pentose	1.09	0.02	0.08
	Sucrose	4.69	0.07	

Table D11: Area, concentration and total sugars yield after 30 min pretreatment with 3% $Ca(OH)_2$ at different power (W).

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugar yield (g/g)
150	Pentose	0.26	0.00	0.03
	Hexose	3.38	0.02	
	Cellubiose	0.14	0.00	
600	Pentose	9.67	0.05	0.08
	Hexose	7.28	0.03	
750	Pentose	1.51	0.03	0.19
	Hexose	9.54	0.16	

Table D12: Area, concentration and total sugars yield after 60 min pretreatment with 3% $Ca(OH)_2$ at different power (W)

Power (W)	Type of sugar	Concentration (g/L)	Sugar yield (g/g)	Total Sugars yield (g/g)
600	Pentose	5.04	0.14	0.24
	Hexose	3.38	0.09	
750	Pentose	4.30	0.11	0.15
	Hexose	1.86	0.05	