Use of Amaranth as feedstock for bio-ethanol production

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ABSTRACT

The depletion of fossil fuel reserves and global warming are the two main factors contributing to the current demand in clean and renewable energy resources. Biofuels are renewable energy resources and have an advantage over other renewable resources due to biofuels having a zero carbon footprint and most feedstock is abundant. The use of biofuels brought about major concerns and these include food, water and land security. The use of lignocellulose as bioethanol feedstock can provide a solution to the food, water and security concerns. Biofuels such as bioethanol can be produced from lignocellulose by breaking down the structure of lignocellulose liberating fermentable sugars. Amaranth lignocellulose has a potential to be used as a feedstock for bioethanol production because amaranth plants has a high yield of biomass per hectare, require very little to no irrigation and have the ability to withstand harsh environmental conditions.

The aim of this study was to investigate the viability of amaranth as a feedstock for bioethanol production by using alkaline assisted microwave pretreatment. Alkaline pretreatment of amaranth using $Ca(OH)_2$, NaOH and KOH at various concentrations (10-50 g kg^{-1} of alkaline solution in water) was carried out at different energy input (6-54 kJ/g). The pretreated broth was enzymatically hydrolysed using Celluclast 1.5L, Novozyme 188 and Tween 80 at pH 4.8 and 50^{o}C for 48 hours. The hydrolysate was further fermented to ethanol using *Saccharomyces cerevisiae* at a pH of 4.8 and 30^{o}C for 48 hours. The effect of microwave pretreatment on amaranth lignocellulose was evaluated using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The monomeric sugars and ethanol were quantified using high performance liquid chromatography (HPLC).

A maximum sugar yield of 0.36 g/g of biomass was obtained for pretreatment with 30 g kg^{-1} $Ca(OH)_2$ solution in water, 0.24 g/g of biomass was obtained for pretreatment with 50 g kg^{-1} NaOH solution in water and 0.21g/g of biomass was obtained for pretreatment with 50 g kg^{-1} KOH solution in water at 32 kJ/g of energy input. After enzymatic hydrolysis the yields increased to 0.43 g/g, 0.63 g/g and 0.52 g g^{-1} of biomass for $Ca(OH)_2$, KOH and NaOH pretreated biomass respectively. The highest ethanol yield obtained was found to be 0.18 g/g of biomass from fermentation of KOH pretreated broth. The ethanol yield obtained from fermentation of $Ca(OH)_2$ and NaOH pretreated broth was 0.13 g/g of biomass and 0.15 g/g of biomass respectively. The results showed that an increase in concentration of alkaline solution and an increase in energy input liberate more sugars. A decrease in biomass loading

was found to increase the total sugar yield. Pretreatment with KOH was found to liberate more pentose sugars than the other alkaline solutions. The morphological changes shown by the SEM images showed that microwave irradiation is effective in breaking the structure of amaranth lignocellulose. The structural changes shown by the FTIR also validated that alkaline bases were effective in breaking the lignin, cellulose and hemicellulose linkages and liberating more sugars in the process. This work has demonstrated the enormous potential that amaranth lignocellulose has on being a feedstock for bioethanol production.

Keywords: Amaranth, pretreatment, microwave, lignocellulose, alkaline

DECLARATION

I, Nqobile Xaba hereby declare that I am the sole author of this dissertation; the work contained in this dissertation is my own and it has not been submitted to any other university.

Nqobile Xaba

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Dear Dad: Here is another masterpiece in your honour. It is one of my great work and achievement I hope you're proud of it as I am. It goes without saying that we parted ways

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"Life is like a piano, anyone can play a song through meaningless repetition but it takes passion to play a masterpiece"

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

g	: gram
L	: litre
mL	: millilitre
µL	: micro litre
mg	: milligram
kg	: kilo gram
^{o}C	: Degrees Celsius
W	: Watt
nm	: nanometre
s	: seconds
min	: minutes
h	: hours
%	: percent
K	: kelvin
MPa	: Megapascal
eV	: electron volts
Hz	: hertz
MHz	: megahertz
GHz	: gigahertz
rpm	: rounds per minute
kJ/g	: kilo Joule per gram
g/g	: grams per gram

g/L	: grams per litre
mg/L	: milligrams per litre
mL/min	: milligrams per minute
wt%	: weight percentage
w/v	: weight per volume
M	: Molar/moles per litre
FPU/g	: filter paper units per gram
UN	: United Nations
FAO	: Food and Agricultural Organisation
WHO	: World Health Organisation

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

General Introduction

1.1 Introduction

It is estimated that the world population rises by atleast 1.1% per year which is approximately 75 million people per annum resulting in approximately 7 billion people currently residing on planet earth (UN, 2011). Unfortunately, this world population increase brought about a lot of social and economic developments (Saxena *et al.*, 2009). The dependence of humans on energy systems such as electricity, heat and transport fuel was also very much stressed due to increase in demand (Saxena *et al.*, 2009). These energy systems were harnessed from reservoirs including coal, oil and natural gas (fossils) (Saxena *et al.*, 2009). The problem associated with fossil fuel resources is that they are currently depleting and fossil derived fuels have a major effect on the global warming crisis (Cheng & Timilsina, 2011; Nigam & Singh, 2011; Saxena *et al.*, 2009). As a result there is a global demand for substitute energy resources that are economically viable, environmentally friendly and most importantly, renewable (Nigam & Singh, 2011). Such energy resources have been around for decades even though they were not being utilised to their full capacity and these include, hydroelectric, wind, solar and biomass (Saxena *et al.*, 2009).

Biomass is the fourth largest energy provider after coal, petroleum and natural gas and is accountable for approximately 10-14% of the world's energy, and is considered a viable option to reduce the current demand on fossil derived energy resources (Saxena *et al.*, 2009). Utilisation of biomass for energy is favoured by compelling reasons, including that it is renewable, sustainable and eco-friendly (Nigam & Singh, 2011; Saxena *et al.*, 2009). A wide variety of sources constitute to biomass, including wastes (agricultural and municipal), forests, and the edible and non-edible plants (Cheng & Timilsina, 2011; Nigam & Singh, 2011; Saxena *et al.*, 2009). Biomass can be used to provide electricity, heat and fuel (Saxena *et al.*, 2009). Current research is focusing on the production of biofuels such as bioethanol and biodiesel. Biofuels such as bioethanol has a potential of replacing fossil derived fuels or to be used as an additive (Nigam & Singh, 2011).

1.2 History and world production of biofuels

Fuel ethanol has been around as far back as the early nineteenth century when in 1826, the first engine to operate on ethanol was built by Samuel Murray (Demirbas, 2007; Demirbas *et al.*, 2009). Later in 1896 Henry Ford made a car that can operate on pure ethanol (Demirbas *et al.*, 2009). From then onwards, ethanol fuel became popular in Europe and the United States (Demirbas *et al.*, 2009) . The first world war brought about a decrease in ethanol fuel used due to increase in production costs until it blossomed in the 1970s due to the world oil crisis (Mussato *et al.*, 2010). Brazil also had started producing bioethanol from sugar cane due to overproduction, such that in 1984 Brazilian cars were using hydrated bioethanol fuel (96% bioethanol) (Mussato *et al.*, 2010). The United States of America (USA) started around 1980 to produce bioethanol and since has been leading the world. USA uses up to 85% ethanol blends in petrol in specially designed vehicles by Ford, Chrysler and General Motors (Mussato *et al.*, 2010). Biofuels production increased from 4.4 to 50.1 billion litres globally from the year 1980 to 2005 (Nigam & Singh, 2011). Currently, the world production of biofuels is still increasing (Sims *et al.*, 2012) and bioethanol is the most utilised biofuel (Mussato *et al.*, 2010).

Southern African countries are still catching up on biofuels. The South African biofuels industrial strategy drafted in 2007 states that 2% of petrol needs to be replaced by bioethanol (Department of Minerals and Energy, 2007). South Africa (SA) uses approximately 12 billion litres per annum of petrol and this means we require 240 million litres of bioethanol to be produced to make the E2 blend (Herrington, 2012). The main producers of bioethanol in SA are NCP, Illovo, Glendale and USA distiller producing approximately 186 million litres (Department of Minerals and Energy, 2007). South Africa already uses 2% blend of petrol using petroleum based ethanol (Mussato *et al.*, 2010). African countries such as Malawi and Swaziland have up and running biofuel companies and Malawi already uses 15% bioethanol blend in their fuel (Herrington, 2012). Therefore, South Africa is still far behind other African countries in bioethanol production.

1.3 Challenges to bioethanol production

The global biofuels industry has grown and a number of developments have occurred, but there are a few challenges that still need to be dealt with. The first one involves feedstock which is the primary topic that always comes to anyone's mind concerning biofuels (Cheng & Timilsina, 2011; Nigam & Singh, 2011). Feedstock involves some environmental and

economic concerns such as land use and food security (Cheng & Timilsina, 2011; Viikari *et al.*, 2012). Bioethanol producers need to move away from first generation feedstock (maize, sugar cane) towards second generation feedstock (lignocellulose) (Mussato *et al.*, 2010). The lignocellulose to be used also needs to be able to give high sugar conversion yields as well as have high content of cellulose and hemicellulose, and low lignin content (Cheng & Timilsina, 2011) (Sanchez & Cardona, 2008). Current research to achieve this is being done by genetically modifying the lignocellulose plants to contain less lignin (Cheng & Timilsina, 2011). The overall bioethanol production needs to be cost effective (Cheng & Timilsina, 2011; Viikari *et al.*, 2012). This entails reducing the amount of energy required in the process and using and recycling of by-products and other reagents (Cheng & Timilsina, 2011). Converting both cellulose (glucose) and hemicellulose (xylose) to bioethanol is also important, because xylose is the major component of hemicellulose (Cheng & Timilsina, 2011). Converting xylose is difficult, but research on genetically engineered micro-organisms that can ferment xylose is being done (Cheng & Timilsina, 2011).

1.4 Motivation

The use of lignocellulose in the production of biofuels provides a solution to the food versus fuel debate which is a current issue when it comes to biofuels production. Amaranth *(Amaranthus cruentus)* is a small-seeded grain crop (Viglasky *et al.*, 2008). It can be characterized as a high-energy multipurpose plant. It can grow anywhere in the world and it is a short cycle plant that is resistant to drought and salinity as well any contamination by radioactive dust or other contaminants (Viglasky *et al.*, 2008). It is also highly nutritious, with its grain and leaves as food sources. Amaranth lignocellulose can be utilized as an alternative feedstock for ethanol production because it has over 60 species that can be grown and used (Viglasky *et al.*, 2008). Using amaranth lignocellulose for the production of bioethanol will have less impact on land and water use since only the inedible parts of the plant will be used for bioethanol production. The production of ethanol from amaranth lignocellulose will not use more arable land only for energy production, because the amaranth that is grown will be used for both food (grain starch) and energy (bioethanol from lignocellulose). Therefore, amaranth is worth investigating as a feedstock for ethanol production. Amaranth lignocellulose has a potential to be used as a viable bioethanol production feedstock in South Africa because amaranth only grows as a volunteer crop and cultivating it specifically for bioethanol production will not threaten the availability of food

sources in this country; it will add to the current food crops. A pretreatment method is needed inorder to convert amaranth to bioethanol because of its recalcitrant nature. Microwave irradiation is an attractive pretreatment method because it will rapidly break down amaranth lignocellulose releasing sugars. Microwave pretreatment will also reduce the energy of the overall production process, therefore making the process cost effective. Combining microwave with alkali salts will accelerate the breaking down of amaranth lignocellulose. Alkali pretreatment is well known for its economic viability (alkali salts are cheap) and their ability to liberate sugars while supressing the inhibiting effect of lignin. The combination of alkali pretreatment and microwave will offer tremendous advantage of liberating more sugars from amaranth lignocellulose in an economically viable process.

1.5 Aim

- The aim of the project is to investigate and show the viability of amaranth as a sustainable feedstock for bioethanol production in South Africa using microwave irradiation as a pretreatment method.

1.6 Objectives

- Quantify components of amaranth lignocellulose
- Develop and use microwave irradiation as a pretreatment method to liberate sugars from amaranth lignocellulose.
- Quantify and convert cellulose and hemicellulose from amaranth lignocellulose into fermentable sugars
- Investigate the effect of parameters such as time, power and concentration of base on the sugar yield during microwave pretreatment and hydrolysis.
- Investigate the conversion of fermentable sugars liberated from amaranth lignocellulose to ethanol using suitable micro-organisms

1.7 Scope of the study

A general introduction to biofuels is provided in Chapter 1. Current literature on biofuels production process focussing on bioethanol production processes (pretreatment and fermentation) is provided in Chapter 2. In Chapter 2 amaranth is also discussed, highlighting its uses and its potential as a feedstock for bioethanol production. Details of the experimental procedure and analytical methods used for the production of bioethanol in this study are

provided in Chapter 3. The results obtained during the evaluation of pretreatment parameters during microwave alkali pretreatment of amaranth are provided and discussed in Chapter 4. The results obtained during enzymatic hydrolysis and fermentation of microwave alkali pretreated amaranth are presented and discussed in Chapter 5. The conclusion and recommendation on the work done on pretreatment, hydrolysis and fermentation of amaranth is provided in Chapter 6.

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CHAPTER 2

Literature Review

2.1 Introduction to biofuels

Renewable energy resources have recently received unprecedented attention in research (Sanchez & Cardona, 2008), because of the limited fossil fuel reserves as well as the implications brought on by the use of fossil derived fuels. Biomass is considered a viable renewable energy resource, because it is sustainable and can be developed in the future and has very low sulphur content (Demirbas, 2007). Additionally, the greenhouse gases produced by biofuels are renewable since it was used to grow the plant in the first place, hence no additional carbon dioxide is released into the air as in the case of fossil fuels (Balat, 2011). Biomass is used to make fuels such as bio-ethanol and bio-diesel amongst many. Fuels that are derived from biomass are referred to as bio-fuels. The advantages of bio-fuels include; wide availability, less impact on environment than fossil fuels and biodegradability (Demirbas, 2007).

Ethanol can be produced from biomass feedstock. Ethanol is commonly produced as a by-product during petrol production through fossil fuels in the petroleum industries. This ethanol is further used to produce ethyl acetate and the remaining pure ethanol is used to blend with petroleum. Ethanol is blended with petrol from as low as 2% (E2) to as high as 100% (Sun & Cheng, 2002). Due to the increase in greenhouse gases associated with fossil derived fuels, it has become apparent that bioethanol should be used to blend with petrol in higher quantities and also ethanol on its own should be used as fuel (Scarlat & Dallemand, 2011). The use of ethanol on its own as fuel can reduce the net carbon dioxide emission by a 100% (on a life cycle basis) and high ethanol blends decreases the emission of volatile organic compounds. Ethanol blends containing up to 10% ethanol can be used in any petroleum based engine without modification (Addison, 2012).

The biggest challenge in production of biofuels is finding the proper feedstock to use. The largest producers of bioethanol in the world are the United States of America (USA) and Brazil (Limayem & Ricke, 2012). The USA produces ethanol from maize and Brazil from sugarcane (Limayem & Ricke, 2012). The feedstock that these two countries (USA and Brazil) are using is also food sources; maize is used to make maize meal and sugar cane is

used in sugar production. In South Africa, production of biofuels from maize was banned for food security reasons; therefore we need to find feedstock that does not compete with food sources.

2.2 Feedstock used in biofuels production

Bioethanol can be produced from a wide range of feedstock; these include sugars such as sugar cane, sugar beet, sweet sorghum and fruits. Starch has also been used to produce bioethanol as early as the 1900s with the most important and economical sources being maize, wheat, rice, potatoes, cassava and sweet potato. Biofuels produced from sugars and starch is referred to as first generation biofuels. First generation biofuels have long been established. The limiting factor of first generation biofuels is that they compete directly with food sources and there are also some concerns on available arable land to grow plants just for the sole purpose of producing biofuels. This has led to a shift in research towards second generation biofuels where lignocellulose and non-food crops are used as feedstock for bioethanol production. Second generation production refers to the use of soft and hard woods, straws, pulp, agricultural residues and municipality waste for bioethanol production. Unlike first generation production, second generation production uses the non-edible parts of the plant to produce bioethanol. Therefore, using second generation production does not compete with food sources and also avoids the issue of using arable land, because only residues that remain after the food has been harvested from the plants are used for fuel production. (Balat, 2011).

2.2.1 Starch to ethanol

Starch is the major carbohydrate storage accounting for 70 to 72% by weight in crops such as maize, wheat, cassava and other starchy material such as barley. The main component of starch is α-glucose chains and it is referred to as amylose or amylopectin depending on the chain length of α-glucose. Starch is converted to bioethanol by first disintegration of the α-glucose chains to dextrin by α-amylase enzymes. Glucoamylase is the enzyme that subsequently converts the dextrin to D-glucose as shown on the chemical reaction below. (Gnansounou & Dauriat, 2005).

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \qquad \text{(Equation 1)}$$

2.2.2 Disaccharides to ethanol

Sugar crops are the most widely used feedstock in bioethanol production (Sanchez & Cardona, 2008). Sugar cane juice or sugar molasses can be used (Sanchez & Cardona, 2008). Sugar cane contains sucrose that is converted to fructose and glucose by micro-organisms such as *saccharomyces cerevisiae* (Sanchez & Cardona, 2008). Sucrose is enzymatically hydrolyzed using invertase to glucose and fructose by the chemical reaction below (Equation 2) (Gnansounou & Dauriat, 2005). The produced glucose and fructose are further fermented by zymase to produce bioethanol (Gnansounou & Dauriat, 2005) (Equation 3). Both invertase and zymase are enzymes found in yeast (Gnansounou & Dauriat, 2005). (Gnansounou & Dauriat, 2005).

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6 \quad \text{(Equation 2)}$$

Sucrose Glucose Fructose

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad \text{(Equation 3)}$$

Glucose (or fructose) Ethanol Carbon dioxide

2.2.3 Lignocellulose to ethanol

Lignocellulose is a bipolymer that is widely available in the world. It accounts for approximately 50% of the world's biomass (Sanchez & Cardona, 2008). Due to its abundance, producing valuable products such as bioethanol is one of the most promising technologies for the future. Lignocellulosic materials contain appreciable amounts of fermentable sugars and there are currently two main processes available by which lignocellulose can be converted to bioethanol, i.e. biochemical and thermochemical processes (Limayem & Ricke, 2012) .

Lignocellulose can be placed into four categories in the following manner: (1) forest residues, (2) municipal residues, (3) waste paper, and (4) crop residues. Rice straw is lignocellulose material that has an annual production of 731 million tons spread in Africa, Asia, Europe, America and Oceania. Rice straw alone produce up to 205 billion litres of bioethanol per annum while the remaining lignocelluloses result in the production of up to 442 billion litres per annum. These figures indicate the impact that producing bioethanol from lignocellulose could have in the world. (Balat, 2011)

2.2.4 Structure of Lignocellulose

One of the most important factors considered in the lignocellulosic biomass conversion to bioethanol is its composition. The composition of lignocellulosic biomass is a vital factor, because it determines how much carbohydrates is available to produce bioethanol. Composition varies with type of biomass, species and where the species originate (Agbor *et al.*, 2011). Generally, lignocellulose contains 48 wt.% C, 6 wt.% H, and 45 wt.% O, the rest being inorganic components (Balat, 2011). Lignocellulose is made up of cellulose, hemicellulose, and lignin (Figure 2.1) (Agbor *et al.*, 2011; Gnansounou & Dauriat, 2005; Limayem & Ricke, 2012; Menon & Rao, 2012) as major components (90% dry matter) the remaining part being ash and extractives (Balat, 2011). Celluloses and hemicelluloses are tightly bonded to the lignin by covalent and hydrogen bonding (Limayem & Ricke, 2012). Cellulose and hemicellulose are converted to sugars and then fermented to make bioethanol (Balat, 2011). Lignin does not contain any hydrolysable sugars and can therefore be removed for other uses (Balat, 2011).

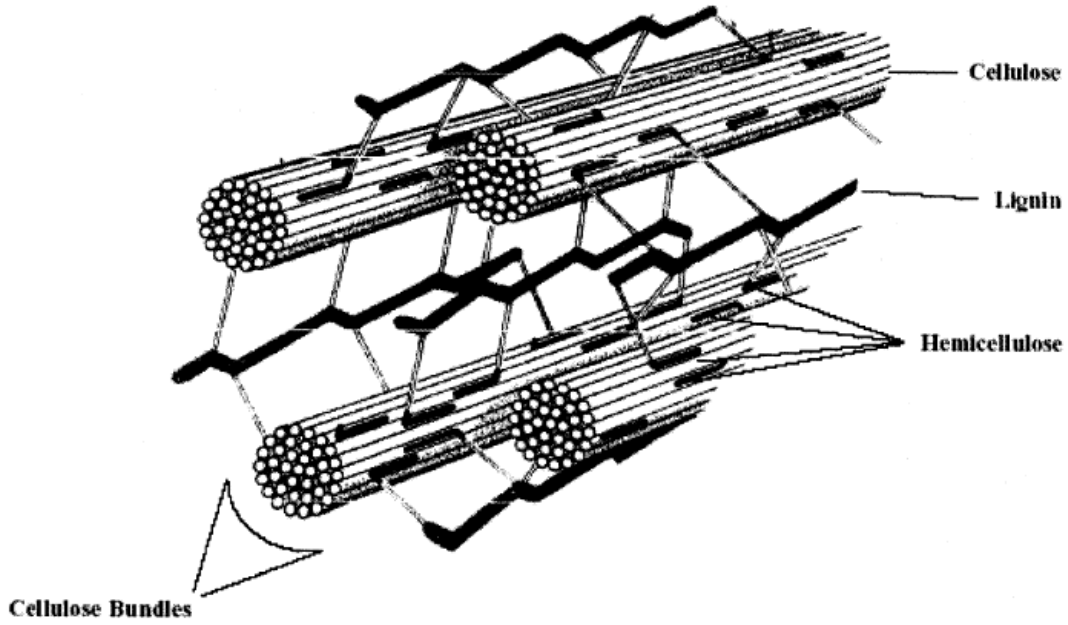

Figure 2.1: Structure of plant cell wall showing lignocellulose (Gnansounou & Dauriat, 2005).

2.2.4.1 Cellulose

Cellulose is the major component of the plant cell wall (Balat, 2011) and lignocellulose often contains approximately 40 to 60% of cellulose (Gnansounou & Dauriat, 2005). It functions

as a structural support component and it is also found in fungi, algae and bacteria (Agbor *et al.*, 2011). Cellulose is crystalline and is not branched. Cellulose contains ordered β-D-glucopyranose functional groups joined together by β-(1,4) glycosidic bonds (Agbor *et al.*, 2011; Limayem & Ricke, 2012). Cellobiose is two joined glucose molecules and cellobiose is the main repeating unit of cellulose (Figure 2.2) (Agbor *et al.*, 2011). The hydroxyl groups found in cellulose cross link with other hydroxyl groups in cellulose chains (200-300) (Agbor *et al.*, 2011), resulting in the formation of microfibrils (Limayem & Ricke, 2012; Menon & Rao, 2012). These microfibrils make cellulose recalcitrant(Limayem & Ricke, 2012). The linear structure of cellulose is caused by the hydrogen bonds within the microfibrils and the crystallinity arises as a result of interchain hydrogen bonds (Agbor *et al.*, 2011).

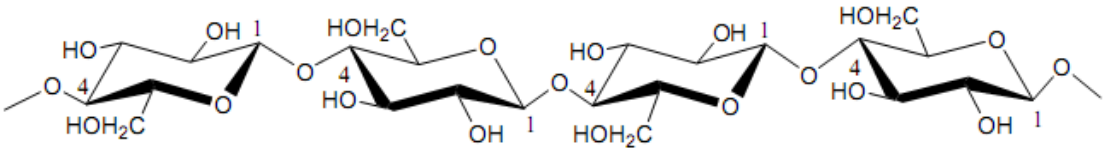

Figure 2.2: Chemical structure of cellulose (Klemn *et al.*, 1998)

2.2.4.2 Hemicellulose

Lignocellulose contains approximately 20 to 40% of hemicellulose (Balat, 2011; Gnansounou & Dauriat, 2005) . Unlike cellulose, hemicellulose is branched and non-crystalline polymers of pentose, hexose and acetylated sugars (Agbor *et al.*, 2011; Balat, 2011; Limayem & Ricke, 2012). It is made up of xylose, arabinose, mannose, glucose, and galactose (Agbor *et al.*, 2011; Balat, 2011; Limayem & Ricke, 2012). Like most lignocellulose, hemicellulose composition is different for each biomass (Agbor *et al.*, 2011). The major component in grass is xylan while softwood is composed of glucomannan. Xylan is a heteropolysaccharide with backbone chains of 1, 4-linked β-D-xylopronase units (Figure 2.3) (Agbor *et al.*, 2011; Klemn *et al.*, 1998). Xylans contain compounds including xylose, arabinose, glucoronic acids, acetic acid and ferulic acid (Agbor *et al.*, 2011).

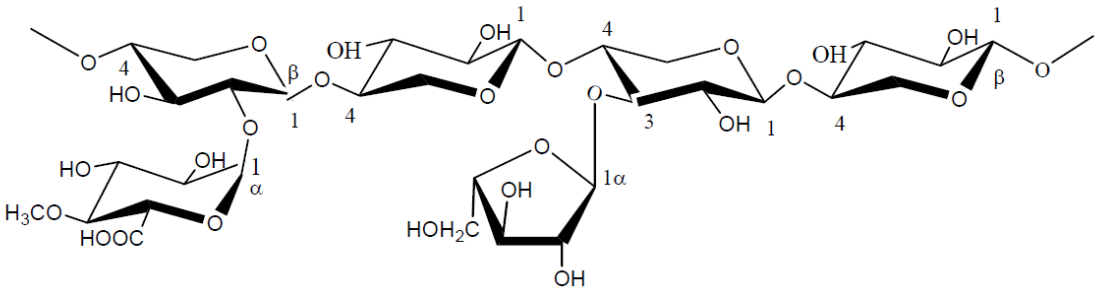

Figure 2.3: Structure of hemicellulose (Klemn *et al.*, 1998).

2.2.4.3 Lignin

Lignin comprises the smallest part of lignocellulose and makes up approximately 10 to 25% of lignocellulose (Gnansounou & Dauriat, 2005). It is found in plant cell walls and its function is to provide strength to the cell wall and protects it from microbial attack as well as facilitating transportation of water in vascular terrestrial plants. Lignin is a macromolecule with a molecular weight of 10 000 Da (Limayem & Ricke, 2012; Sun & Tomkison, 2002). There are three types of phenyl alcohol units that make up the structure of lignin, namely *trans* ρ-coumaryl, coniferyl and sinapyl alcohols (Agbor *et al.*, 2011; Hatfield & Fukushima, 2005; Sun *et al.*, 2000). These phenyl units vary with the type of biomass e.g. the sinapyl and coniferyl alcohol units are found in hardwood whereas softwood contains the coniferyl unit (Sun *et al.*, 2000). The β-ether inter-unit bonds make lignin very hard to be digested by enzymes (Sun *et al.*, 2000).

The removal of lignin is important in the conversion of lignocellulosic biomass to bioethanol. Lignin affects enzymatic hydrolysis by adsorption of the enzymes on the surface of lignin, binding to the cellulase enzyme which results in the formation of lignin-cellulase complexes, and it also contains toxic phenolic compounds that can kill microorganisms (Agbor *et al.*, 2011). Thus, pretreatment of lignocellulosic feedstock to remove lignin is crucial for the effective production of bioethanol. In addition to bioethanol production, removed lignin can be used to provide self-sustaining energy and it can also be used to make other useful products such polyurethane foam amongst many (Limayem & Ricke, 2012).

2.3 Amaranth

Amaranth is the most underexploited and underutilised crop (Teutonico & Knorr, 1985). It is very high in nutrition and this is what caused interest in amaranth plants in the last four

decades (Tucker, 1986). Amaranth is an ancient crop that dates as far back as 4000 years B.C. with the earliest record being that of grain amaranth namely *A. cruentus* recorded in Mexico (Bressani, 2003; Teutonico & Knorr, 1985). The Aztec people used amaranth as their main food crop in the 1400s until the arrival of the Spanish conquistadors in the beginning of the 1500s that banned the use of amaranth because its use was associated with religious practices (Bressani, 2003; Department of Agriculture Forestry & Fisheries, 2010; Myers, 2002; O'Brien & Price, 1983; Teutonico & Knorr, 1985; Tucker, 1986). Over the many years it was treated as a weed and it was distributed to other parts of the world as a weed, an ornament or grain (Bressani, 2003). Amaranth reappeared in the 1970s in the United States of America when research into amaranth began anew. (Department of Agriculture Forestry & Fisheries, 2010; Myers, 2002; Tucker, 1986). Thereafter, other places including Africa, India and Nepal started using amaranth grain or leaves as food crop (Department of Agriculture Forestry & Fisheries, 2010; Myers, 2002; Tucker, 1986). Farming of amaranth has also emerged in other places, including China, South America, Russia, Eastern Europe, and the Mexico (Department of Agriculture Forestry & Fisheries, 2010; Myers, 2002; Tucker, 1986).

Figure 2.4: *Amaranthus cruentus* (O'Brien & Price, 1983)

2.3.1 Plant Description

Amaranth plants (Figure 2.4) grow upright and they are approximately 1.5 to 3.0 m in height (Bressani, 2003). There is variation of colour of flower, stem and leaf across amaranth species (Department of Agriculture Forestry & Fisheries, 2010). The most common colour across the species is maroon and crimson (Department of Agriculture Forestry & Fisheries, 2010; Myers, 2002). The variations are as a result of difference in species, growth and environment (Bressani, 2003).

The stem is vertical, usually carved, thick and looks more like that of sunflower (Department of Agriculture Forestry & Fisheries, 2010). The leaves vary in size, shape (lanceolate, ovate or elliptic) and number within the different species (Bressani, 2003). The leaves are alternate in all the plants (Department of Agriculture Forestry & Fisheries, 2010). The flowers are small and usually green in colour. In the plant they occur as elongated dense clusters at the branch tip. The flowers have small spikes that can be white, green or purple (Department of Agriculture Forestry & Fisheries, 2010). Amaranth has small seeds that can be black, gold or cream in colour, but those of grain type are cream in colour (Bressani, 2003; Department of Agriculture Forestry & Fisheries, 2010). Amaranth is 1-1.5 mm in diameter with a mass of 0.6-1.3 mg per seed and lenticular in shape (Bressani, 2003). Amaranth can produce 49 to 89 g of grain (50000 to 100 000 seeds) (Bressani, 2003).

Amaranth plants are one of the rare C_4 dicots and it is this characteristic that gives amaranth the ability to adapt in different environments (Bressani, 2003; Mlakar *et al.*, 2010; Tucker, 1986). Amaranth photosynthesizes via the C4 pathway allowing it to efficiently use carbon dioxide resulting in decreased water loss (Bressani, 2003; Mlakar *et al.*, 2010; O'Brien & Price, 1983; Tucker, 1986) and allowing the plant to be able to withstand high temperature (20^oC-40^oC) and survive with minimum water without wilting. The required soil temperature for the germination of amaranth seeds and optimum growth is between 18^oC and 25^oC air temperature (Department of Agriculture Forestry & Fisheries, 2010; O'Brien & Price, 1983). Amaranth plants cannot tolerate being inundated with water and severe shortages in water causes early flowering and restricts leaf development. It grows well in well drained and fertile soil (Department of Agriculture Forestry & Fisheries, 2010; O'Brien & Price, 1983). Growth is favoured at soil pH of 6.4 and vegetable amaranth is negatively affected by soil pH between 4.7 and 5.3 (Department of Agriculture Forestry & Fisheries, 2010).

The name amaranth is a Greek word for everlasting, immortal or non-wilting (Mlakar *et al.*, 2010). Amaranth has 60 to 70 species, 40 of these species originate in America and 400 variations of the species are found all over the world (Bressani, 2003; Department of Agriculture Forestry & Fisheries, 2010; Mlakar *et al.*, 2010; O'Brien & Price, 1983; Tucker, 1986). Most of these amaranth species are weeds (Tucker, 1986). Amaranth is in the order *Caryophyllales*, the family *Amaranthaceae*, sub family *Amaranthoideae*, the genus *Amaranthus* and the section *Amaranthus* (Mlakar *et al.*, 2010) Amaranth is divided into two broad groups namely vegetable amaranth and grain amaranth based on animal and human consumption (Mlakar *et al.*, 2010; O'Brien & Price, 1983).

2.3.1.2 Vegetable amaranth

Currently, amaranth is consumed more as a vegetable than grain (Bressani, 2003). *A. tricolar* is the main type of vegetable amaranth; this species is native in south East Asia (Bressani, 2003; Teutonico & Knorr, 1985). Other vegetable amaranth species are *A. hybridus* (used in Latin America) and *A. cruentus* (used in Africa) (Bressani, 2003; Teutonico & Knorr, 1985). *A. cruentus* is a grain type but is also used as a leafy vegetable in Africa (Teutonico & Knorr, 1985).

2.3.1.3 Grain amaranth

Grain amaranth is referred to as a pseudo cereal type of grain crop and it consists of three main types of species (Mlakar *et al.*, 2010; O'Brien & Price, 1983). The first being *A. hypochondriacus* which is commonly known as prince's feather and this species is cultivated in Mexico (Bressani, 2003; Mlakar *et al.*, 2010; Teutonico & Knorr, 1985; Tucker, 1986). The second is *A. cruentus* which includes bush green and red amaranth, and it is grown in Guatemala (Bressani, 2003; Mlakar *et al.*, 2010; Teutonico & Knorr, 1985). The third is *A. caudatus* that has two subspecies namely *caudatus* (love-lie bleeding) and *mantegazzianus* (Inca wheat) (Bressani, 2003; Mlakar *et al.*, 2010; Teutonico & Knorr, 1985). *A. caudatus* is mainly cultivated in Peru and Bolvaria (Bressani, 2003; Teutonico & Knorr, 1985).

2.3.2 Uses of amaranth

Amaranth grain is rich in starch, amino acids and fats. The leaves are rich in proteins, vitamins and minerals. Amaranth leaves and grain are used as vegetables and some other amaranth species (*Amaranthus spinosus* and *Amaranthus viridis*) are used in healing of snake bite wounds (Viglasky *et al.*, 2008). Amaranth starch is used in bakery, pasta and biscuit production (Tenywa, 2012; Viglasky *et al.*, 2008). It is used in beverages, sauces and

porridge (Mlakar *et al.*, 2010; O'Brien & Price, 1983). Amaranth has very diverse uses all over the world. In Mexico they use it to make Algeria candies by popping the seeds and molasses (O'Brien & Price, 1983; Teutonico & Knorr, 1985). In India the seeds are cooked with rice or used to make candy (Teutonico & Knorr, 1985). In Nepal it is ground and mixed with flour to make gruel (Teutonico & Knorr, 1985). *A. cruentus* is mostly used as vegetable in Africa (Teutonico & Knorr, 1985). The indigenous foods of Mozambique include *A. caudatus, A. gracilis, A. graecizans* and *A. spinosus* (Teutonico & Knorr, 1985). Nigerians use amaranth to make soup and in West Africa it is used to make sauce or served over vegetables (Teutonico & Knorr, 1985). Other uses include non-toxic dyes from the red pigment in amaranth and amaranth oil is used in skin cosmetics (Teutonico & Knorr, 1985).

2.3.3 Composition and Nutritional Value

The composition of amaranth changes as a result of the variations in species type, climate, cultivation practices and sampling method used Bressani (2003) (see Table 2.1). The plants were found to contain 70-94% moisture and 6-30% dry matter. Proteins obtained ranged from 18-38% and total lipids between 1.3% and 10.6%. The protein found was high compared to 14% or less found in wheat and this protein is of very high quality (Tucker, 1986). The amino acid content in amaranth protein is well balanced and is very close to the optimum protein reference pattern in the human diet which is set by FAO/WHO (Bressani, 2003; Mlakar *et al.*, 2010; O'Brien & Price, 1983). Amaranth protein contains a high percentage of lysine, an essential amino acid that our bodies cannot produce, with lysine in amaranth being double the amount found in wheat and almost triple that found in maize (Bressani, 2003; O'Brien & Price, 1983; Tenywa, 2012; Tucker, 1986). Amaranth contains approximately 5.4 to 24.6% crude fibre and 7.6 to 22.2% ash. Variations in composition of amaranth plants are a result of the age of the plant (Bressani, 2003).

Amaranth also has other compounds that accumulate with growth including nitrates, oxalate, tannins and phylate (Bressani, 2003; O'Brien & Price, 1983). Amaranth contains minerals including calcium, magnesium, phosphorus, potassium, sodium, iron, copper, zinc, manganese and sulfur and vitamins including thiamine, riboflavin, vitamin C as well as carotene, folic acid, biotin and nicotinic acid (Bressani, 2003; O'Brien & Price, 1983; Tenywa, 2012).

Table 2.1: Compositional analysis of amaranth (grams per 100g)*(Bressani, 2003)

	Vegetable	Grain	Forage
Moisture	85±4.4	9.9±2.0	87.8±0.76
Dry matter	15.0±4.4	90.1±2.0	12.2±0.76
Protein (N ×6.25)	24.1±4.2	15.2±1.7	19.2±5.6
Total lipids	3.8±0.68	7.0±1.6	2.9±1.3
Crude fiber	14.9±3.7	6.2±3.2	16.6±6.2
Dietary fiber		13.6±4.8	
Ash	17.7±1.6	3.3±0.5	19.0±3.9
Carbohydrate	42.9±4.6	62.1±7.6	43.8±8.6
Amylose	-	6.1±1.2	-
Energy (calories per 100g)	284	336	337
Metabolic energy	-	12.2	-
Nitrate	0.55±0.19	-	-
Oxalate	4.5±1.8	-	5.86±1.89
Phytate	-	1.03±1.16	-
Tannins	-	0.18±0.14	-
Cell walls	-	-	63.5±7.7
Neutral detergent Fiber	-	-	43.4±10.9
Acid detergent fiber	-	-	34.6±15.0
Acid detergent lignin	-	-	5.2
Cellulose	-	-	23.4
Hemicellulose	-	-	20
In vivo digestion (%)	-	-	60.0±4.1

*Data are given on a dry-weight basis except moisture

2.3.4 Amaranth in South Africa

In South Africa, amaranth naturally occurs as a wild crop after the first rains. Amaranth is planted to be used for food and also to obtain seeds for stocking (Department of Agriculture Forestry & Fisheries, 2010). Amaranth grows in Limpopo, North West, Mpumalanga, KwaZulu-Natal and Eastern Cape (Department of Agriculture Forestry & Fisheries, 2010; Mnkeni *et al.*, 2007). There are no available figures of the level of production (Department of Agriculture Forestry & Fisheries, 2010).

The consumption of amaranth in South Africa is low despite it being a highly nutritious plant. The leaves are consumed as a vegetable by Xhosa women in the eastern cape; Xhosa man do not eat amaranth because they believe that they will become feminine and do not eat amaranth because they believe that eating purple or red leaves would make them insane . Overall, the limited use of this crop has been caused by superstition and the limited knowledge that rural people have of it. (Mnkeni *et al*., 2007)

Various studies on amaranth have been done in agronomy and food science by Universities including the University of Free State, University of Fort Hare, North West University and University of Pretoria as well as the research institute namely the Agricultural Research Council. A study conducted by Mnkeni and co-workers (2007) to evaluate the nutritional quality of vegetable and seed amaranth in South Africa found that the leaves contained high ascorbic acid and nitrates. The seeds were found to contain high amounts of manganese, iron and zinc and this study was based in the Eastern Cape (Mnkeni *et al*., 2007). Another study was carried out by Blodgett and co-workers (2007) to determine whether the growth of endolyptic fungi on *Amaranthus hybridus* was a result of the soil and watering practices which concluded that soil had an influence on the fungal colonies found. This study was based in Potchefstroom, North-West Province, South Africa. Bello and co-workers (2011) conducted a study on how water supply and harvesting frequency affects *Amaranth cruentus* production in semi-arid areas and this study was based in Bloemfontein, Free State Province South Africa. Bello and co-workers (2011) was able to prove that the production of amaranth was able to increase when continuously harvested and also that in addition to the rainfall, small amount of water for irrigation increases production regardless of the rainfall (Bello *et al.*, 2011). Amaranth was one of the leafy vegetables included in the study done by Voster and co-workers (2007) that evaluated the importance of these leafy vegetable in South Africa (KwaZulu-Natal, Eastern Cape and Limpopo). It was concluded that food security in rural South African regions is dependent on leafy vegetables and that in order to sustain these crops, conservation practices needs to be employed (Voster *et al.*, 2007).

The evaluation of amaranth as a potential feedstock for biofuels production has not been done in South Africa until now. A few people have conducted such studies around the world but none of these studies have actually shown figures of bioethanol production (Akond *et al.*, 2013a; Akond *et al.*, 2013b; Godin *et al.*, 2013; Viglasky *et al.*, 2008). The composition of amaranth to determine whether or not it is possible to produce biofuels from amaranth was evaluated (Godin *et al.*, 2013; Viglasky *et al.*, 2009; Viglasky *et al.*, 2008). A recent study

conducted by Akond and co-workers (2013a) that characterised 35 species of *amaranthacae* evaluating biomass yield, cell wall components and morphology found that the heights of the plants had direct correlation with dry matter, a weak correlation with composition of cell wall and lignin has a negative influence on cellulose and hemicellulose. These species showed diversity in morphology, cell wall yield and biomass yield and this is important for development of biofuels crop (Akond *et al.*, 2013a). Another study that Akond and co-workers (2013b) did on genotypic variations and cell wall polymers of *amaranth* and *celosia* genera concluded that *Amaranthus cruentus* had the lowest lignin content and highest cellulose content compared to the other 35 species investigated. Therefore, it was concluded that *A. cruentus* is a potential biofuels feedstock (Akond *et al.*, 2013b). Early studies on amaranth as a potential biofuels feedstock were carried out by Viglasky (2008) who also stated that *A. cruentus* should be utilised for biofuels production. A similar results was found by Godin and co-workers (2013) who evaluated potential biofuel feedstocks using 1059 species of 49 different plants and amaranth amongst them.

The studies have shown that in South Africa amaranth is not being used to its full potential, either as a food crop or in renewable energy production (Bello *et al.*, 2011; Mnkeni *et al.*, 2007; Voster *et al.*, 2007). Research studies on the potential of this crop for biofuels production are underway around the world and in South Africa using different conversion routes. Amaranth lignocellulose can be converted to biofuels via two ways and these are the thermochemical process and the biological process.

2.4 Conversion Routes of Lignocellulose to Biofuels: Thermochemical conversion methods

During thermochemical conversion methods, biomass is treated at high temperatures in the presence of oxygen or without oxygen to break down the structure of biomass. There are four main methods namely combustion, gasification, pyrolysis and liquefaction.

2.4.1 Combustion

Combustion is one of the oldest methods of harnessing energy from biomass and it involves burning of biomass in open air (Goyal *et al.*, 2008). The energy is obtained in the form of heat and electricity (Srirangan *et al.*, 2012). During combustion, a chemical reaction occurs between excess oxygen and biomass, producing heat, water and carbon dioxide (Goyal *et al.*, 2008). Combustion at a temperature range of 800°C–1000°C produces hot gases (Goyal *et*

al., 2008). Various process equipment can be used for combustion, including furnaces, stoves, boilers and steam turbines (Goyal *et al*., 2008; Speight, 2011). Combustion is used domestically for heat and cooking, and it is used industrially for generation of steam for turbines and boilers (Goyal *et al*., 2008). The advantages of this process is that the feedstock is cheap and exist is large quantities (Srirangan *et al.*, 2012). The drawback is that the feedstock cannot be readily combusted; it needs to be modified (Goyal *et al.*, 2008). Meaning that it needs to have low water content therefore requires drying (Speight, 2011; Srirangan *et al.*, 2012), it also needs chopping and grinding before it can be combusted and this increases the processing cost (Goyal *et al.*, 2008). Another concern is that direct combustion of contaminated municipal wastes may release toxic pollutants therefore negating the clear technology associated with the process (Srirangan *et al.*, 2012)

2.4.2 Gasification

Gasification involves the conversion of biomass in oxygen at temperatures between 800°C and 900°C to form a gaseous mixture of hydrogen, carbon dioxide, carbon monoxide and other compounds along with tars, chars, inorganic constituents and ash as shown in the reaction below (Demirbas, 2009; Goyal *et al.*, 2008; Speight, 2011; Srirangan *et al.*, 2012). Methane is another product that is formed by thermal splitting of the organic material (Goyal *et al.*, 2008).

$$C_6H_{12}O_6 + O_2 + H_2O \rightarrow CO + CO_2 + H_2 + other\ products \quad \text{(Equation 4)}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{(Equation 5)}$$

The composition of the gas depends on the composition of the feedstock, the gasification process and the gasifying agent (Demirbas, 2009) The resulting bio-syngas formed can be converted to liquid fuels by the Fisher-Tropsch Synthesis or used to make higher value chemicals (Speight, 2011; Srirangan *et al.*, 2012). The drawbacks to this process is that the tars and chars are hard to clean and can also poison the catalysts used during fuel synthesis (Srirangan *et al.*, 2012). A typical thermochemical lignocellulosic plant is as shown in Figure 2.5.

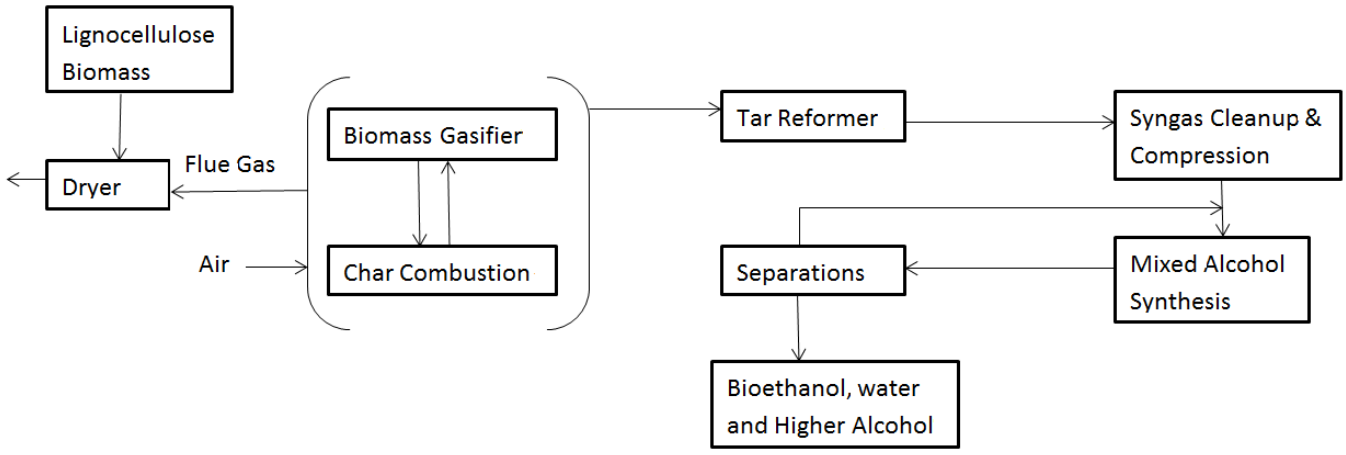

Figure 2.5: Thermochemical conversion pathway of lignocellulose to bioethanol (Srirangan *et al.*, 2012)

2.4.3 Pyrolysis

Pyrolysis is the rapid decomposition of biomass in an inert environment producing gaseous products and chars by treating lignocellulose at temperatures of 350°C to more than 800°C (Balat, 2011; Demirbas, 2009; Goyal *et al.*, 2008; Speight, 2011; Srirangan *et al.*, 2012). At low temperatures, the decomposition is slow and forms less volatile products (Sun & Cheng, 2002). The amount of products formed and their quality depends on the reaction temperature, pressure, heating rate and reaction time (Goyal *et al.*, 2008; Srirangan *et al.*, 2012). Factors that determine the variation of each product formed are the composition of biomass, heating rate, temperature and residence time (Goyal *et al.*, 2008; Srirangan *et al.*, 2012). Slow pyrolysis promotes the formation of chars whereas fast and flash pyrolysis will promotes the formation of bio-oils (Goyal *et al.*, 2008; Srirangan *et al.*, 2012). The quality bio-oils produced from pyrolysis needs further purification to ensure that the oils can be used as transportation fuels (Srirangan *et al.*, 2012).

2.4.4 Liquefaction

Liquefaction involves the decomposition of biomass in the presence of a catalyst into light molecules (Demirbas, 2009; Srirangan *et al.*, 2012). The reaction is in a liquid phase and is pressurized (10-20 MPa) at low temperatures (250°C–350ºC) (Srirangan *et al.*, 2012). The light molecules formed are unstable and reactive and re-polymerize to form heavy oily compounds (Demirbas, 2009; Srirangan *et al.*, 2012). This reaction is accompanied by other undesirable side reactions and also the formation of chars (Srirangan *et al.*, 2012). This can be prevented by the addition of hydrogen and organic solvents as well as catalysts such as

alkaline hydroxides and carbonates (Srirangan *et al.*, 2012). Drawbacks to the use of liquefaction is that the reactors are complex and expensive (Srirangan *et al.*, 2012).

2.5 Conversion Routes of Lignocellulose to Biofuels: Biochemical conversion of lignocellulose to bioethanol

Biochemical conversion methods make use of microorganisms to assimilate feedstock (biomass) and convert it to bioethanol (Srirangan *et al.*, 2012). The major benefits of a biochemical pathway is that it does not produce any toxic waste into the environment and only minor by-products are formed (Srirangan *et al.*, 2012). The drawback is that it can be very slow, therefore will take longer to complete the reaction (Srirangan *et al.*, 2012). A typical biochemical conversion process has three major steps, namely pretreatment, hydrolysis and fermentation (Figure 2.6) (Xu *et al.*, 2011b). During pretreatment, hemicellulose is broken down to reducing sugars and it causes cellulose to be susceptible to hydrolysis by decreasing cellulose crystallinity (Lin & Tanaka, 2006; Xu *et al.*, 2011b). In the second step, cellulose is hydrolyzed to its reducing sugars using enzymes (Limayem & Ricke, 2012). Lastly, the produced sugars are fermented by micro-organisms to bioethanol (Limayem & Ricke, 2012).

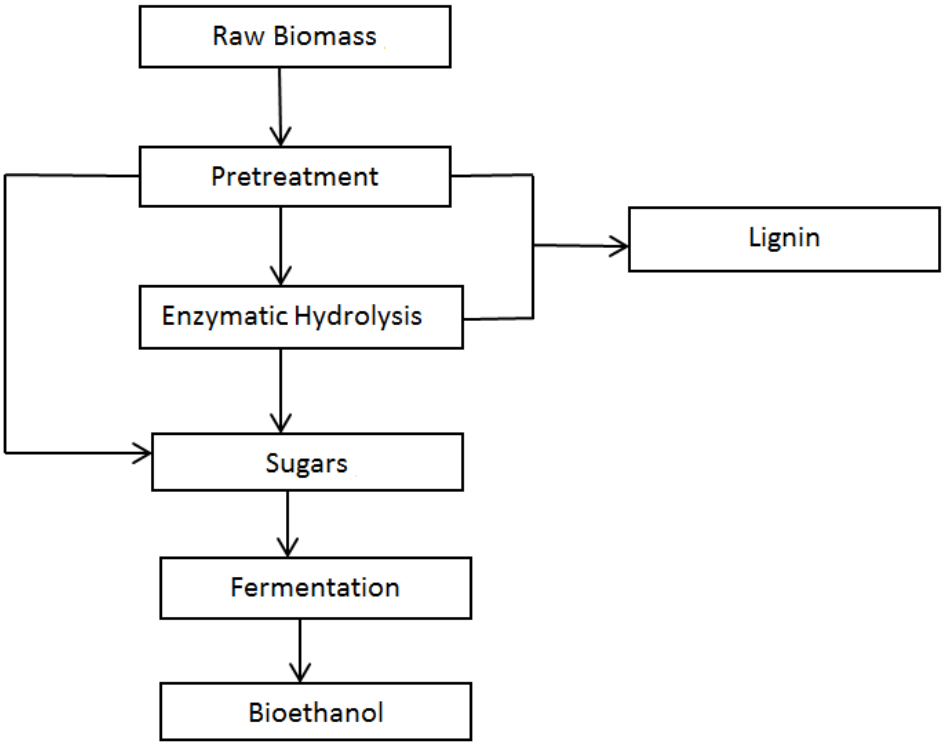

Figure 2.6: General pathway for conversion of biomass to bioethanol (Xu *et al.*, 2011b).

2.5.1 Pretreatment

Pretreatment is one of the vital steps in bioethanol production. A good and effective pretreatment is necessary to expose and break down the cellulose and hemicellulose (Figure 2.7) so that sugars can be released easily during hydrolysis (Kumar *et al.*, 2009; Limayem & Ricke, 2012). These sugars are then further fermented to bioethanol. A good pretreatment procedure aims to be cost effective, enhance the formation of sugars during hydrolysis, prevent the loss of any carbohydrates and also prevent the formation of any by-products that can hinder hydrolysis or fermentation (Sun & Cheng, 2002). There are different techniques which can be applied in biomass pretreatment and these are physical pretreatment (comminution and irradiation), physico-chemical pretreatment (steam explosion, carbon dioxide explosion and liquid hot water), and chemical pretreatment (alkaline, acid, organosolv process and ozonolysis).

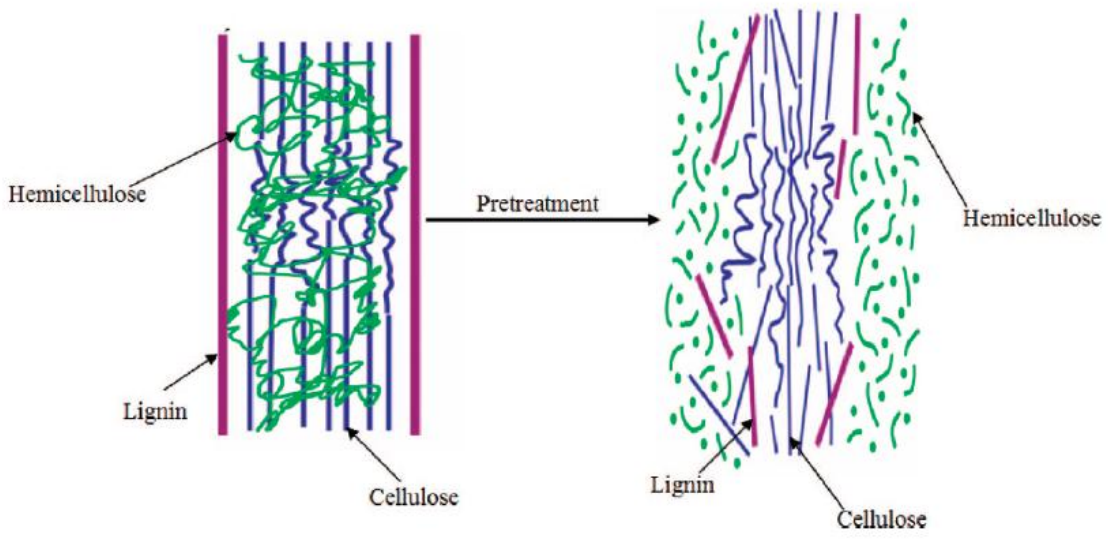

Figure 2.7: The effect of pre-treatment on the structure of lignocellulose in bio-fuel production (Kumar *et al.*, 2009).

2.5.1.1 Comminution

Biomass is comminuted in order to reduce crystallinity of cellulose, increase the surface area, decrease the degree of polymerization, to promote the digestibility of biomass and to make biomass more susceptible to enzymatic hydrolysis (Agbor *et al.*, 2011). This involves chipping, grinding, shredding and milling of biomass in order to reduce its size (Agbor *et al.*, 2011). The size of biomass will vary according to the type of pretreatment used, for instance chipped biomass has an average particles size of 10-30 mm whereas ground and milled

biomass has an average particle size of approximately 0.2 to2 mm (Sun & Cheng, 2002). Previous studies have shown that biomass particles sizes of below 0.4 mm has no effect on the rates and yields of hydrolysis (Chang *et al.*, 1997). Chipping is normally used so as to limit heat and mass transfer (Agbor *et al.*, 2011). Grinding and milling are more suitable for cellulose crystallinity and particle size reduction (Agbor *et al.*, 2011). The energy required for any physical pretreatment to be used in dependent on the particle size needed, biomass characteristics and the degree of lignocellulose crystallinity (Agbor *et al.*, 2011).

2.5.1.2 Microwave Irradiation

Irradiation refers to the application of gamma rays, electron beam, ultrasonic and microwaves (Taherzadeh & Karimi, 2008). Irradiation can be used to improve enzymatic hydrolysis as well as to break down hemicellulose and to make cellulose susceptible towards hydrolysis (Taherzadeh & Karimi, 2008)

Microwaves are found between the infrared and radio frequency in the electromagnetic spectrum (see Figure 2.8) and range from 0.3 GHz to 300 GHz (Bundhoo *et al.*, 2012; Motasemi & Ani, 2012; Tabil *et al.*, 2011). When substances are exposed to microwaves, they can absorb, reflect or transmit microwaves and it is this absorbed microwave energy that is converted to heat within thin material (Bundhoo *et al.*, 2012). Microwaves are primarily used for heating for domestic use; they have also been applied in samples digestion, extraction of analytes and in organic synthesis to accelerate chemical reactions (Segneanu *et al.*, 2013). Microwaves are unable to induce chemical reactions because they cannot break chemical bonds (Yin, 2012). This is caused by the fact that the energy of the microwave photon at the operating frequency of all microwaves of 2450 MHz is 0.0016 eV, which is much lower than the energy required to break a chemical bonds (Yin, 2012).

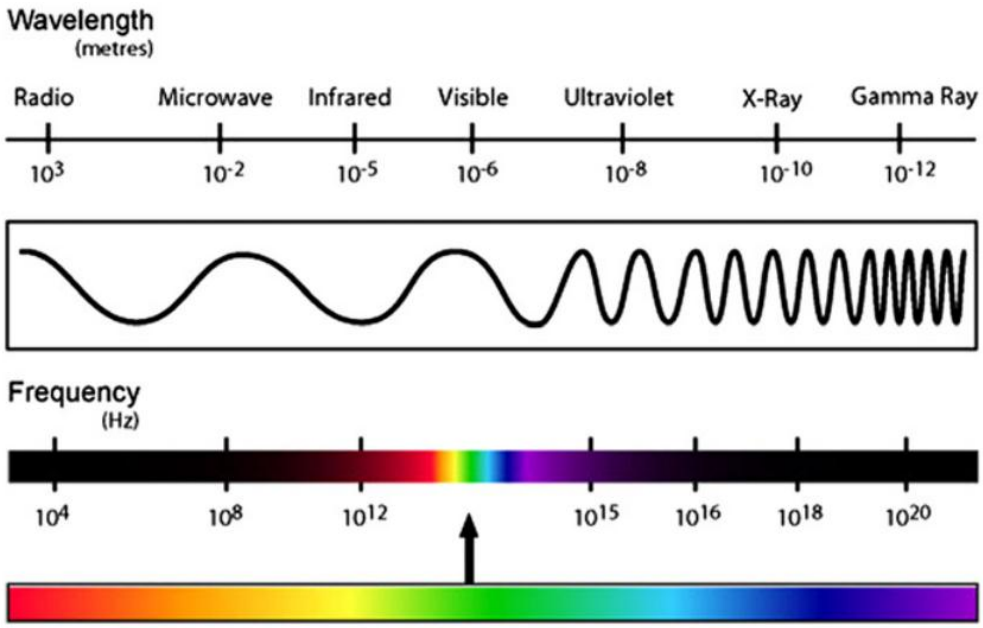

Figure 2.8: The electromagnetic spectrum (Motasemi & Ani, 2012).

Microwaves are able to break down the structure of lignocellulose by thermal effects or non-thermal effects (Bundhoo *et al.*, 2012; Tabil *et al.*, 2011). Microwave pretreatment involves soaking of biomass in a chemical solution and subjection of the broth to microwave irradiation (Alvira *et al.*, 2010). It is assumed that the microwaves will create "hot spots" that over time create an explosion (Bundhoo *et al.*, 2012; Janker-Obermeier *et al.*, 2012; Tabil *et al.*, 2011). It is this explosion that breaks down the lignocellulose structure by thermal effects (Bundhoo *et al.*, 2012) . The non-thermal effects occur when the polar side chains of the sell wall change their structure thereby accelerating the destruction of the crystal structure of biomass (Bundhoo *et al.*, 2012; Tabil *et al.*, 2011).

Microwave is now often used instead of conventional heating. The methods of conventional heating are conduction, convection or radiation (Bundhoo *et al.*, 2012). Microwave is a much more attractive method because it directly heats the reaction mixture, therefore it is more efficient; it offers shorter reaction times therefore saves time and have low energy consumption (Motasemi & Ani, 2012; Xue *et al.*, 2010; Yin, 2012). The energy efficiency of microwaves also arises on the fact that large particles can be used, less grinding chopping is necessary, which saved on energy costs (Bundhoo *et al.*, 2012). Conventional heating on the other hand has quite a few disadvantages. These include the loss of heat to surroundings, heat transfer resistance, only a portion of heat supplied is used, continuous supply of heat damage reactor walls, heating is rapid and takes longer, promoting side reaction with

undesired by products, and extensive physical pretreatment is required to reduce particle size (Bundhoo *et al.*, 2012; Yin, 2012). In biomass pretreatment, the common form of conventional heating used is the autoclave. Studies have been done by Haque and co-workers (2013) on alkali pretreatment of *M. sinensis* at boiling temperature (105^{o}C) and low pretreatment time (10 min) using NaOH (0.75-2.5%). A concentration of 2.5% NaOH was found to be the most effective in liberating 73-87% of reducing sugars and removing 74% of lignin (Haque *et al.*, 2013). Similar studies on alkaline pretreatment in an autoclave have been reported (McIntosh & Vancov, 2010) (McIntosh & Vancov, 2011; Sheikh *et al.*, 2013). Hu and Wen (2008) compared the use of microwave-assisted alkali pretreatment of switch grass to alkali pretreatment using conventional heating. It was found that microwave heating gave a yield of 34.5 g/100 g biomass of total sugars which was 53% higher compared to that obtained for conventional heating Hu & Wen (2008).

Microwave irradiation has been applied in different fields. In waste water treatment, it is used in pretreatment of sludge for production of methane in order to increase rate of reaction and to increase the yield of methane (Saha *et al.*, 2011). The sludge was pretreated at a temperature range of 50-175^{o}C in a time range of 20-130 minutes. Microwave treatment was found to increase methane production by 90% compared to the control (Saha *et al.*, 2011). Jones and co-workers (2002) reviewed the application of microwave heating in soil remediation, waste processing, minerals processing and activated carbon regeneration in great detail. Appleton and co-workers (2005) also reviewed the exploitation of microwave technology in waste processing and treatment in an energy efficient way.

Microwave irradiation is almost always combined with a chemical pretreatment and this is when it is more effective. A case where it was not combined with any chemicals was reported by Nikolic and co-workers (2011) where corn meal in water was irradiated at 80 W and 96^{o}C from 1 to10 min, after it was enzymatically liquefied in a water bath (85^{o}C) for 1 hour and then subjected to simultaneous saccharification and fermentation (SSF) (Nikolić *et al.*, 2011). The yield of ethanol was found to be 9.91% (w/w) and a theoretical ethanol yield of 92.27% on the corn meal that was subjected to SSF for 44 hours (Nikolić *et al.*, 2011). The combination of microwave irradiation with acid pretreatment is reported by Chen and co-workers (2012) where sugarcane bagasse of 10%wt. (w/v) was pretreated with sulphuric acid (0 to 0.02 M) at 180 W for 30 minutes. The results showed that 40-44% of bagasse degrades during pretreatment, 80-98% of hemicellulose is hydrolysed and 0.005 M sulphuric acid was found to produce the maximum glucose and xylose (Chen *et al.*, 2012). Another study by

Monteil Rivera and co-workers (2012) used microwave in conjunction with 85% (v/v) ethanol and 0.5 N sulphuric acid at a time range of 0-30 minutes at 120^{o}C to extract lignin in triticale straw. The highest lignin content obtained was 91% when using 92% ethanol, 0.64 N sulphuric acid, and 148^{o}C which was optimised using response surface methodology (Monteil-Rivera *et al.*, 2012). Other studies done by Zhu and co-workers (2006) used microwave pretreatment in combination with acid, alkali and hydrogen peroxide in rice straw, and Liu and co-workers (2010) used microwave pretreatment in aqueous glycerol in Japanese cedar wood chips.

Most of the work that has been done on the use of microwaves to break down lignocellulose has shown that microwave alkali combination is more effective in breaking down the lignocellulose plant structure than acid broths (Alvira *et al.*, 2010). Janker-Obermeir and co-workers (2012) used microwave irradiation in combination with alkali treatment to solubilize hemicellulose and lignin in wheat straw, and NaOH (2-5 wt%) was the base used for pretreatment for 10 to 60 minutes at 60 to140 ºC with a 10 wt% biomass loading. More than 80% hemicellulose and 90% lignin were recovered with minor degradation products formed while the cellulose was kept intact (Janker-Obermeier *et al.*, 2012). Similar studies on alkaline microwave pretreatment have been done by Zhu and co-workers (2005), Xu and co-workers (2011a) and Singh and co-workers (2011). Tsubaki and Azuma (2013) conducted studies on total tea fractionation using microwave alkali pretreatment of green tea residue, 1% NaOH was used for pretreatment in 5 minutes at a temperature range of 120-200^{o}C using 5wt% biomass loading. This method was able to successfully separate pectic polysaccharides, protein, phenolic compounds and aliphatic compounds; a conversion of 89% of cellulose to glucose was obtained during hydrolysis (Tsubaki & Azuma, 2013). Similar studies were done by Vani and co-workers (2012) using cotton plant residue, Lai and Idris (2013) using oil palm trunks and fronds, and Boonsombuti and co-workers (2013) using corncob. The reported studies discussed above all show diversity in the feedstocks that have been used in microwave irradiation pretreatment. This shows that microwave pretreatment can have a huge impact and has success in renewable energy production.

2.5.1.3 Ultrasonic Irradiation

The application of ultrasonic energy to pretreat lignocellulose is relatively new (Yunus *et al.*, 2011). Ultrasound waves are frequencies equal to 20 kHz or higher in the electromagnetic spectrum (Bundhoo *et al.*, 2012). The principle governing ultrasonic irradiation is termed cavitation. This is when a liquid is exposed to ultrasound; the energy waves will form areas

of low pressure and high pressure resulting in a cycle able to overcome the attraction forces in the liquid (Bundhoo *et al.*, 2012). This causes formation of bubbles that bursts with the liquid resulting in severe temperature and pressure (Bundhoo *et al.*, 2012). Ultra-sonification is more of a physical process and the idea is that it would disrupt the structure of hemicellulose and decrystallize cellulose so that they are easily accessed by enzymes (Yunus *et al.*, 2011). Ultrasonic irradiation was used to dissolve lignin in wheat straw using alkali pretreatment where pretreatment without the use of ultrasonic irradiation gave a range of 43%-47% within 5 to 30 minutes and when ultrasound was used, 50% of lignin was dissolved in 35 minutes (Sun & Tomkison, 2002) . Yunus and co-workers (2011) investigated the effect of ultrasonic pretreatment on acid hydrolysis of oil palm empty fruit bunch. It was found that the highest xylose yield was found to be 58% when the biomass was exposed to ultrasonic irradiation for 45 min at 90% amplitude and a xylose yield of 22% was obtained without the use of ultrasonic irradiation (Yunus *et al.*, 2011).

2.5.1.4 Steam explosion

Steam explosion uses high pressure saturated steam to treat biomass followed by a rapid reduction in pressure and then the biomass undergoes explosive decompression (Sun & Cheng, 2002). This is usually carried out at temperature range of 160-260^{o}C (Agbor *et al.*, 2011). Steam explosion breaks down the lignin and hemicellulose using high temperature and therefore increases cellulose hydrolysis (Agbor *et al.*, 2011). It is affected by parameters such as the residence time, temperature, size of biomass and moisture (Menon & Rao, 2012). Low amounts of sulphuric acid and carbon dioxide can be added in-order to promote enzymatic hydrolysis (Menon & Rao, 2012). This method is more effective for hard woods than softwoods. Its main advantage is that there is no recycling eliminating environmental costs. It is limited by the fact that it destructs a portion of xylan fraction; it does not completely break down the lignin carbohydrate matrix and can also produce inhibitory compounds that reduce micro-organism activity during hydrolysis and fermentation (Agbor *et al.*, 2011).

2.5.1.5 Ammonia fiber explosion (AFEX)

This is often referred to as the AFEX process and it is one of the alkaline physico-chemical pretreatments. It is very much similar to steam explosion described above except that the AFEX produces only the solid part of the pretreated biomass whereas the steam explosion produces a slurry (Taherzadeh & Karimi, 2008). During this process the biomass is exposed to liquid ammonia at high temperature and pressure followed by an abrupt reduction of

pressure (Balat, 2011). The loading in the AFEX process is usually 1 to2 kg of ammonia per 1 kg of dry biomass, at a temperature range of 60-90^{o}C for 10 to 60 minutes and a pressure of above 3 MPa (Menon & Rao, 2012). The main parameters that are controlled in the AFEX process include ammonia loading, temperature, water loading, pressure and time (Menon & Rao, 2012).

The AFEX process significantly improves the fermentation of some crops and grasses (Menon & Rao, 2012). Lignocellulosic materials such as rice straw, sugar beet pulp, sugar cane bagasse, corn stover, switch grass amongst many have been used in the AFEX process (Balat, 2011). The AFEX process like other alkaline pretreatment will break up the structure of lignin and thereby remove the linkages between lignin and carbohydrates (Agbor *et al.*, 2011). These carbohydrates can then be easily accessed by enzymes for hydrolysis. It also causes the biomass to swell, thereby decreasing the degree of polymerization and recrystallizes cellulose (Agbor *et al.*, 2011). One of the merits of using the AFEX process is that no by-products are formed. Furans and any phenolic fragments of lignin that may be deposited on the cellulosic surface can be washed with water (Taherzadeh & Karimi, 2008). The pitfalls of the AFEX process is that it is not effective in biomass with a high lignin content, it also does not solubilize hemicellulose as the other pretreatments such as the dilute acid method and the ammonia used needs to be recycled for cost and environmental concerns (Taherzadeh & Karimi, 2008).

2.5.1.6 Carbon dioxide (CO_2) explosion

This process operates under the same principle as described above for steam explosion and ammonia fiber explosion. The difference being that it uses carbon dioxide instead of steam or ammonia. Supercritical carbon dioxide is used; it is available at low costs, non-toxic, non-flammable, easy recoverable after extraction and is environmentally friendly (Taherzadeh & Karimi, 2008). The addition of co-solvents such as ethanol-water or acetic acid-water in CO_2 explosion can promote further removal of lignin (Taherzadeh & Karimi, 2008). The higher pressure for carbon dioxide is necessary for faster and more efficient penetration of the CO_2 molecules into the cellulosic materials (Sun & Cheng, 2002). This process was used as early as 1982 by Dale and Moreira (1982) in the pretreatment of alfara (4 kg CO_2/kg fiber at the pressure of 5.62 MPa) which yielded 75% glucose in 24 hours of enzymatic hydrolysis. This glucose yield was lower than that obtained when using steam and ammonia fiber explosion but higher than that obtained using enzymatic hydrolysis without pretreatment. A study

conducted by Park and co-workers (2001) showed that as high as 100% glucose yields have been obtained when CO_2 explosion is coupled with enzymatic hydrolysis.

2.5.1.7 Liquid hot water (LHW) pretreatment

This is one of the oldest hydrothermal methods that have been used in the pulp industry where lignocellulose material is cooked in hot water (Balat, 2011). The LHW pretreatment exposes biomass in high pressured hot water (Balat, 2011). This process takes place at a temperature range of 473–503 K (Balat, 2011), the time is optimized according to the used temperature and it may be from seconds to hours (Fig. 2.9) (Girio *et al.*, 2012). Figure 2.9 represents the water phase diagram for water based pretreatments, which shows the regions where the LHW occurs as well as other water pretreatments such as steam explosion that occurs in supercritical conditions (Girio *et al.*, 2012). Approximately 20-40% of total mass get into solution in the LHW process (Balat, 2011). This process has very high recovery of hemicellulose (Taherzadeh & Karimi, 2008). Cellulose and lignin are less affected but it does make cellulose more susceptible to hydrolysis by enzymes (Taherzadeh & Karimi, 2008). The solid phase is rich in cellulose and lignin whereas the liquid phase is rich in hemicellulose (pentose sugars) with less inhibitor (Girio *et al.*, 2012).

The main advantage of LHW over steam explosion is the elevated pentose recovery and there is less formation of inhibitors (Taherzadeh & Karimi, 2008). General advantages of using LHW pretreatment is that there is no corrosion of hydrolysis reactors because there are no chemicals added, size reduction of biomass is also not necessary (Taherzadeh & Karimi, 2008). The main pitfall of the LHW pretreatment is that when it is used for ethanol fermentation, the hemicellulose in solution is in its oligomeric form (Girio *et al.*, 2012).

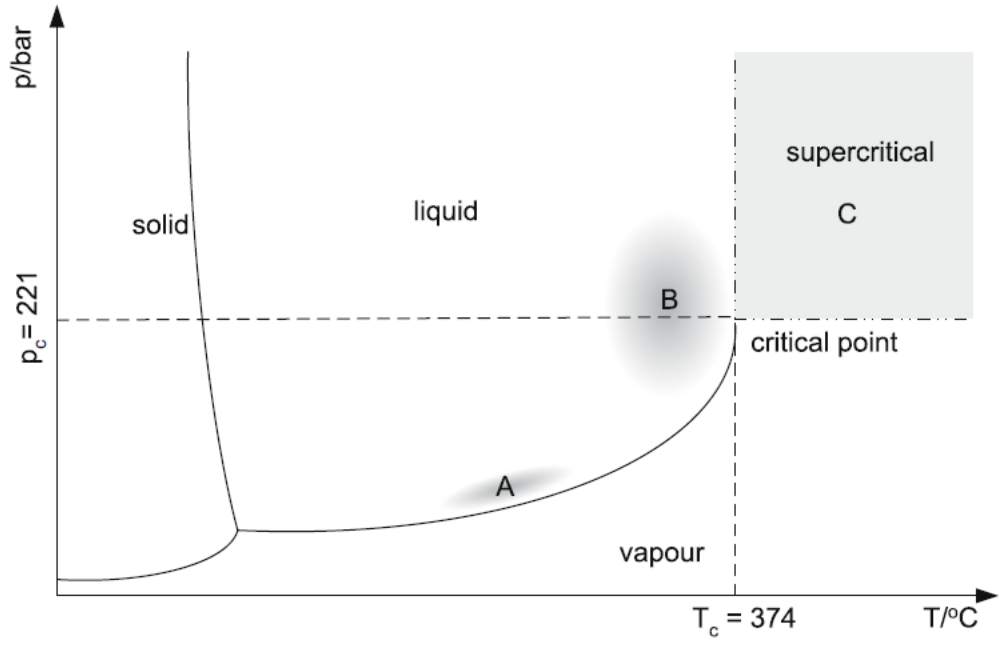

Figure 2.9: Water phase diagram showing various ranges of water base pretreatments as a function of temperature and pressure. (A) LHW, autohydrolysis; (B) subcritical conditions; (C) supercritical conditions (Girio *et al.*, 2012).

2.5.1.8 Alkaline pretreatment

This process uses alkaline solutions including bases such as sodium hydroxide, calcium hydroxide (lime), potassium hydroxide and ammonia (Taherzadeh & Karimi, 2008). These bases breaks down the lignocellulose and removes the lignin, it also dissolves the hemicellulose leaving only cellulose (Ong, 2004). The removal of lignin enhances hydrolysis and increase sugar yield. The bases basically cause the destruction of the lignin structure by causing swelling, breaking the ester bonds between the cellulose, hemicellulose and lignin (Sun & Cheng, 2002). This mode of action reduces cellulose crystallinity and the degree of polymerisation (Sun & Cheng, 2002). Pretreatment is done at low temperature and pressure, residence times are longer and the concentration of base used is high (Taherzadeh & Karimi, 2008).

Sodium hydroxide has been the alkali most studied (Menon & Rao, 2012). Its mode of action is to degrade the lignin structure and make cellulose and hemicellulose more susceptible to enzymes for hydrolysis (Menon & Rao, 2012). Lime, like most bases uses an aqueous solution of $Ca(OH)_2$ for biomass pretreatment at low temperature and pressure (Agbor *et al.*, 2011). This type of pretreatment removes the acetyl groups and also removes lignin (Agbor

et al., 2011). There are two types of lime pretreatment that have been investigated, namely the short term and long term (Balat, 2011). During short term lime pretreatment, biomass is boiled at a temperature range of 358-408 K with a lime loading of 0.1g $Ca(OH)_2$/g dry biomass from 1 to3 h (Balat, 2011). The lime loading is the same for long term pretreatment except that biomass is boiled at a temperature range of 313-328 K from 3-4 weeks in an open vessel (Balat, 2011). The benefits of using lime over other bases such as sodium hydroxide, potassium hydroxide or ammonia is that lime is cheaper, safe to use and it can be easily recovered from a solution as calcium carbonate. The drawbacks of pretreating with lime is that lime does not work well in the pretreatment of softwoods, because it does not effectively remove the lignin, large quantities of water are needed for washing up and it needs to be neutralised. This makes the preceding steps difficult to do and also increases the production costs (Agbor *et al.*, 2011).

Ammonia uses a process called ammonia recycle percolation where a solution of ammonia is used to pretreat biomass at high temperature (170^{o}C) (Girio *et al.*, 2012). This process only affect hemicellulose sugars solubilising approximately 40-60% and does not affect cellulose (Girio *et al.*, 2012). It also removes the lignin and has low selectivity (Girio *et al.*, 2012). Its mode of action is the same as that of lime and sodium hydroxide.

Oxidising agents can be combined with alkaline solutions in order to enhance the pretreatment. For instance, hydrogen peroxide (H_2O_2) is added to the alkaline solution during pretreatment. In alkaline peroxide pretreatments the biomass is immersed in water (with a pH range of 11-12 adjusted with sodium hydroxide) comprising H_2O_2 (Balat, 2011). The biomass can be soaked for as long as 6-24 hours at room temperatures (Balat, 2011). Alkaline peroxide pretreatment enhances enzymatic hydrolysis by effectively removing the lignin (Taherzadeh & Karimi, 2008). Applications of alkaline peroxide includes it use in removing waxes, silica and water proof cutins in plant tissues (Taherzadeh & Karimi, 2008).

2.5.1.9 Acid pretreatment

This pretreatment method is used to break down lignocellulose and elevates enzymatic hydrolysis. There are two main types of acid pretreatment, namely the concentrated and dilute acid method. Amongst the acids used such as nitric acid, hydrochloric acid, phosphoric acid and fluoroacetic acid, sulphuric acid is most commonly used. (Girio *et al.*, 2012).

The concentrated acid pretreatment process allows for both cellulose and hemicellulose to be hydrolysed (Girio *et al.*, 2012). This process is operated at temperatures less than 40^{o}C and the concentration of acid used is 30-70% (Taherzadeh & Karimi, 2008). The benefits of using this concentrated acid is that its low operating temperature reduces operating costs and very little sugar degradation products are produced (Girio *et al.*, 2012). The pitfalls of this process is the corrosion of reactors, recovering the acid is necessary and uses a lot of energy and large quantities of gypsum are produced during neutralisation of hydrolyzate (Taherzadeh & Karimi, 2008). These mentioned pitfalls causes high maintenance costs and therefore depleting the commercial interest of this process (Taherzadeh & Karimi, 2008).

The dilute acid process basically breaks down hemicellulose and hydrolyses it to xylose and arabinose. The hemicellulose is dissolved while the lignin and cellulose stay as solids (Ong, 2004). This process converts almost all the hemicellulose (80-100%), lignin is less affected and makes cellulose prone to enzymatic hydrolysis (Taherzadeh & Karimi, 2008). Sulphuric acid as the most commonly used acid has been used to pretreat switchgrass, poplar, corn stover, and spruce (Menon & Rao, 2012). The dilute acid method is also used in the production of furfural (Menon & Rao, 2012). The dilute acid process uses mild conditions compared to the concentrated acid process (Sun & Cheng, 2002). The dilute acid process has very high conversion yields of xylose to xylan. This makes this process more economically viable since a third of lignocellulose carbohydrates are xylan (Sun & Cheng, 2002).

The dilute acid process consists of two types, namely the high and low temperature process (Sun & Cheng, 2002). The high temperature process is a continuous flow process operating at temperatures greater than 160^{o}C with solid loadings of 5-10% (weight of substrate/weight of reaction mixture) (Sun & Cheng, 2002). The low temperature process is a batch process operating at temperatures less than 160^{o}C with a solid loading of 10-40%. The high temperature process has higher solid loadings than the low temperature process (Sun & Cheng, 2002). The pretreatment time varies from a few seconds to minutes depending on the type of biomass and temperature (Agbor *et al.*, 2011).

The benefits of the dilute acid method are that it hydrolyses the hemicellulose to its monomeric sugars, renders the cellulose more susceptible to enzymatic hydrolysis, and removes any heavy metal contaminant associated with biomass. The pitfalls of this method are the corrosion of reactors causing an increase in operating costs, the pretreated broth needs to be neutralised for fermentation, neutralising with calcium hydroxide causes the formation

of gypsum with reverse solubility, sugar degradation products are also produced that inhibit fermentation and it is necessary to reduce the size of the biomass used. (Balat, 2011).

2.5.1.10 Organosolv process (OP)

The organosolv process uses organic solvents such as acetone, ethanol and methanol amongst many in combination with inorganic catalysts (sulphuric acid, hydrochloric acid) to break down lignocellulose (Ong, 2004). Organic acids (oxalic acid, acetylsalicylic acid and salicylic acid) can also be used as catalysts with organic solvents or as their aqueous solutions (Agbor *et al.*, 2011). The catalysts are added in order for the process to operate at low temperature or to promote the removal of lignin (Taherzadeh & Karimi, 2008). During this process, biomass is heated in water containing the solvent dissolving lignin and hemicellulose, and leaving the solid phase with only cellulose (Taherzadeh & Karimi, 2008). The lignin can be extracted from the solvent and it is of very high purity. Operating temperature depends on the solvent used and can be from room temperature to 205^{o}C (Girio *et al.*, 2012).

Some factors to be considered when choosing the solvent are the solvent price and the ease with which it can be recovered; these factors are the ones driving the economics of this process. For instance, the use of low molecular weight ethanol is much more preferable than using ethyl glycol or tetrahydrofurfuryl alcohol with higher boiling points. (Taherzadeh & Karimi, 2008).

The benefits of using the organosolv process is that it has very high selectivity is a sense that it can separate fractions of hemicellulose, cellulose and high purity lignin (Agbor *et al.*, 2011). It is very good at pretreating biomass that is rich in lignin such as soft woods. Operating costs are easily reduced by recovering the organic solvent by distillation and recycling (Agbor *et al.*, 2011). The process requires less energy because size reduction of biomass is not necessary for good cellulose conversion (Agbor *et al.*, 2011). The drawbacks include the fact that chemicals and the catalysts are expensive, sometimes even more that the other well-known pretreatment processes (Agbor *et al.*, 2011). Solvents used can hinder the growth of micro-organisms used during the preceding steps including enzymatic hydrolysis and fermentation (Sun & Cheng, 2002). Therefore, the used solvents need to be removed and recycled (Sun & Cheng, 2002). The use of volatile organic solvents is a hazard because any leaks in the system can cause fire, explosion, environmental and health concerns (Agbor *et al.*, 2011). The process is expensive and complex (Ong, 2004).

2.5.1.11 Ozonolysis

This is a chemical pretreatment that uses ozone to break down lignocellulose; it specifically degrades lignin as well as part of hemicellulose (Taherzadeh & Karimi, 2008). It has been used to remove lignin in agriculture and forestry waste (Balat, 2011). The important parameters in ozonolysis are the moisture content, particle size as well as concentration of ozone in the gas flow (Taherzadeh & Karimi, 2008). The benefits of using ozonolysis is that it does not form any inhibitors, it is the most effective way to remove lignin, all pretreatments are conducted at room temperature and pressure (Sun & Cheng, 2002), and the pretreated biomass and broth does not contain any acidic, basic or toxic material (Taherzadeh & Karimi, 2008). The downside of using this method is that it requires large quantities of ozone and is therefore expensive (Balat, 2011).

2.5.2 Hydrolysis

During hydrolysis, cellulose and hemicellulose polymer sugars are broken down into monomeric form by the use of acids (dilute or concentrated) and enzymes. The sugar monomers are then fermented to bioethanol. Hydrolysis of pre-treated biomass produces higher yields (> 90%) than that of biomass that has not been pre-treated (< 20%). There are mainly two types of hydrolysis namely chemical and enzymatic hydrolysis (Table 2.2). (Hamelinck *et al.*, 2005).

Hydrolysis of lignocellulose results in a wide variety of products (Figure 2.10). Cellulose is broken down to glucose and glucose can further degrade into 5-hydroxymethyl furfural (HMF). Hemicellulose degrades by first forming xylan which is broken down to xylose. When the temperature and pressure is too high, xylose forms furfural. Hemicellulose also degrades to mannose, acetic acid, galactose and glucose. Xylan also breaks down to form water, methanol, formic, acetic and propionic acids, hydroxy-1-propanone, hydroxy-1-butanone and 2-furfuraldehyde. Lignin degrades to form phenolic compounds. (Balat, 2011).

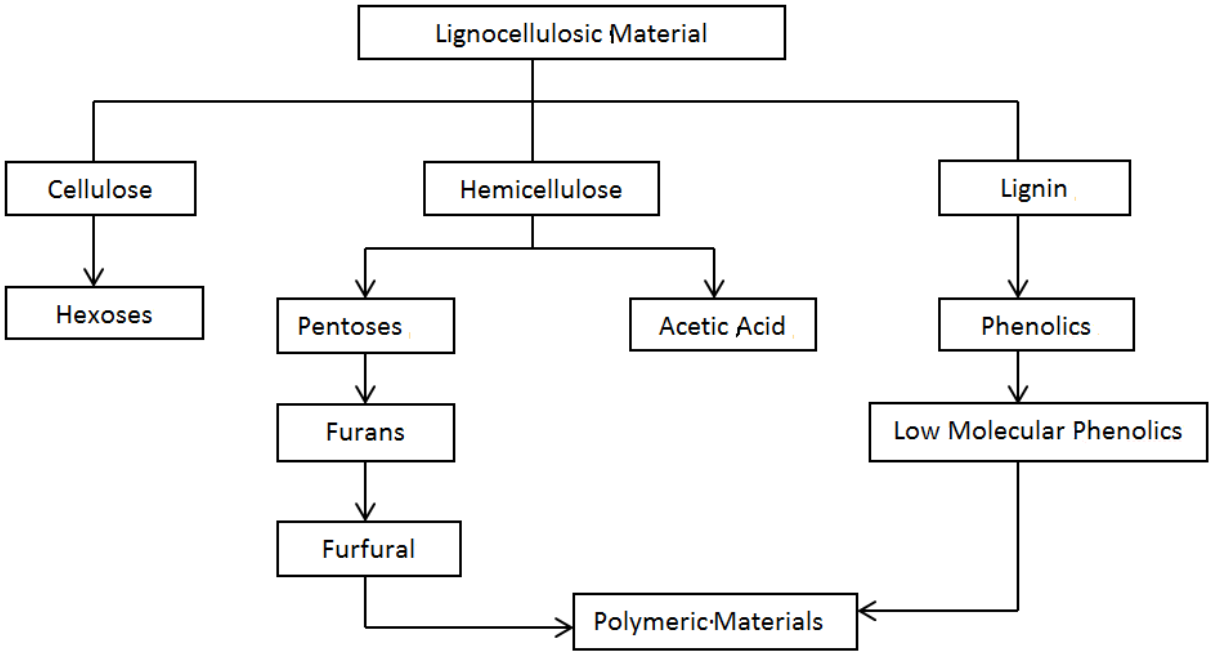

Figure 2.10: The degradation products of lignocellulose during hydrolysis (Balat, 2011).

2.5.2.1 Chemical hydrolysis

Chemical hydrolysis uses sulphuric acid or hydrochloric acid to convert hemicellulose and cellulose to their sugar constituents using specified temperature, pressure and time (Balat, 2011). There are two types of acid hydrolysis namely the dilute and concentrated acid hydrolysis (Limayem & Ricke, 2012).

Dilute acid hydrolysis can be used in a single stage or in a two stage process. In the two stage process, the first stage uses mild conditions to produce pentoses (xylose and arabinose) while the second stage uses harsher conditions to break down the stronger cellulose fibres to produce hexoses (glucose, galactose and mannose) (Cheng & Timilsina, 2011). The first stage breaks down the cellulose and milder conditions are used so that the reaction would not continue. This is done to prevent the formation of sugar degradation products such as furfurals otherwise their formation reduces the sugar yield.

The single stage dilute acid hydrolysis requires temperatures between 200-400^{o}C using a concentration range of 1-3% (Limayem & Ricke, 2012). Under these conditions degradation products of glucose into HMF and the degradation of xylose to furfural occurs (Limayem & Ricke, 2012). A two stage acid hydrolysis can be employed to avoid the formation of unwanted by products. The first stage is mild and uses a temperature of 190^{o}C with an acid concentration of 0.7% for 3 min to obtain pentose sugars (Sanchez & Cardona, 2008). The

second stage is a much harsher treatment of the solids at 215^{o}C using a concentration of 0.4% for 3 min to obtain hexoses (Sanchez & Cardona, 2008).

The challenges encountered when using the dilute acid hydrolysis is that it is difficult to increase glucose yields (>70%) while keeping cellulose conversions high with low glucose degradation in an economically viable way. The particle size of the biomass used must be reduced in order for an effective continuous process. (Balat, 2011).

Concentrated acid hydrolysis uses sulphuric acid in a concentration range of (70-90%) to break down hemicellulose (Balat, 2011). The biomass needs to be dried first before the concentrated acid is added (Balat, 2011). Concentrated acid hydrolysis has longer reaction times than the dilute hydrolysis and usually has high yields (>90%) (Balat, 2011). Hemicellulose and cellulose conversion rates are faster and complete conversion occurs (Balat, 2011). It is suitable for a variety of substrates and it produces minor sugar degradation products. The acid used has to be recycled to make the process more cost effective (Sun & Cheng, 2002). The down side of using this process is that it uses large amounts of acid and recycling is expensive, making this process not commercially applicable (Limayem & Ricke, 2012). Also, environmental and corrosion issues makes it unattractive (Balat, 2011).

2.5.2.2. Enzymatic Hydrolysis

The formation of sugar degradation products (inhibitors) can be prevented by the use of enzymes during hydrolysis. Enzymatic hydrolysis uses the cellulase and hemicellulase enzymes to break down cellulose and hemicellulose to sugars and is 100% selective towards cellulose conversion to glucose. Generally the yields are as high as 75%-85%. The process is very slow (24 to 72 hours). The advantage of using enzymatic hydrolysis is that it is compatible with many pre-treatment methods; it is conducted under mild conditions (pH of 4.8 and temperature of 318-323 K) providing high yield of sugars and corrosion of reactors is not a problem. (Balat, 2011).

Cellulase enzymes are used to hydrolyse cellulose (Gnansounou & Dauriat, 2005). These enzymes can either be produced from bacteria or fungi (Sun & Cheng, 2002). Some of the bacteria that can produce cellulase are *Clostridium, Celluumonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora and Streptomyces* (Bisaria, 1991; Sun & Cheng, 2002), . Fungi has received a lot of attention in terms of research because of the low growth rate exhibited by bacteria and they also need an

aerobic environment for growth (Duff & Murray, 1996; Sun & Cheng, 2002). Cellulase producing fungi are *Sclerotium rolfsii, P. chrysosporium, Trichoderma, Aspergillus, Schizophyllum and Penicillium* (Duff & Murray, 1996; Sternberg, 1976; Sun & Cheng, 2002). However, the most investigated species is *Trichoderma* (Sun & Cheng, 2002). Cellulase is made of three groups namely endoglucanase, exoglucanase or cellobiohydrolase and β-glucosidase (Sun & Cheng, 2002). Cellobiohydrolasae are responsible for the steadily declining the degree of polymerisation in both crystalline and amorphous cellulose (Sanchez & Cardona, 2008). Endoglucanases breaks down cellulose, abruptly decreasing the degree of polymerisation in amorphous cellulose (Sanchez & Cardona, 2008). The glucosidases are responsible for breaking down cellobiose into glucose monomers (Sanchez & Cardona, 2008).

The optimum dosage of cellulase normally used is 10 FPU/g of biomass (Balat, 2011) but changes within the range of 7-33 FPU/g biomass with any change in structure of lignocellulose and biomass loading (Gnansounou & Dauriat, 2005). This dosage gives high yields of glucose between 48-72 hours without increasing the costs of required enzymes (Balat, 2011). The drawback of using cellulase is that the rate of hydrolysis decreases with a decrease in the formation of end product (cellobiose and glucose) (Balat, 2011). Using simultaneous saccharification and fermentation is the solution to the mentioned problem (Balat, 2011).

Hemicellulose is made up of xylan which is a very complex polymer (Balat, 2011). The application for xylan hydrolysis includes ruminal digestion, waste treatment, fuel, chemical production and paper manufacturing (Balat, 2011). Like cellulase, enzymes that can hydrolyse xylan are produced by bacteria and fungi (Balat, 2011), but they are also produced by yeast, marine algae, protozoans, snails, crustaceans and seeds (Girio *et al.*, 2012). These include *Trichoderma* spp., *Penicillium* spp., *Talaromyces* spp., *Aspergillus* spp., and *Bacillus* spp. The enzymes produced are endoxylanase, exoxylanase, β-xylosidase, α-arabinofuranosidase, acetyl xylan esterase and ferulic acid esterase (Balat, 2011). The enzymes can easily break down xylan, because its structure is not crystalline regardless of the fact that hemicellulose hydrolysing enzymes are much larger than those for cellulose hydrolysis (Balat, 2011).

Table 2.2: Comparison of the different types of hydrolysis (Balat, 2011).

Method	Consumables	Temperature (K)	Time	Glucose yield (%)	Available
Dilute acid	<1% H_2SO_4	488	3 min	50-70	Now
Concentrated acid	30-70% H_2SO_4	313	2-6 h	90	Now
Enzymatic	Cellulase	323	1.5 days	75-85	Now-2020

2.5.2.3 Factors affecting hydrolysis

Hydrolysis is affected by many factors which can be classified into two broad categories namely: factors associated with substrate structure and those concerned with the enzymes used (Alvira *et al.*, 2010). The general factors are the substrate, cellulase activity, reaction conditions (temperature, pH, pressure etc.) and product inhibition (Balat, 2011).

When the concentration of substrate is low, the yield and hydrolysis rate becomes low (Balat, 2011). Therefore, increasing substrate concentration increases the yield, but it can trigger substrate inhibition which lowers the yield and hydrolysis rate (Sun & Cheng, 2002). The interaction between the substrate and the cellulase enzymes is different for each substrate and is dependent on the structural factors, namely: surface area, lignin content, degree of cellulose polymerisation and crystallinity (Hamelinck *et al.*, 2005).

Cellulose crystallinity and the degree of polymerisation are crucial during hydrolysis but these factors alone do not account for the challenges encountered with lignocellulosic substrates (Alvira *et al.*, 2010) Endoglucanases reduce the degree of polymerisation by pruning the amorphous cellulose chains (Alvira *et al.*, 2010). The smaller the particle sizes of the substrate the higher the surface areas and the easier it becomes for enzymes to hydrolyse the substrate (Alvira *et al.*, 2010). Increasing surface area and porosity increases the susceptibility of substrate towards enzymatic hydrolysis (Alvira *et al.*, 2010). Lignin inhibits enzymatic hydrolysis by binding to cellulase enzymes (Alvira *et al.*, 2010; Limayem & Ricke, 2012). This effect can be minimized by the addition of surfactants such as Tween and polyethylene glycol amongst many (Alvira *et al.*, 2010; Lee *et al.*, 2008; Limayem & Ricke, 2012)

Increasing enzyme dose improves hydrolysis rate but also increases the cost of the process (Sun & Cheng, 2002). The enzymes are usually immobilized on a support and can then be recycled and this is beneficial because it increases the rate of hydrolysis and the yield while keeping the production cost low (Sun & Cheng, 2002). The presence of cellobiose decrease the activity of cellulase and this can be prevented by the addition of more β-glucosidases when hydrolysing, removing the sugars as they are formed or using simultaneous saccharification and fermentation (Limayem & Ricke, 2012; Sun & Cheng, 2002).

2.5.3 Fermentation

Fermentation is traditionally known to make use of micro-organisms or yeast to convert C-6 sugars to ethanol under anaerobic conditions (Balat, 2011). The main challenge in fermenting sugars produced from lignocellulose is that it contains large amounts of xylose that needs special micro-organisms for fermentation (Srirangan *et al.*, 2012). A lot of research is currently being carried out in this field. Fermentation of xylose is important so as to make the fermentation of lignocellulose economically more viable (Srirangan *et al.*, 2012). As a result of this issue, no industrial scale microbial fermentation plants for lignocellulose are in operation (Srirangan *et al.*, 2012). Mostly bioethanol plants in operation are sugar cane and corn based.(Srirangan *et al.*, 2012) Fermentation of hexose and pentose sugars produces bioethanol and carbon dioxide (Limayem & Ricke, 2012) as the main by-product as shown in equations 6 and 7:

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \quad \text{(Equation 6)}$$

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad \text{(Equation 7)}$$

The type of fermentation process to be used is dependent on the kinetic properties of microorganisms, the type of lignocellulosic hydrolysate and the economic viability of the process. The fermentation process can either be continuous, batch or fed batch. The batch process is the simplest and it is a closed system. All the reagents are added into the reactor vessel and nothing else can be added after the microorganisms have been added. In the fed batch process, a fresh feedstock is added at certain time points and it enables an increase in cell concentration to its maximum, extended cell lifetime, and the product formation to it maximum. Maintenance of important parameters such as pH and temperature can be done in batch processes. In a continuous process, the hydrolysate is continuously introduced to a stirred vessel with the micro-organisms. (Balat, 2011).

2.5.3.1 Microorganisms used in bioethanol fermentation

The type of micro-organisms used in the conversion of reduced sugars to bioethanol includes bacteria, fungi and yeast (Kuhad *et al.*, 2011). Two of these micro-organisms are further discussed below because these are the commonly used micro-organisms during fermentation.

Saccharomyces cerevisiae is yeast that is most often used in bioethanol production. Its popularity is due to the fact that it can withstand high concentrations of ethanol and is not affected by the inhibitors, it has a high growth rate and a good fermentative capacity (Mussato *et al*., 2010). It can produce approximately 18% ethanol of the hydrolysate. *S. cerevisiae* can ferment hexoses and not the pentose sugars (Xu *et al.*, 2011b). As a result research is being carried out to find the micro-organisms that can ferment both hexose and pentose sugars (Xu *et al*., 2011b). Modified *S. cerevisiae* by genetic manipulation can ferment pentose sugars (Xu *et al.*, 2011b).

Zymmomonas mobilis is a bacterium that can also ferment hexose sugars and the only micro-organism that can anaerobically metabolise glucose (Lin & Tanaka, 2006). It gives higher ethanol yields (2.5 ×) than *S. cerevisiae* (Lin & Tanaka, 2006). It can withstand up to 120 g/l ethanol and can produce yields 5–10% more ethanol per fermented glucose (Lin & Tanaka, 2006). A superior recombinant *Z. mobilis* produced through genetic engineering is able to assimilate pentose sugars e.g. xylose (Xu *et al.*, 2011b).

2.5.3.2 Fermentation techniques

There are four main types of fermentation techniques that can be utilised when fermenting lignocellulose. These are Separate hydrolysis and fermentation (SHF), Simultaneous saccharification and fermentation (SSF), Simultaneous saccharification and co-fermentation (SSCF) and Direct microbial conversion (DMC) or Consolidated biomass processing (CBP) (Balat, 2011; Menon & Rao, 2012; Sanchez & Cardona, 2008; Srirangan *et al.*, 2012). Each technique is unique and can be applied to biomass conversion processes depending on the expected outcome.

Separate hydrolysis and fermentation is a sequential process (Srirangan *et al.*, 2012), hydrolysis of the pretreated broth occurs in a separate step to hydrolysis to reducing sugars followed by fermentation of the hydrolysate by micro-organisms in the second step to produce bioethanol (Menon & Rao, 2012). The main advantage of this process is that hydrolysis and fermentation occurs in their optimum conditions (Menon & Rao, 2012; Sanchez & Cardona, 2008; Srirangan *et al.*, 2012). The drawback is that the process is labour

intensive and enzymatic hydrolysis can be inhibited by the end product (Srirangan *et al.*, 2012).

During the Simultaneous saccharification and fermentation SSF process, hydrolysis and fermentation occurs at the same time (Sun & Cheng, 2002). The enzymes and the micro-organisms are added in the same reactor vessel, glucose formed during hydrolysis is instantly fermented by the micro-organisms to bioethanol (Sanchez & Cardona, 2008). The main challenge in carrying out this process is that the conditions (pH, temperature, substrate concentration) for fermentation and hydrolysis needs to be the same, Therefore hydrolysis does not occur at its optimum (Balat, 2011; Sanchez & Cardona, 2008). The use of thermotolerant yeast strains enables the fermentation temperature to be closer to the optimum enzyme temperature (Menon & Rao, 2012). The benefits of using SSF are as follows: (1) able to convert the sugars that inhibit cellulase activity at the same time increasing the rate of hydrolysis; (2) requires low enzyme dose, increased yields; (3) the instant removal of glucose and production of bioethanol reduce the need for sterile environment; (4) sugar concentration in reactor is always low therefore enzyme inhibition is significantly reduced; (5) reaction times are low as well as less volume of reactor is needed (Balat, 2011; Sun & Cheng, 2002).

The inability of *S. cerevisiae* to ferment pentose sugars has caused further research on micro-organism that can assimilate pentoses (Limayem & Ricke, 2012; Sanchez & Cardona, 2008). The Simultaneous saccharification and co-fermentation SSCF process was designed to rectify the deficiencies of the SSF process. SSCF uses genetically engineered microbes to ferment both hexose and pentose sugars (Balat, 2011; Menon & Rao, 2012). In this process, cellulose and hemicellulose are hydrolysed and the formed reduced sugars are co-fermented in the same reactor vessel (Balat, 2011).

Direct microbial conversion (DMC) involves the production of cellulase or hemicellulase, enzymatic hydrolysis and fermentation of the reduced sugars to bioethanol at the same step (Balat, 2011; Limayem & Ricke, 2012; Menon & Rao, 2012). This is the most advanced fermentation of lignocellulose technique to date (Balat, 2011). The benefits of using DMC are reduced production costs, easy operation and few reactors used (Balat, 2011). The drawbacks of DMC includes reduced ability of the micro-organism to withstand bioethanol, formation of by products (acetate, lactate) that will results in reduced bioethanol yields and insufficient accumulation of the hydrolysate (Balat, 2011). The microbial culture that has a

combination of substrate utilisation and product formation needed for this process has not been discovered (Menon & Rao, 2012), therefore research on this subject is still needed.

2.6 Conclusion

Energy security is a very important factor and biofuels are one of the most promising alternatives in the current energy crisis. A lot of research still needs to be done on the conversion of biomass to bioethanol so as to make this process more attractive and easier to apply on industrial scale. This includes improving pretreatment, hydrolysis and fermentation. Most of the issues accompanying bioethanol production are associated with economics and the environment (Gnansounou & Dauriat, 2005). In order to resolve these issues, the production process needs to be cost effective by carrying out an economic analysis. Waste production and used energy needs to be minimised. The use of irradiation and chemical pretreatments seems to be the most promising method for bioethanol conversion and therefore needs to be further investigated for different feedstock.

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CHAPTER 3

Materials and Methods

3.1 Introduction

In this chapter, the process for converting amaranth to bioethanol will be explained and described in detail. The experimental procedure used is given in section 3.2 which includes determination of composition of amaranth, pretreatment, hydrolysis and fermentation. The analyses that were done are given in section 3.3. The references used are given in 3.4.

3.2 Materials

3.2.1 Chemicals

The chemicals used in this study have been listed in Table 3.1 below together with the supplier name and purity. All the chemicals were purchased in South Africa.

Table 3.1: Chemicals used in this study.

Name	Supplier	Purity (%)	Uses
Potassium Hydroxide (KOH)	Associated Chemical Enterprises (Pty) Ltd	98	Pretreatment
Calcium Hydroxide ($Ca(OH)_2$)	Associated Chemical Enterprises (Pty) Ltd	98	Pretreatment
Sodium Hydroxide (NaOH)	Associated Chemical Enterprises (Pty) Ltd	98	Pretreatment
D(+)-Cellobiose	Sigma-Aldrich	98	Standard used in calibration curve for HPLC analysis
D(+)-Glucose	Associated Chemical Enterprises (Pty) Ltd	98	Standard used in calibration curve for HPLC analysis
D(+)-Sucrose	Associated Chemical Enterprises (Pty) Ltd	98	Standard used in calibration curve for HPLC analysis
D(+)-Fructose	Associated Chemical Enterprises (Pty) Ltd	98	Standard used in calibration curve for HPLC analysis
D(+)-Mannose	Associated Chemical	98	Standard used in calibration curve

	Enterprises (Pty) Ltd		for HPLC analysis
D(+)-Xylose	Associated Chemical Enterprises (Pty) Ltd	98	Standard used in calibration curve for HPLC analysis
D(+)-Arabinose	Associated Chemical Enterprises (Pty) Ltd	98	Standard used in calibration curve for HPLC analysis
D(+)-Galactose	Merck	98	Standard used in calibration curve for HPLC analysis
Hydrochloric acid (HCl)	Associated Chemical Enterprises (Pty) Ltd	32	Adjusting the pH
Trisodium citrate dihydrate	Merck	99-105	Buffer component
citric acid	Sigma-Aldrich	99-102	Buffer component
Tween 80	Merck	n/a	Surfactant used in enzymatic hydrolysis
celluclast 1.5 L	Novozymes	n/a	Enzyme used in hydrolysis
Novozym 188	Novozymes	n/a	Enzyme used in hydrolysis
Ethanol	Rochelle Chemicals	99.8	Standard used in calibration curve for HPLC analysis

3.2.2. Feedstock

Amaranth (*Amaranthus cruentus*) was obtained from Agricol Research Company (ARC) in Potchefstroom (26°43'37.5"S 27°04'48.2"E), North West province (RSA). The first batch was harvested in February 2012 and the second batch was harvested in November 2012. Both these batches were used in this study. Harvesting of the plants was done by hand. The plants were cut into stem and roots; they were washed under running water, dried in the over at 50^{o}C to a moisture content of approximately 10%. The dried amaranth roots and stem were milled separately using a hammer mill to particle size of < 2 mm. The milled amaranth was stored in sealed plastic bags at room temperature (19^{o}C). The handling and processing of amaranth to prepare the feedstock for use experiments is shown in Figure 3.1.

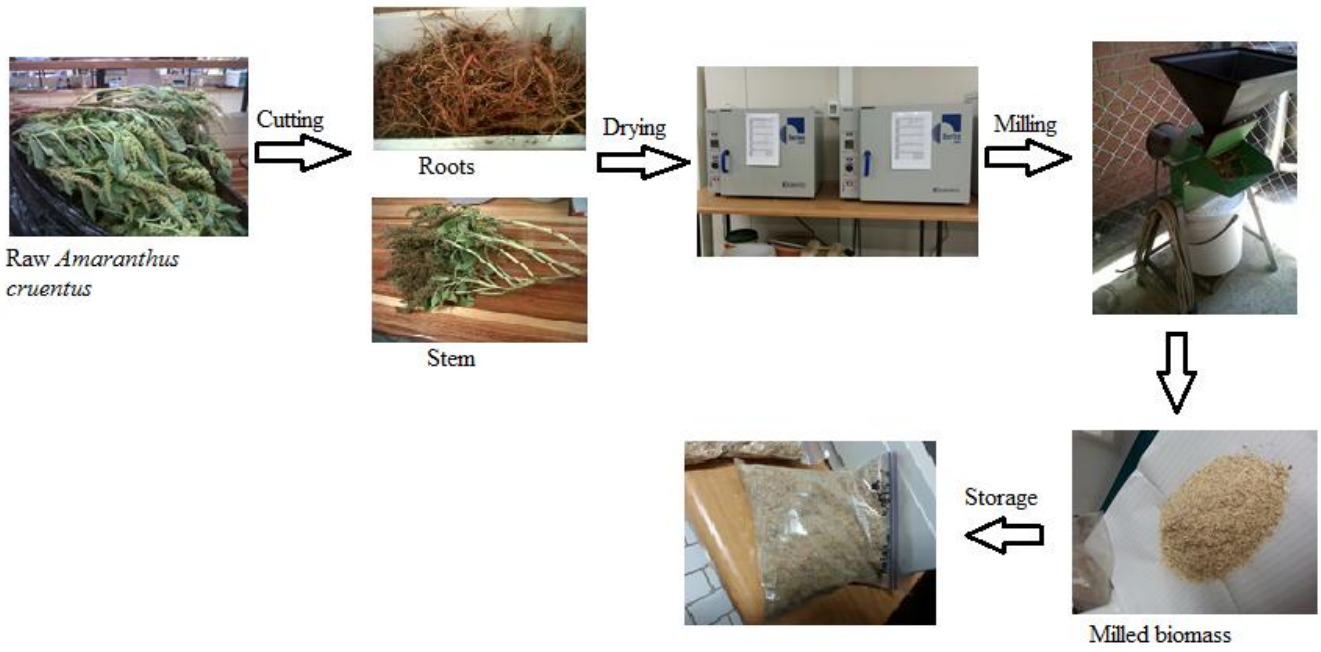

Figure 3.1: Physical pretreatment of amaranth

3.2.3. Micro-organisms

Saccharomyces cerevisiae was used in this study to ferment the obtained sugars to bioethanol. This was obtained as a commercial baker's yeast. The yeast cells were first revived by dissolution in 10 mL of hydrolysate, shaking for 10 minutes and then this broth was added to the rest of the hydrolysate to be fermented.

3.2.4. Preparation of buffer

The buffer was prepared by first making up solutions of citric acid and trisodium citrate. A mass of 21 g of citric acid was dissolved in 1000 mL of deionised water in a reaction bottle and set aside. Then, a mass of 29.4 g of trisodium citrate was dissolved in 1000 mL of deionised water in a reaction bottle and set aside. In a separate 1000 mL reaction bottle, Citric acid solution (230 mL) was mixed with trisodium citrate solution (270 mL) and 500 mL of water was added. This made up the 0.05 M trisodium citrate buffer with a pH of 4.8. A mass of 10 g of sodium azide was then dissolved in the buffer solution. The method used was similar to that used by Adney and Baker (1996).

3.3. Experimental procedure

3.3.1. Compositional analysis

The composition of amaranth plant parts (root, stem and leaves) was done in order to determine the amount of available sugars (cellulose and hemicellulose) that can be converted to bioethanol. The composition of this starting material was done by the Agricultural Research Council analytical services in Pretoria (ARC-Irene Analytical Services) to determine the acid soluble lignin, ash, protein, cellulose and hemicellulose content using the method

3.3.2. Production of bioethanol from amaranth lignocellulose

The production of bioethanol from amaranth was achieved by first pretreating biomass to reduce the crystallinity of cellulose and expose cellulose and hemicellulose for enzymatic hydrolysis. The second step was enzymatic hydrolysis of the pretreated biomass to decompose complex sugars to monomeric sugars that can be fermented to ethanol. The last step was the fermentation of the obtained sugars to bioethanol. These steps are shown in the schematic diagram below (Figure 3.2) and each step is described in detail below. The experimental procedure that is followed in this study is similar to that carried out by Keshwani (2009).

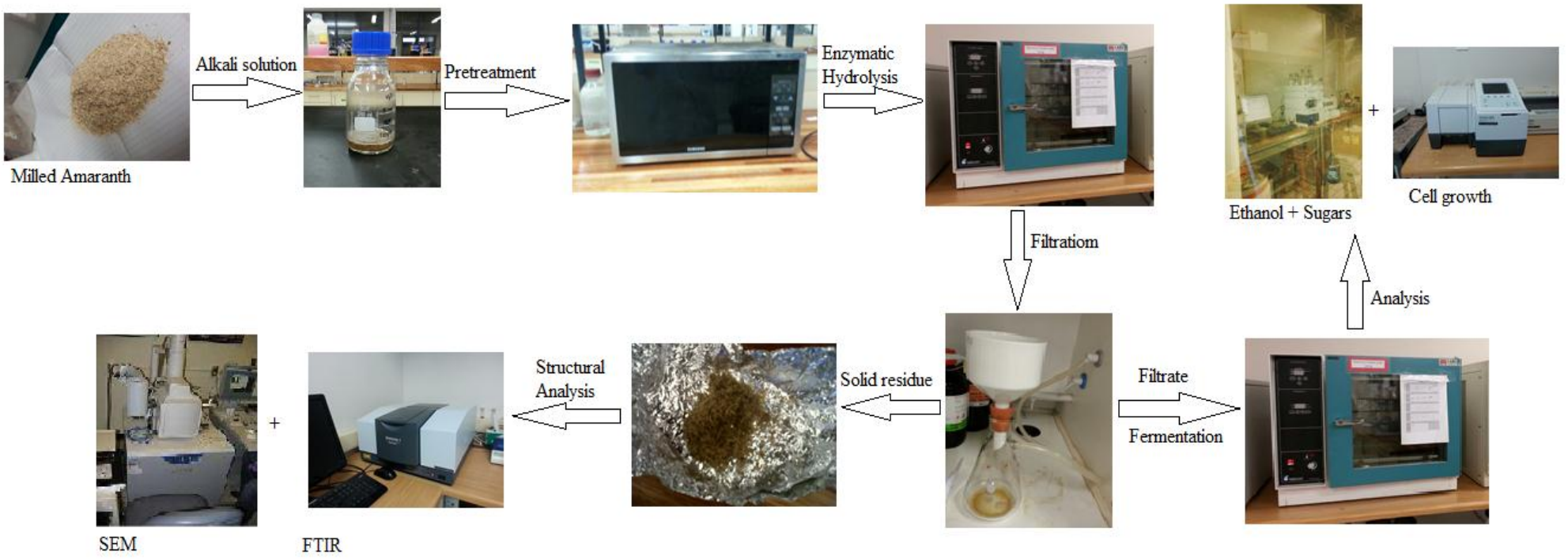

Figure 3.2: Pathway for the production of bioethanol from amaranth lignocellulose.

3.3.3. Pretreatment

A mass of 5 g of amaranth roots was weighed and 100 mL of alkaline solution was added into a 500 mL reaction bottle. This was then placed in microwave and heated. The bottle was carefully removed from the microwave and allowed to cool. The contents were filtered under vacuum; the filtrate was decanted into centrifuge tubes and its pH was adjusted to pH 7 using HCl. The solid residue was dried at 105^{o}C overnight and stored in glass vials for further analysis. A Samsung domestic microwave (Figure 3.3) oven was used in all experiments with a variable power setting in a range of 100 W to 900 W. All pretreatments were done in triplicates.

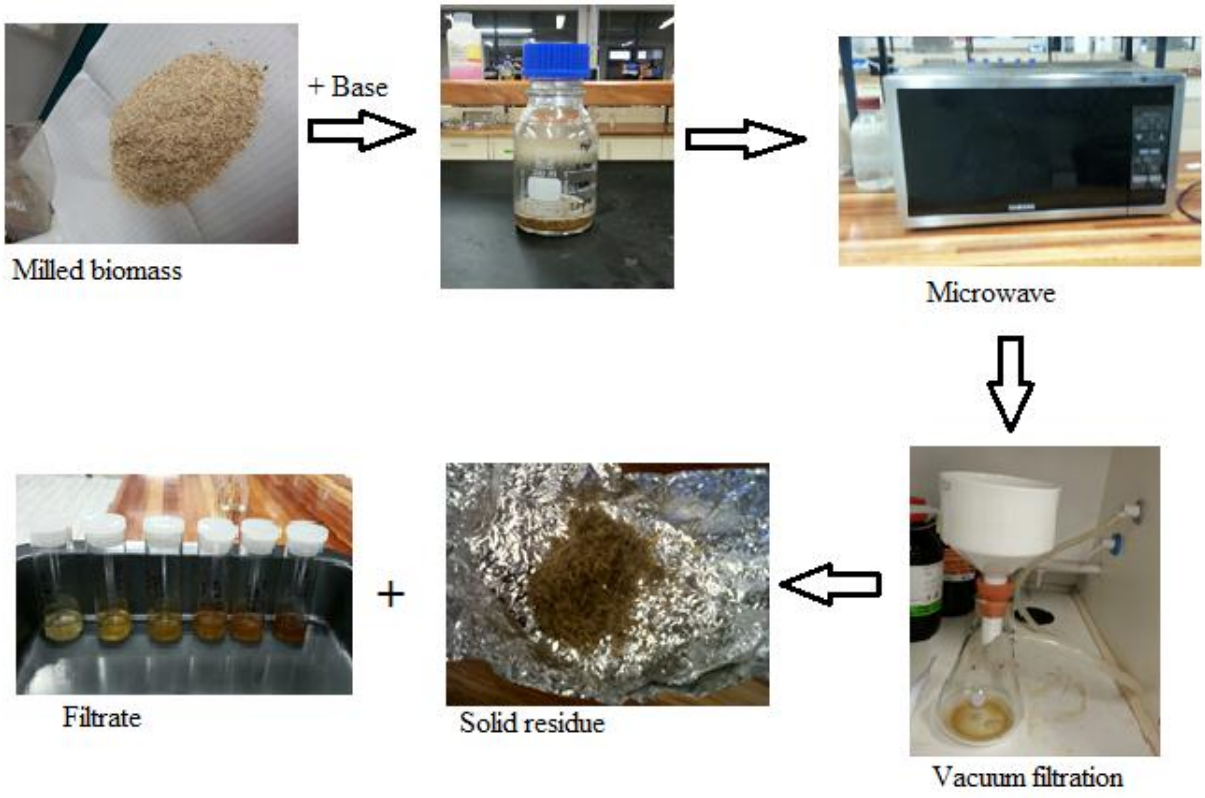

Figure 3.3: Experimental procedure followed in the pretreatment of amaranth feedstock

3.2.3.1. Evaluation of parameters

Various pretreatment parameters were evaluated for their effect on the obtained sugar yield. The effect of type of base, concentration of the base, biomass loading and power input was investigated during pretreatment. Three bases were used during pretreatment, i.e KOH, NaOH and $Ca(OH)_2$. The concentration of each base used was 10, 20, 30 and 50 g kg^{-1}. Pretreatment power input used was 6, 10.8, 12, 18, 21.6, 24, 32.4, 36, 43.2 and 54 kJ/g of

biomass as calculated in Appendix B Section B1. The loading of biomass that is often used pretreatment is 5%(w/v) as was the case in this study. This loading was varied to 3% to see what influence it has on the yield during pretreatment. All the variables are summarised in Table 3.2.

Table 3.2: Summary of parameters evaluated for microwave pretreatment

Base	NaOH, KOH, $Ca(OH)_2$
Concentration of base (g kg^{-1})	10, 20, 30, 50
Power (W)	100, 180 , 300
Time (minutes)	5, 10, 15, 20
Biomass loading (% w/v)	3, 5

3.3.4. Enzymatic hydrolysis

Enzymatic hydrolysis was done to ensure that all the sugars are released and to further release sugar monomers from recalcitrant cellulose polymers. This was done by adding celluclast (0.24 mg/L), novozyme (0.25 mg/L) and tween 80 (1.25 g/L) into the hydrolysate containing the pretreated biomass in trisodium citrate buffer containing sodium azide. The sodium azide was added to prevent microbial growth (Wang *et al.*, 2010). The buffer was used to keep the pH of the solution constant at pH 4.8. Sterilised 500 mL reaction bottles were used during hydrolysis. This was then placed in an incubator at 50^{o}C, shaking at 150 rpm for 48 hours. A sample was taken at 3 hour intervals for the first 12 hours and then at 12 hour intervals for the remaining time. After 48 hours, the hydrolysate was filtered under vacuum, the filtrated was further used in fermentation and the solid residue was dried and further analysed. The method used was similar to that described by Mcintosh and Vancov (2010).

3.3.5. Fermentation

Fermentation was carried out using a method similar to that used by Chaudhary and co-workers (2012) .The hydrolysate obtained through pretreatment which gave the highest sugar yields were fermented using *Saccharomyces cerevisiae (S. cerevisiae)* in the form of baker's yeast. This fermentation was done in sterilised 500 mL reaction bottles. The amount of yeast used was 5 g/L at a temperature of 30^{o}C in an incubator at 120 rpm. Samples were taken at chosen time intervals and these were analysed for ethanol, sugars and cell density.

3.4. Instrumental Analysis

3.4.1. Quantitative Analysis

3.4.1.1. High performance Liquid chromatography (HPLC)

This analysis was done following the procedure outline by Sluiter and co-workers (2008). Standard solutions for calibration were prepared by carefully weighing each compound (glucose, sucrose, mannose, galactose, arabinose, fructose, xylose and ethanol) into a 100 mL volumetric flask and dissolving it in deionised water. Each standard was further diluted 5 folds and these were filtered using 0.45 µm membrane filters into HPLC vials. These were analysed using HPLC and the obtained data was used to plot calibration curves (appendix A).

Total sugars and sugar monomers were analysed after pretreatment, hydrolysis and fermentation. Ethanol was also analysed after fermentation. This was done by taking the filtrate of each step, diluting it 10 fold and filtering it through a 0.45 µm membrane filter into HPLC vials. The prepared samples were then analysed by HPLC. The parameters of the instrument used are shown in Table 3.3

Table 3.3: Instrument parameters used for HPLC analysis

Instrument name	Agilent Technology systems 1200 series
Column	Shodex SP0810 Sugar column
Column temperature	80°C
Detector	Refractive index
Refractive index detector temperature	55°C
Injection volume	5 µL
Flow rate	1.00 mL/min
Mobile phase	Water

3.4.1.2. Ultraviolet spectroscopy (UV)

Cell density in the broth was measured during fermentation by measuring the absorbance of the fermented samples at 600 nm using a UV spectrophotometer (SHIMADZU) as described by Amartey and Jeffries (1996) .

3.4.2. Qualitative analysis

3.4.2.1. Fourier Transform Infra-red spectroscopy (FTIR)

The effect of the different pretreatments on the structure of amaranth was analysed with FTIR spectrophotometer (SHIMADZU) by mixing 3 mg ground samples of amaranth with 300 mg of KBr. The transmittance of the sample was measured in the range of 400 cm^{-1} to 5000 cm^{-1} with 25 scans per sample. Both unpretreated amaranth and the pretreated samples were analysed. The method used is similar to that used by Binod and co-workers (2012).

3.4.2.2. Scanning electron microscopy

The extent of structural destruction of the biomass was analysed on the unpretreated amaranth and the pretreated amaranth using a scanning electron microscope (FEI Quanta 250 FEG) as described by Goshadrou and Karimia (2011). The samples were prepared by coating amaranth biomass in a copper grid.

3.5 References

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Amartey, S. & Jeffries, T. 1996. An improvement in Pichia stipitis fermentation of acid-hydrolysed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. *World Journal of Microbiology and Biotechnology,* 12 (3):281-283.

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Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. NREL/TP-510-42623.

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CHAPTER 4

Microwave Assisted Pretreatment

4.1. Introduction

The results on the production of ethanol from amaranth lignocellulose biomass will be presented and discussed in this chapter. A compositional analysis of the amaranth used in this study is provided in Section 4.2. The parameters that affect sugar yield during alkali pretreatment, including the concentration of the base, the microwave irradiation power input and the biomass loading will be evaluated in section 4.3. The effect of enzymatic hydrolysis on the sugar yield will be investigated in section 4.4. The fermentation results will be discussed in Section 4.5 and the references are given in Section 4.6.

4.2. Compositional analysis of amaranth

The compositional analysis of raw amaranth was done by the Agricultural Research Council analytical services (Irene laboratories) and is shown in Table 4.1; this was done in order to assess the potential use of amaranth as bioethanol feedstock.

Table 4.1: Chemical composition of amaranth root, stem and leaves on a dry basis.

Component	Amaranth Biomass Composition (wt %)		
	Roots	Stem	Leaves
Dry matter	93.89	93.12	91.09
Moisture	6.11	6.88	8.91
Protein	6.81	8.94	15.98
Fat	3.02	1.05	3.03
Ash	15.12	19.44	15.65
Neutral detergent fibre (NDF)	60.02	44.47	36.32
Acid detergent fibre (ADF)	47.53	34.15	16.39
Acid detergent lignin (ADL)/lignin	11.18	7.84	3.76
Cellulose	36.35	26.31	12.63
Hemicellulose	12.49	10.32	19.93

The cellulose and hemicellulose values in Table 4.1 are similar to those found in literature (Akond *et al.*, 2013a; Akond *et al.*, 2013b; Godin *et al.*, 2013; Viglasky *et al.*, 2009). The amaranth used in this study contains a higher amount of ash than that reported in literature, but this is expected as ash content varies across species due to differences in soil where the plants are cultivated as well as differences in cultivation practices (Viglasky *et al.*, 2009). The theoretical sugar yield based on cellulose and hemicellulose was found to be 48.84 g/100g dry amaranth biomass in roots, 36.63 g/100g dry amaranth biomass in the stem and 32.56 g/100 g dry amaranth biomass in leaves. The roots were used in all the experiments because of the high theoretical sugar yield.

4.3 Pretreatment with Calcium hydroxide ($Ca(OH)_2$)

The influence of different parameter on the total sugar yield during pretreatment with $Ca(OH)_2$ was investigated. All pretreatments were done using a domestic microwave. The microwave power used in the study was varied between 100 and 300 W and these power settings were chosen to ensure that the biomass were not burned during the experiments and to minimise loss in volume due to evaporation. The effect of parameters such as energy input (microwave power and pretreatment time), the concentration of base, the type of base and biomass loading on the total as well as 5 and 6- carbon sugar yield were evaluated. The energy input was calculated as shown in Appendix B section B2 and the obtained values are shown in Table B2.1. The biomass loading was kept constant at 5 g per 100 g base solution. Amaranth biomass was pretreated with varying concentrations of $Ca(OH)_2$ with the power setting kept constant at 180W resulting in an energy input in the range of 11-43 $kJ.g^{-1}$ biomass. Pure water, without the addition of a base was used as a control in all experiments.

4.3.1 The effect of $Ca(OH)_2$ concentration

The effect of different $Ca(OH)_2$ concentration at different energy input on the total sugar yield is shown in Figure 4.1. The experimental error obtained for $Ca(OH)_2$ pretreatment was calculated to be 3.3% at a 95% confidence level and shown in Table B3.1 in appendix B.

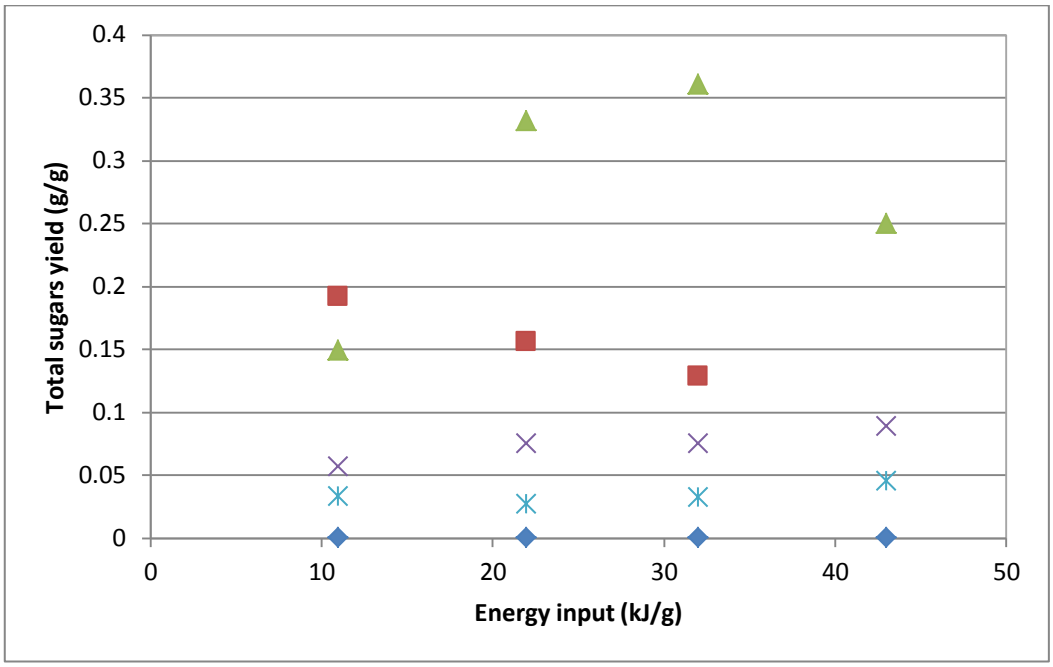

Figure 4.1: Total sugars of microwave assisted pretreatment of $Ca(OH)_2$ at different concentrations at 180 W (■ 5% $Ca(OH)_2$, ▲ 3% $Ca(OH)_2$, × 2% $Ca(OH)_2$, Ж 1% $Ca(OH)_2$, ♦ Control).

From Figure 4.1 it can be seen that all pretreatments resulted in sugar yields that are significantly higher than the control sample. Pretreatment with a 30 $g.kg^{-1}$ $Ca(OH)_2$ solution in water resulted in the highest sugar yield of 0.36 g sugar. g^{-1} biomass. This corresponds to a conversion efficiency of 73.7% based on the theoretical yield. Pretreatment with 50 $g.kg^{-1}$ $Ca(OH)_2$ solution in water resulted in sugar yields that were lower than those obtained when pretreating with 30 $g.kg^{-1}$ $Ca(OH)_2$ solution in water and decreased with increase in energy input. $Ca(OH)_2$ was able to release more sugars by breaking the ester bonds cross linking lignin and hemicellulose therefore breaking the recalcitrant structure of lignocellulose. The breaking of these ester bonds results in fractions that are rich in hemicellulose and cellulose polymers. The lower yields obtained during pretreatment with 50 $g.kg^{-1}$ $Ca(OH)_2$ solution in water are due to sugar degradation and also at high $Ca(OH)_2$ concentrations some of the alkali is converted to irrecoverable salts such as acetates.

4.3.2. The effect of power density on total sugar yields

The effect of microwave power density on total sugar yields was investigated by pretreating the biomass at constant concentration of 30 g. kg^{-1}in water solution for 15 minutes while varying the power from 100W to 300W. The influence of power input over different pretreatment times (power density) is shown in Figure 4.2.

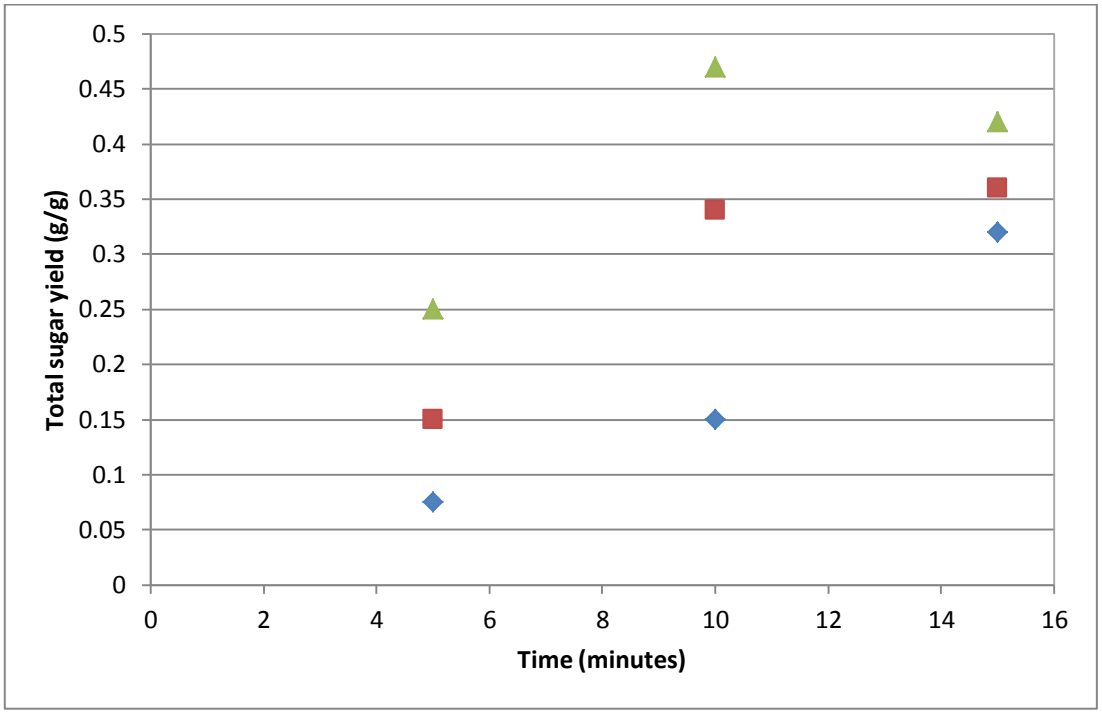

Figure 4.2: The effect of microwave irradiation power on total sugar yield (♦-18 $kJ.g^{-1}$, ■-32.4 $kJ.g^{-1}$, ▲-54 $kJ.g^{-1}$)

From Figure 4.2, it can be seen that an increase in power input at constant time results in a higher total sugar yield and that an increase in pretreatment time results in increase in sugar yield. The highest sugar yield obtained was 0.47 g/g of biomass in 10 minutes at 300 W (36 $kJ.g^{-1}$) and this corresponds to an almost complete conversion based on the theoretical sugar yield. When the power is increased, more energy is supplied to break the strong covalent ester bonds that crosslinks lignin and hemicellulose. This causes a disruption in cellulose structure because lignin is the primary structure of cell walls and holds all cells together. The increased energy also breaks the intermolecular hydrogen bonds crosslinking cellulose polymers (via hydroxyl groups) forming the microfibrils (Agbor *et al.*, 2011; Limayem & Ricke, 2012). The microfibrils make cellulose recalcitrant. The breaking of these bonds causes a decrease in cellulose crystallinity, therefore cellulose becomes more accessible and sugars are easily released. The degradation of the cell walls during microwave pretreatment

is shown in the scanning electron microscope images in Figure 4.3. The images clearly show the disruption that occurs in vessel elements found in primary cell walls resulting in destabilising the structure of cellulose. The structure is destabilized by the breaking of bonds (ester bonds and hydrogen bonds) during pretreatment as described above which results in the release of sugars. The SEM images showed that the unpretreated amaranth had a smooth layer with just a few cracks from milling and therefore all the plant material is still intact, whereas the pretreated image shows that the $Ca(OH)_2$ was able to expose the inner vessel element in primary and secondary walls of the plant. These vessels are made up of cellulose, hemicellulose and lignin.

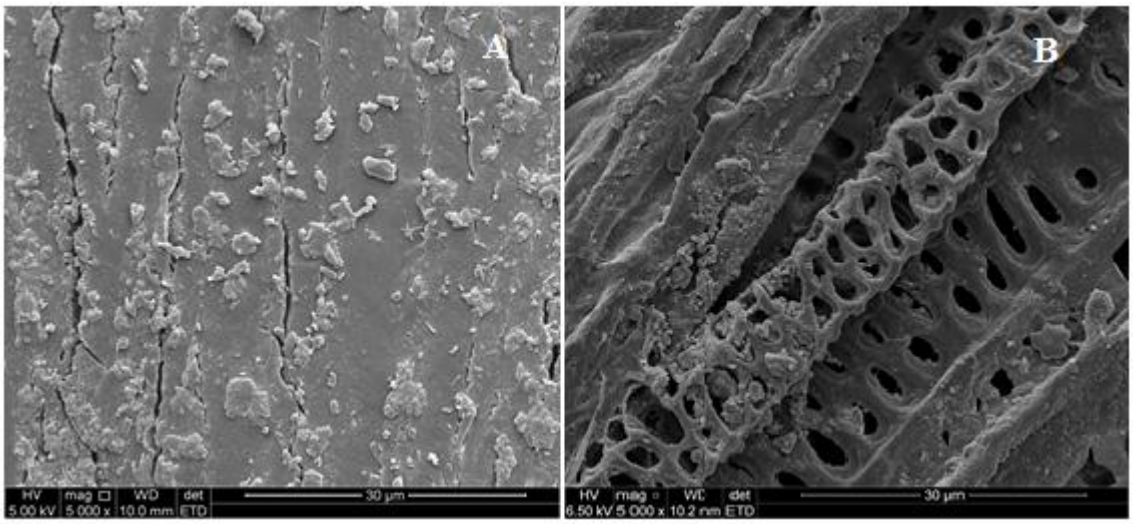

Figure 4.3: Scanning electron microscope images of unpretreated biomass (A) and $Ca(OH)_2$ pretreated biomass (B)

4.3.3. The effect of biomass loading on total sugar yield

The effect of biomass loading on the total sugar yield was investigated using a 50 g kg $^{-1}$ $Ca(OH)_2$ solution in water at different power inputs and is presented in Figure 4.4.

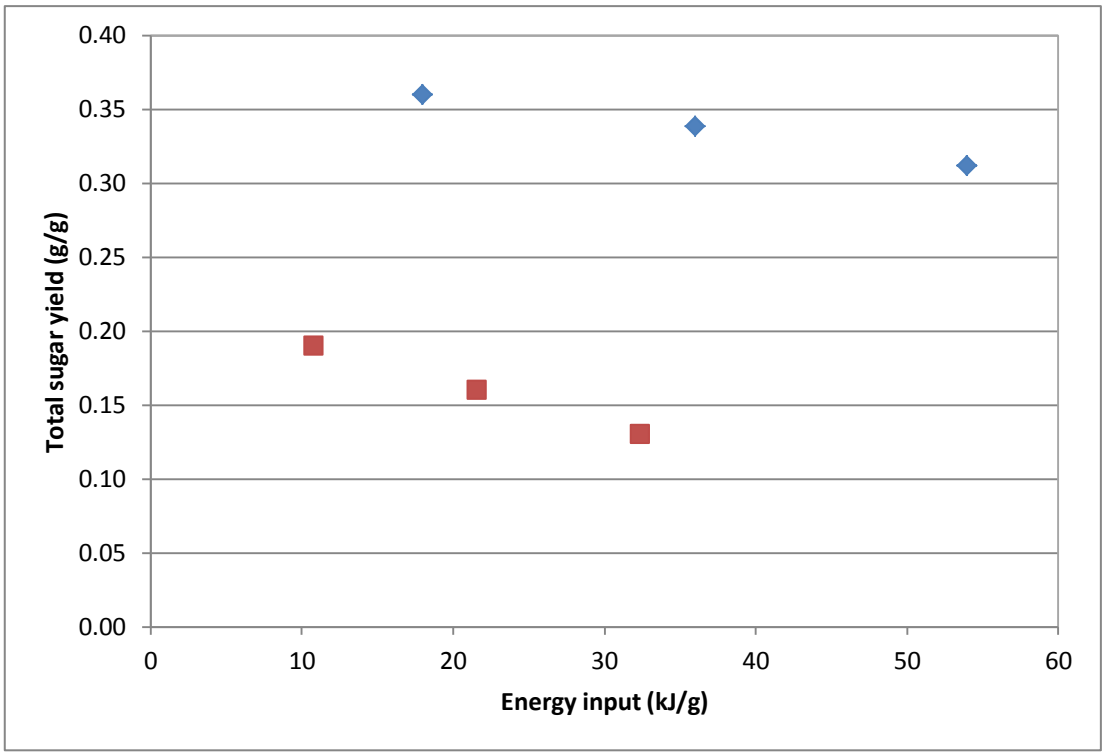

Figure 4.4: The effect of biomass loading on total sugar yield at 180W using 50 g kg $^{-1}$ $Ca(OH)_2$ solution in water (♦- 30 g biomass kg^{-1} $Ca(OH)_2$ solution, ■-.50 g biomass kg^{-1} $Ca(OH)_2$ solution)

Figure 4.4 shows that sugar yield increases with a decrease in biomass loading. The maximum total sugar yields obtained were 0.19 g/g and 0.37 g/g for a biomass loading of 50 and 30 g kg^{-1} $Ca(OH)_2$ solution at a power input of 10.8 and 18 kJ/g respectively. When the biomass loading is low the solution to be pretreated is less viscous because there is less solids in solution. This means that the solids in solution can move freely and also are easily accessible to react with the alkaline solution increasingly promoting structural disruption (breaking of bonds) and thus more sugars are released. Also at low biomass loading there is more energy supplied per biomass increasing the effect of microwave irradiation. The total sugars were found to decrease with increase in energy input and this was due to the many interactions that calcium ions has with the biomass including formation of linkages with biomass by ionic interactions with negatively charged functional groups, binding with lignin, interaction with pectin, cellulose and hemicellulose (Keshwani, 2009). These many side

reactions delay the release of sugars over time. Also a high energy input is accompanied by an increase in temperature, the solubility and pH of $Ca(OH)_2$ decreases with increase in temperature and thus the same alkaline solution at low energy input should increasingly react with biomass compared to the one at a higher energy input when all other variables are kept constant. This effect is more effective when the concentration of $Ca(OH)_2$ is high as is the case in this study.

4.3.4. The effect of $Ca(OH)_2$ pretreatment on the hexoses and pentoses sugar yield

Cellulose and hemicellulose are the major plant components; a good pretreatment is one which is able to break down these polysaccharides to sugar monomers. It is therefore important to access the efficiency of a pretreatment method by its ability to break down either cellulose or hemicellulose. Cellulose is composed of hexoses and hemicellulose has both hexoses and pentoses. The amount of hexoses and pentoses released is important to know because it will direct as to which media (bacteria or yeast) to use for fermentation because some media cannot assimilate pentoses. The effect of Ca(OH)2 pretreatment on the type of pentose and hexose sugars that were released is shown in Figure 4.5 by their proportionality on the column graphs. The results were obtained from biomass pretreatment with 50 g kg^{-1} $Ca(OH)_2$ in water solution at 100 W.

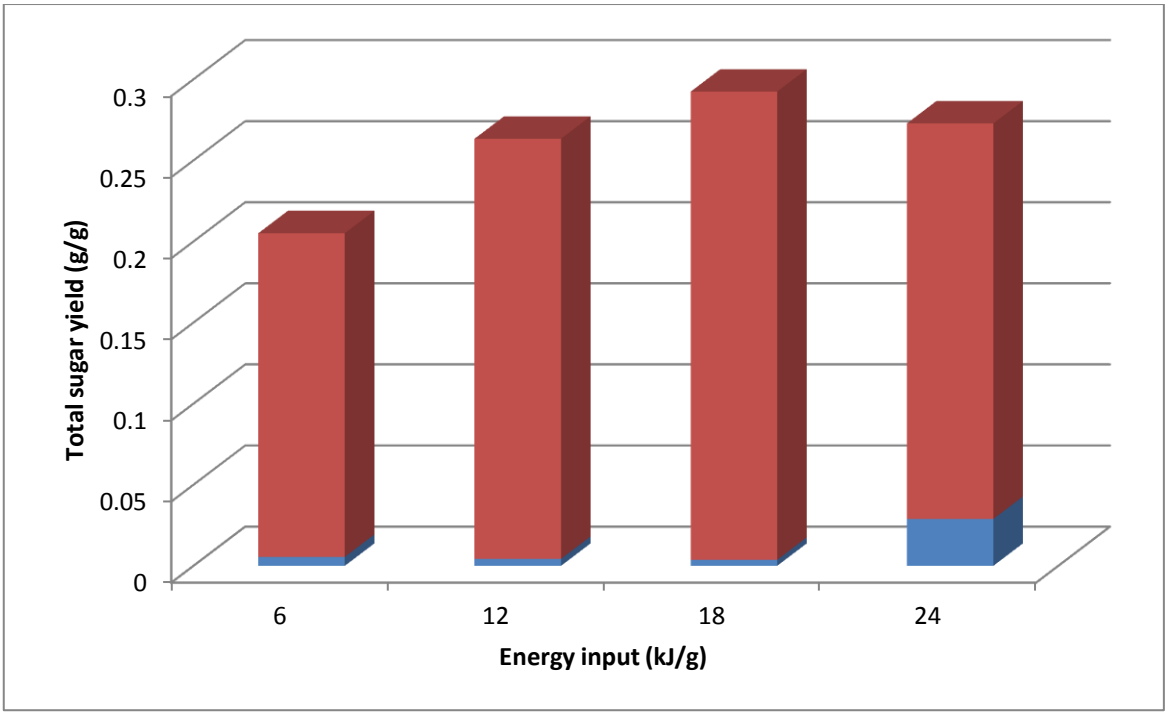

Figure 4.5: The effect of 5% $Ca(OH)_2$ on the type of sugars liberated at 100W (■ pentoses, ■ hexoses).

Figure 4.5 shows that $Ca(OH)_2$ releases more hexoses than pentoses and the highest hexose sugar yield was found to be 0.28 g/g at a power input of 18 kJ/g. The pentose concentration was not significantly different from 6 to 18 kJ/g but was found to be higher at 24 kJ/g (0.029 g/g). These results shows that $Ca(OH)_2$ was more effective at disrupting cellulose chains than hemicellulose. This was supported by the morphological changes shown by the SEM images which showed the disruption and exposure of cellulose. This was further supported by the results obtained when the biomass before pretreatment (A) and after pretreatment (B) was characterised using FTIR shown in Figure 4.6.

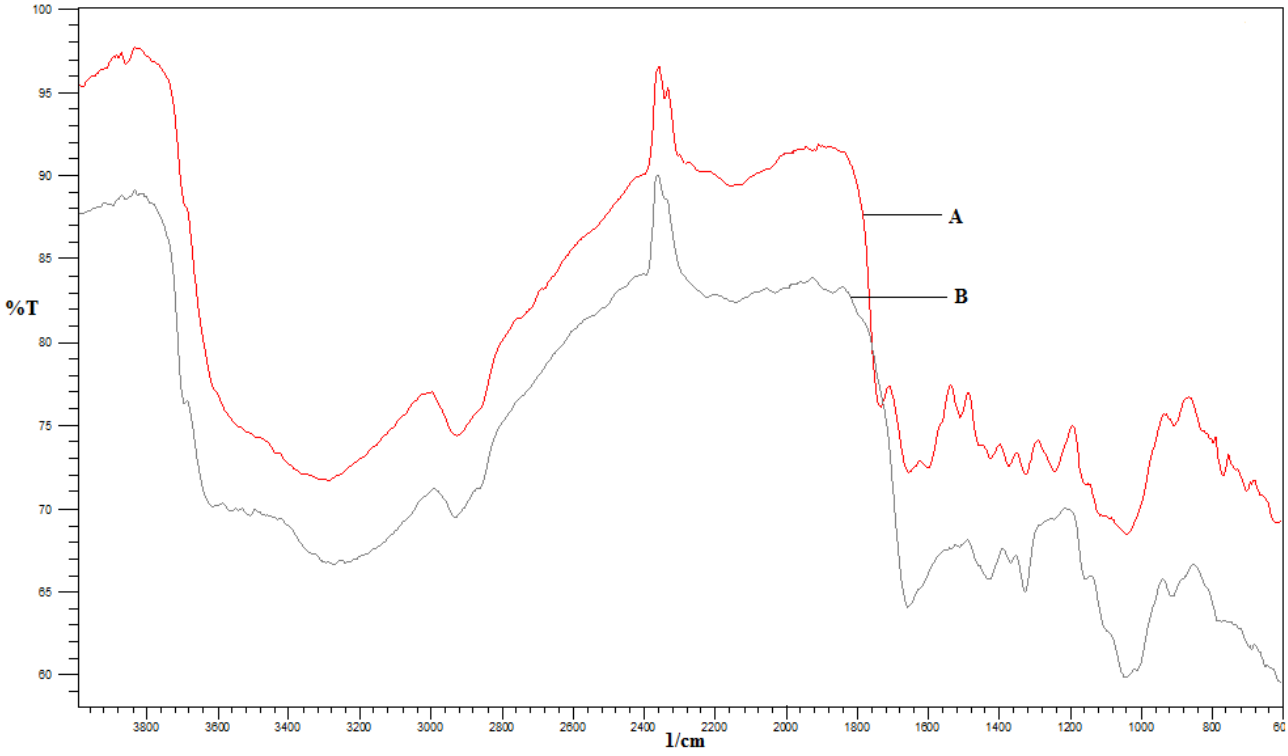

Figure 4.6: FTIR spectra of unpretreated biomass (A) and $Ca(OH)_2$ pretreated amaranth at 180 W (B)

The major peaks observed in the FTIR spectra of the unpretreated amaranth (A) can be defined as shown in Table 4.2 as described by Binod (2012) and Schwanninger (2004).

Table 4.2: Major peaks found in the spectrum of unpretreated amaranth biomass

Peak (cm^{-1})	Functional group	Description
3300	O-H	A broad absorption of the stretching of H-bonded OH group found in cellulose and lignin
2900	C-H	Asymmetric stretching of the methyl groups
2100	$CH_3C=O$	Acetyl group (alkyl group) the terminal groups found in xylan
1700	C=O	Ester carbonyl stretching, this absorption band mainly arises from the carbonyl stretch in unconjugated ketone, ester, or carboxylic groups in carbohydrates and not from lignin
1600	C=C	Stretching vibration in aromatic skeletal of lignin
1400	CH_2	Symmetric stretching
1300	CH_2	Wagging
1201	C-O-H	In plane bending at C-6
1158	C-O-C	Dominates the spectrum of cellulose linkages, the stretching at the β-(1-4) glycosidic linkages.
900	C-O-C	Dominates the spectrum of cellulose linkages, the stretching at the β-(1-4) glycosidic linkages.

The unpretreated spectra and the pretreated spectra were similar but had significant changes indicating structural changes that occurred after pretreatment. These changes were the disappearance of the band at 1700 cm^{-1} and this caused the sharpening of the band at 1600 cm^{-1}. The band at 1700 cm^{-1} is ester, ketone or carboxylic group in carbohydrates. It disappearance signifies that the base was able to break these ester bonds during pretreatment therefore altering the carbohydrate structure. The bands between 1500 cm^{-1} and 1100 cm^{-1} are characteristic of cellulose, and only two are present in the pretreated spectra whereas they were five in the unpretreated spectra. The C-O-H bending peak completely disappeared in the pretreated spectra which signifies the breaking of C-O-H bonds in the cellulose structure. The FTIR results did validate that $Ca(OH)_2$ interact more with cellulose by showing that the most affected peaks were the one associated with cellulose.

4.4 Pretreatment with sodium hydroxide (NaOH)

The influence of different parameter on the total sugar yield during pretreatment with NaOH was investigated. The pretreatments were all done using a domestic microwave. The microwave power used in the study was varied between 100 and 300 W and these power settings were chosen to ensure that the biomass were not burned during the experiments and to minimise loss in volume due to evaporation. The effect of parameters such as energy input (microwave power and pretreatment time), the concentration of base, the type of base and 5 and 6- carbon sugar yield were evaluated. The energy input was calculated as shown in Appendix B section B2 and the obtained values are shown in Table B2.1. The biomass loading was kept constant at 5 g per 100 g base solution. Amaranth biomass was pretreated with varying concentrations of NaOH with the power setting kept constant at 180W resulting in an energy input in the range of 11-43 $kJ.g^{-1}$ biomass. Pure water, without the addition of a base was used as a control in all experiments.

4.4.1. The effect of NaOH concentration

The effect of different NaOH concentration at different energy input on the total sugar yield is shown in Figure 4.7. The experimental error obtained for NaOH pretreatment was calculated to be 2.3% at a 95% confidence level shown in Table B3.1 in Appendix B.

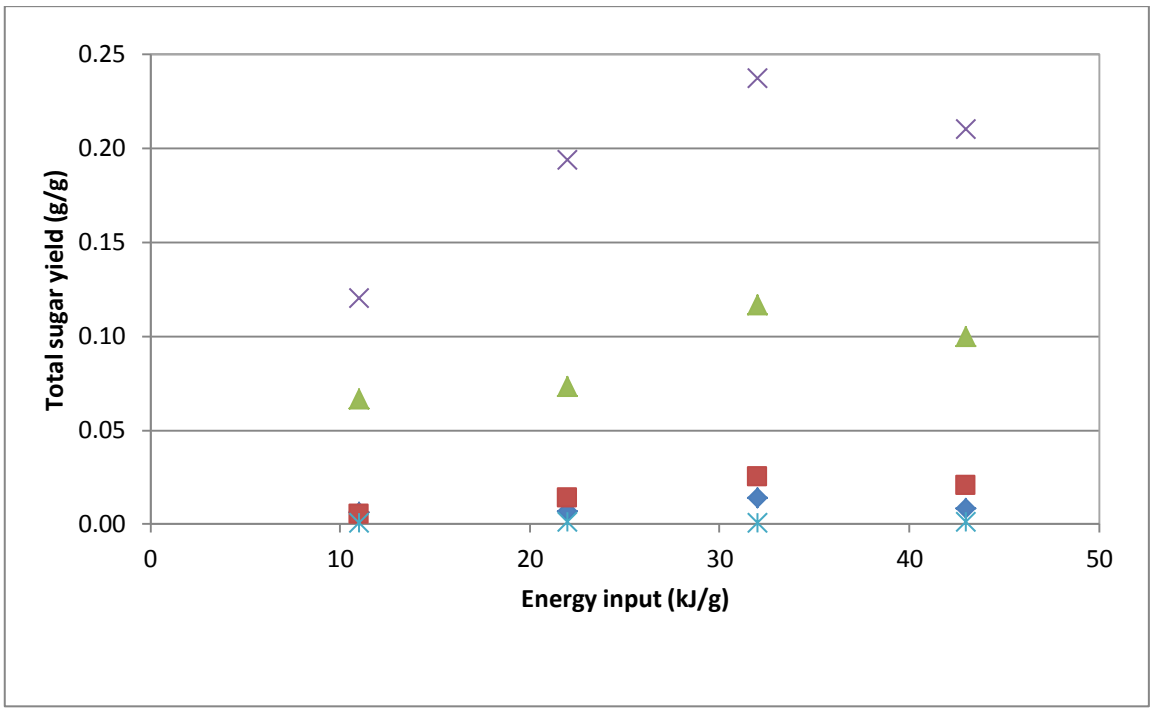

Figure 4.7: Total sugars of microwave assisted pretreatment of NaOH at different concentrations at 180 W (× 5% NaOH, ▲ 3% NaOH, ■ 2% NaOH, ♦ 1% NaOH, Ж control).

Figure 4.7 shows that pretreatment with NaOH resulted in sugar yields that are higher than the control sample. The highest sugar yield obtained was 0.24 g/g of biomass when pretreating with 50 g kg^{-1} NaOH solution in water. This corresponds to a conversion efficiency of 49.1% based on the theoretical yield. NaOH was able to release more sugars by breaking the ester bonds that crosslinks lignin and hemicellulose as well as the hydrogen bonds holding cellulose sheets together, therefore breaking down the structure of lignocellulose. The breaking of these bonds resulted in fractions rich in both hemicellulose and cellulose polymers. An increase in concentration of NaOH resulted in an increase in total sugar yield. This is because an increase in concentration of NaOH also increases the basicity of the solution resulting in more hydroxyl ions available in solution to break the ester and hydrogen bonds keeping together the structure of lignocellulose.

4.4.2 The effect of power density on total sugar yields

The influence of microwave power on total sugar yield was investigated by pretreating the biomass at constant concentration of 50 g kg^{-1} NaOH in water solution for 15 minutes while the power was varied from 100 W to 300W. The effect of the power input over different pretreatment times (power density) is shown in Figure 4.8.

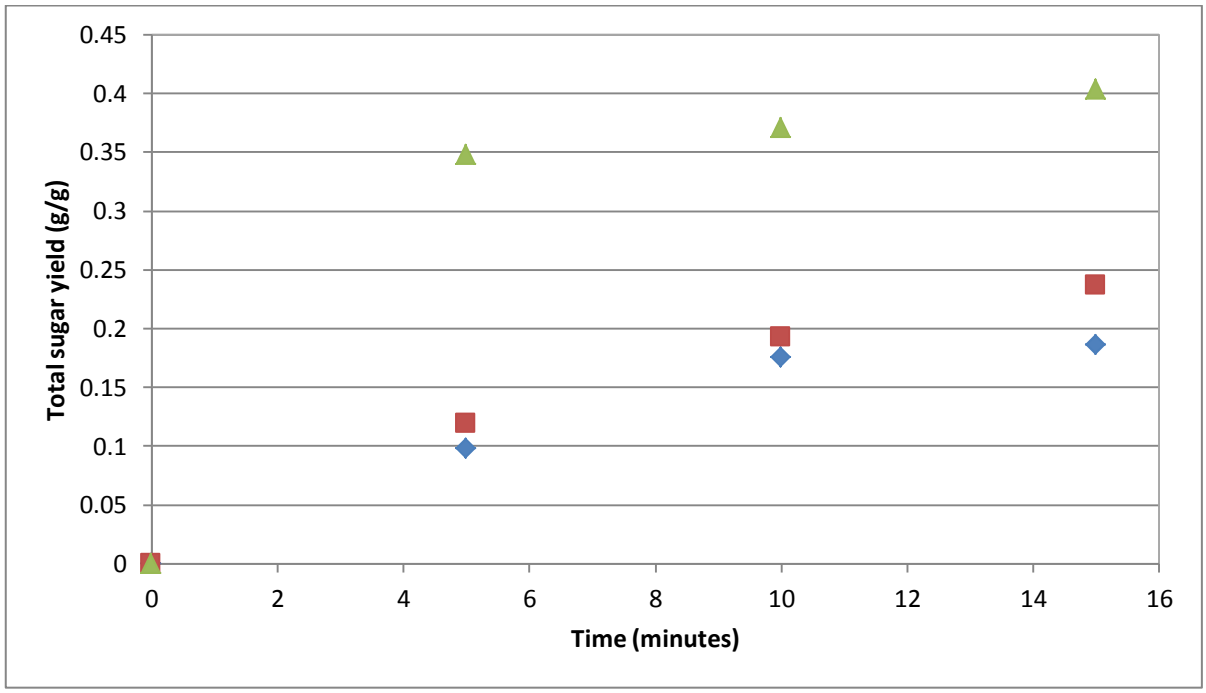

Figure 4.8: The effect of microwave irradiation power on total sugar yield (♦- 18 kJ g^{-1}, ■- 32.4 kJ g^{-1}, ▲- 54 kJ g^{-1})

From Figure 4.8, it can be seen that an increase in power input at constant time results in a higher total sugar yield and also increasing pretreatment time at a constant power input results in an increase in total sugar yield. The highest sugar yield found was 0.40 g/g of biomass in 15 minutes at 300 W (54 kJ g^{-1}) and this corresponds to a conversion efficiency of 81.9% based on the theoretical sugar yield. An increase in power results in more energy being supplied that goes into breaking the ester bonds crosslinking lignin and hemicellulose as well as the hydrogen bonds linking cellulose polymers. When these bonds break, the structure of lignocellulose is also broken because lignin keeps the plant cells together and the hydrogen bonds holds the cellulose sheets together making it crystalline. This result in hemicellulose and cellulose being more susceptible to attach by the alkaline solution, hence more sugars are released. The degradation of the cell walls during microwave pretreatment is shown in the scanning electron microscope images in Figure 4.9. The NaOH pretreated amaranth biomass clearly shows the removal of external fibre which increases the surface area and therefore the polysaccharides (cellulose, hemicellulose and lignin) become more accessible (Binod *et al.*, 2012). The images clearly show the disruption that occurs in vessel elements found in primary cell walls resulting in destabilising the structure of cellulose. The structure is destabilized by the breaking of bonds (ester bonds and hydrogen bonds) during pretreatment as described above which results in the release of sugars. The SEM images showed that the unpretreated amaranth had a smooth layer with just a few cracks from milling and therefore all the plant material is still intact, whereas the pretreated image shows that the NaOH was able to expose the inner vessel element in primary and secondary walls of the plant. These vessels are made up of cellulose, hemicellulose and lignin. Also the NaOH pretreated image has no debris on the surface due to the etching effect of NaOH by dissolving organic some organic material (Binod *et al.*, 2012).

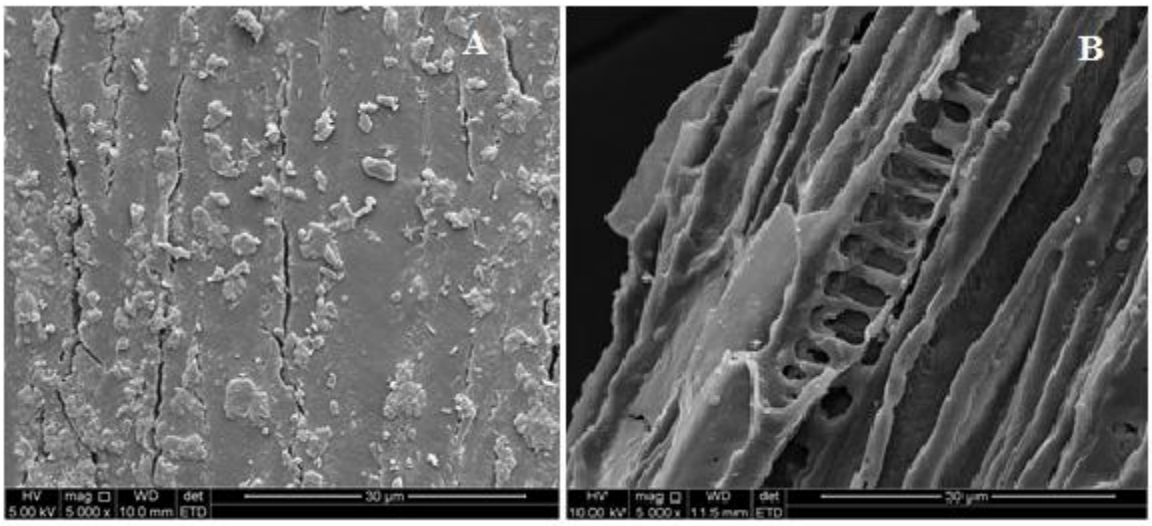

Figure 4.9: Scanning electron microscope images of unpretreated biomass (A) and NaOH pretreated biomass (B)

4.4.3. The effect of NaOH pretreatment on hexose and pentose sugar yield

The influence of using NaOH pretreatment on the pentose and hexose sugars released was investigated by quantifying the total pentose and hexose sugars obtained when the biomass was pretreated with 50 g kg^{-1} NaOH solution in water and the results are shown in figure 4.10.

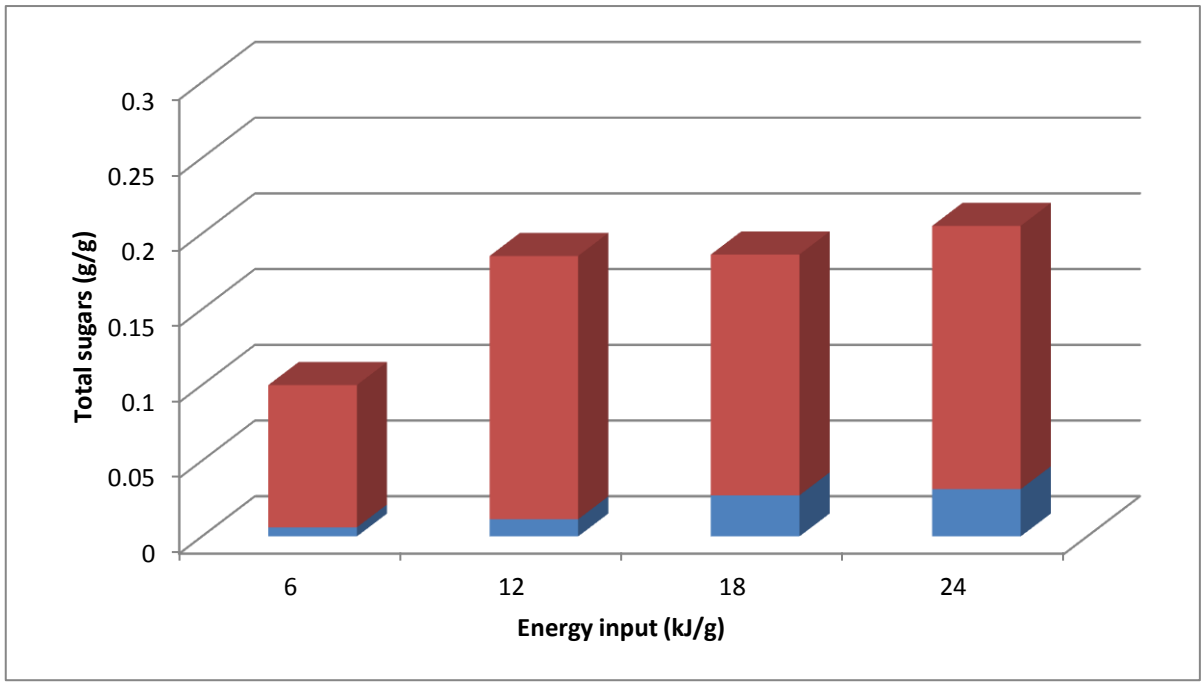

Figure 4.10: The effect of 5% NaOH on the type of sugars liberated at 180W (■ pentoses, ■ hexoses).

From Figure 4.10, it can be seen that more hexose sugars were released than pentose sugars when pretreating with NaOH. The amount of hexose released was constant after 12 kJ/g of energy input and was found to be the highest hexose sugar yield of 0.17 g/g of biomass. The pentose released increased with increase in energy input and was found to be the highest at 24 kJ/g of energy input with a value of 0.031 g/g of biomass. The mechanism of action that is employed by NaOH is the disruption of ester bonds cross-linking lignin and xylan which results in delignification of biomass and the release of cellulose and hemicellulose monomers (Keshwani, 2009; McIntosh & Vancov, 2011). It is this mechanism that is responsible for the increase in pentose sugar yield. The hexose sugars were also released by the breaking of the intermolecular hydrogen bonds found in cellulose. More hexose sugars were released than pentose sugars because hexoses are released from both cellulose and hemicellulose polymers. These findings were supported by the morphological changes shown by the SEM images which showed disruption and exposure of cellulose and hemicellulose. Furthermore, these findings were supported by results obtained when the biomass before pretreatment (A) and after pretreatment (B) were characterised using FTIR (Figure 4.11).

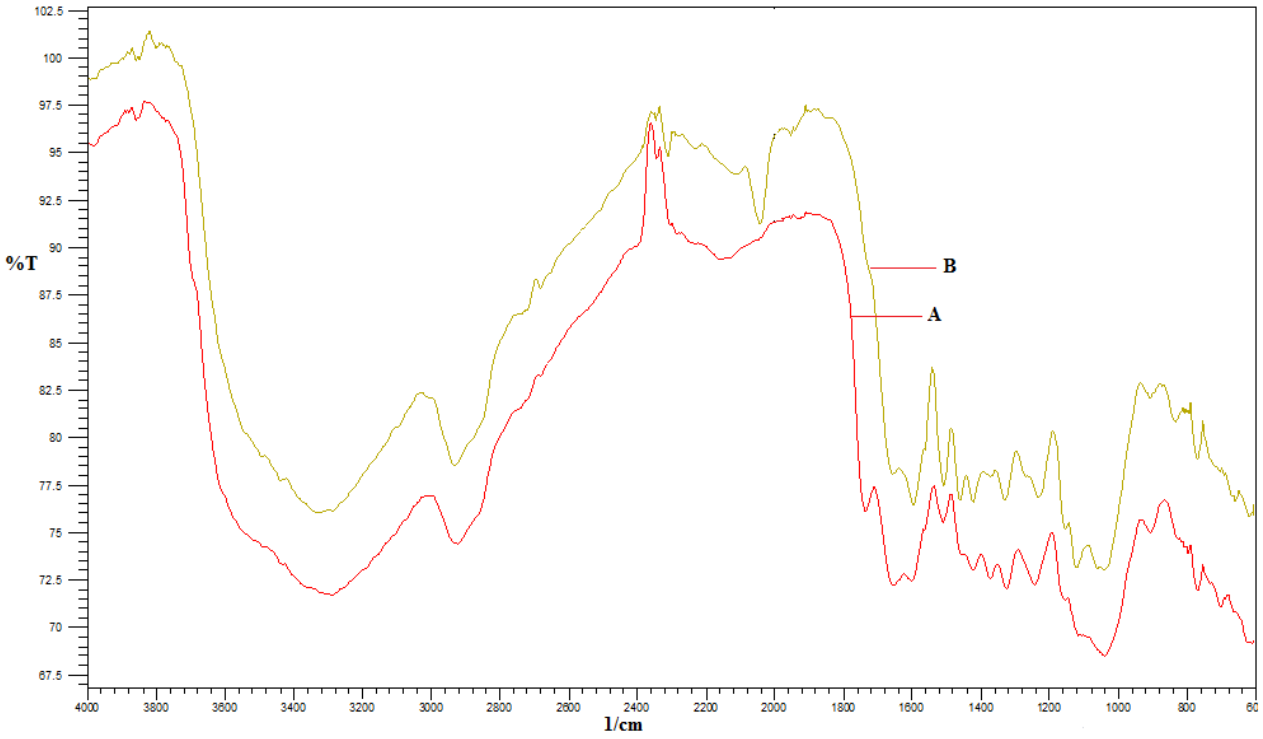

Figure 4.11: FTIR spectra of unpretreated biomass (A) and NaOH pretreated amaranth at 180W (B).

The main peaks that were observed on the FTIR spectra of unpretreated amaranth biomass have been described in detail in Table 4.2. The unpretreated spectra and the pretreated spectra were similar but had significant changes indicating structural changes as a result of pretreatment. The major change observed in the spectra was the shrinking of the broad band found between 2000 cm^{-1} and 2200 cm^{-1}, this is peak represents the acetyl terminal groups that are found in xylan, this peak is very broad in the unpretreated sample but it shrunk and became a little sharp in the pretreated sample. This confirms the interaction of NaOH with hemicellulose; this confirms that NaOH was able to break the ester bonds linking lignin and xylan and in the process disrupting the structure of hemicellulose therefore releasing pentose sugars. The peak at 1050 cm^{-1} was less pronounced in the retreated amaranth sample, it represents cellulose linkages therefore confirms that NaOH also disrupts the structure of cellulose by breaking the hydrogen bonds linking cellulose fibers. The rest of the other major peaks including the broad band at 3300 cm^{-1} representing the O-H group, the peak at 2900 cm^{-1} representing the asymmetric stretching of C-H and the peak at 1700 cm^{-1} for carbonyl group were found in both spectra. This only tells us that there is still residual cellulose, hemicellulose and lignin on the pretreated amaranth biomass.

4.5 Pretreatment with potassium hydroxide (KOH)

The influence of different parameter on the total sugar yield during pretreatment with KOH was investigated. All pretreatments were done using a domestic microwave. The microwave power used in the study was varied between 100 and 300 W and these power settings were chosen to ensure that the biomass were not burned during the experiments and to minimise loss in volume due to evaporation. The effect of parameters such as energy input (microwave power and pretreatment time), the concentration of base, the type of base and 5 and 6- carbon sugar yield were evaluated. The energy input was calculated as shown in Appendix B section B2 and the obtained values are shown in Table B2.1. The biomass loading was kept constant at 5 g per 100 g base solution. Amaranth biomass was pretreated with varying concentrations of KOH with the power setting kept constant at 180W resulting in an energy input in the range of 11-43 $kJ.g^{-1}$ biomass. Pure water, without the addition of a base was used as a control in all experiments.

4.5.1. The effect of KOH concentration

The effect of using different KOH concentration at different energy input for pretreatment of amaranth biomass on the total sugar yield is shown in Figure 4.12. The experimental error

obtained for KOH pretreatment was calculated to be 2.5% at a 95% confidence level and shown in Table B3.1 in Appendix B.

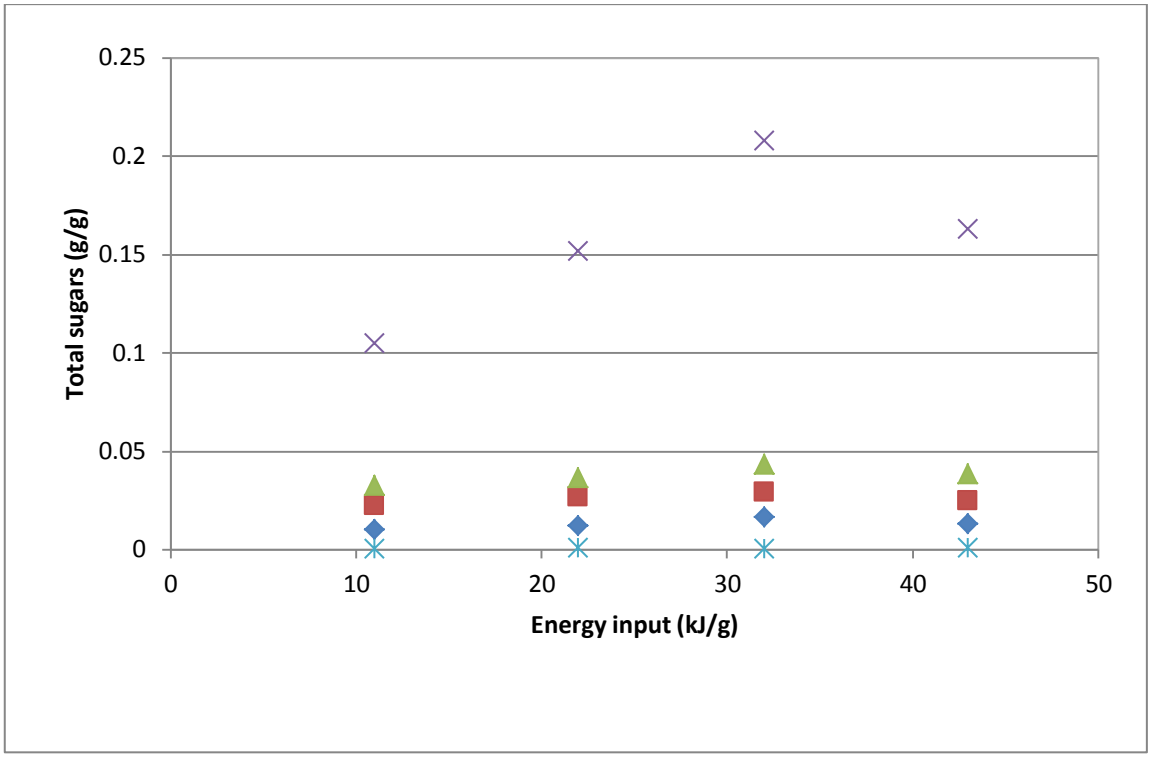

Figure 4.12: Total sugars of microwave assisted pretreatment of KOH at different concentrations at 180 W (× 5% KOH, ▲ 3% KOH, ■ 2% KOH, ♦ 1% KOH, Ж control).

Figure 4.12 shows pretreatment with KOH results in total sugar yields that are significantly higher than the control sample. The highest sugar yield was found to be 0.21 g sugar g^{-1} biomass at 32 kJ/g of energy input. This corresponds to a conversion efficiency of 43% based on the theoretical yield. An increase in concentration of KOH was found to increases the total sugar yield. The total sugar yield for pretreatment with 10, 20 and 30 g kg^{-1} KOH in water solution were all found to be below 0.05 g sugar g^{-1} biomass and this was much lower than that found when 50 g kg^{-1} KOH in water solution was used. This was caused by the inability of lower concentrations of KOH to disrupt and interact with the structure of cellulose or that of hemicellulose. The sugars obtained were released as a result of the breaking of the covalent ester bonds crosslinking lignin and hemicellulose, lignin and cellulose and the hydrogen bonds linking cellulose polymers. When these bonds break, hemicellulose and cellulose polymers become susceptible to KOH attack

4.5.2. The effect of power on reducing sugar yield

The investigation of the effect of microwave power on the sugar yield was done pretreating the biomass at constant concentration of 50 g kg^{-1} KOH in water solution for 15 minutes while microwave power was varied from 100W to 300W. The influence of power input over different pretreatment times is shown in Figure 4.13.

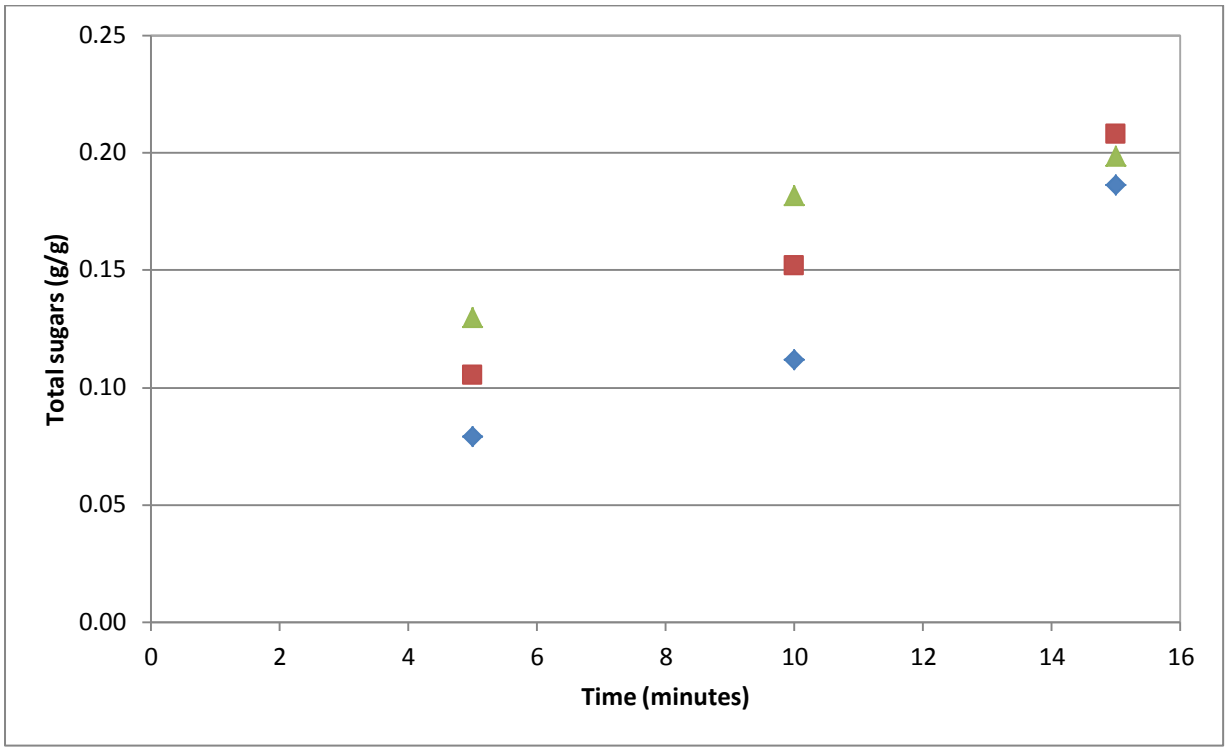

Figure 4.13: The effect of microwave irradiation power on total sugar yield (♦- 18 kJ/g, ■- 32.4 kJ/g, ▲- 54 kJ/g)

From Figure 4.13 it can be seen that an increase in power input at constant time results in a higher sugar yield and that an increase in pretreatment time at constant power input results in an increase in sugar yield. The highest sugar yield obtained was 0.21 g sugar g^{-1} biomass in 15 minutes at 180 W (32.4 kJ/g) and corresponds to a 43% conversion efficiency based on theoretical sugar yield. When power is increased more energy is supplied. More of the energy becomes available to break down the covalent β-(1-4)-glycosidic bonds holding together the structure of cellulose, the hydrogen bonds keeping the cellulose sheets together, the covalent ester bonds that crosslinks lignin and hemicellulose as well as the covalent ester bonds crosslinking lignin and cellulose. All these bonds collectively make lignocellulose recalcitrant and when they break, cellulose and hemicellulose polymers are released and

become more prone to KOH attack. An exception was found when the total sugar yield was found to decrease at 300W and 15 minutes (54 kJ/g) and this may be due to sugar degradation. The degradation of the cell walls during microwave pretreatment is shown in the scanning electron microscope images in Figure 4.14. The images clearly show the disruption that occurs in vessel elements found in primary cell walls. The SEM images showed that the unpretreated amaranth had a smooth layer with just a few cracks from milling and therefore all the plant material is still intact, whereas the pretreated image shows much disordered structure where exposure of vessel elements occurred. KOH pretreatment was able to expose the inner vessel element in primary and secondary walls of the plant. These vessels are made up of cellulose, hemicellulose and lignin. The exposure of these vessels signifies that the structure of the lignocellulose has been destabilised by the breaking of ester bonds, hydrogen bonds and the β-(1-4)-glycosidic bonds which results in the release of sugars.

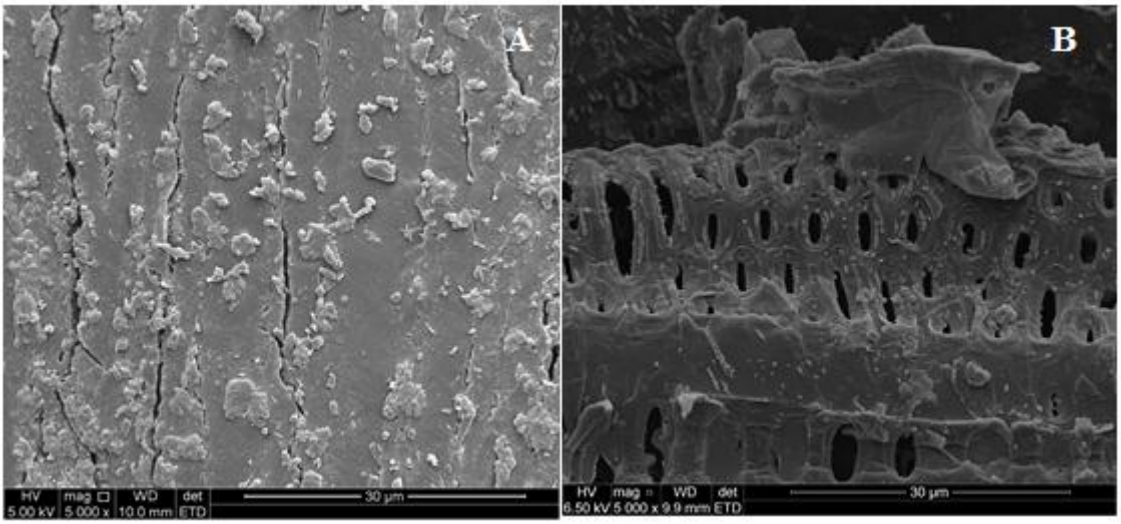

Figure 4.14: Scanning electron microscope images of unpretreated biomass (A) and KOH pretreated biomass (B)

4.5.3 The effect of KOH pretreatment on the hexose and pentose sugar yield

The effect of using KOH pretreatment on the pentose and hexose sugars released was investigated by quantifying the total pentose and hexose sugars obtained when the biomass was pretreated with 50 g kg^{-1} KOH solution in water and the results are shown in figure 4.15.

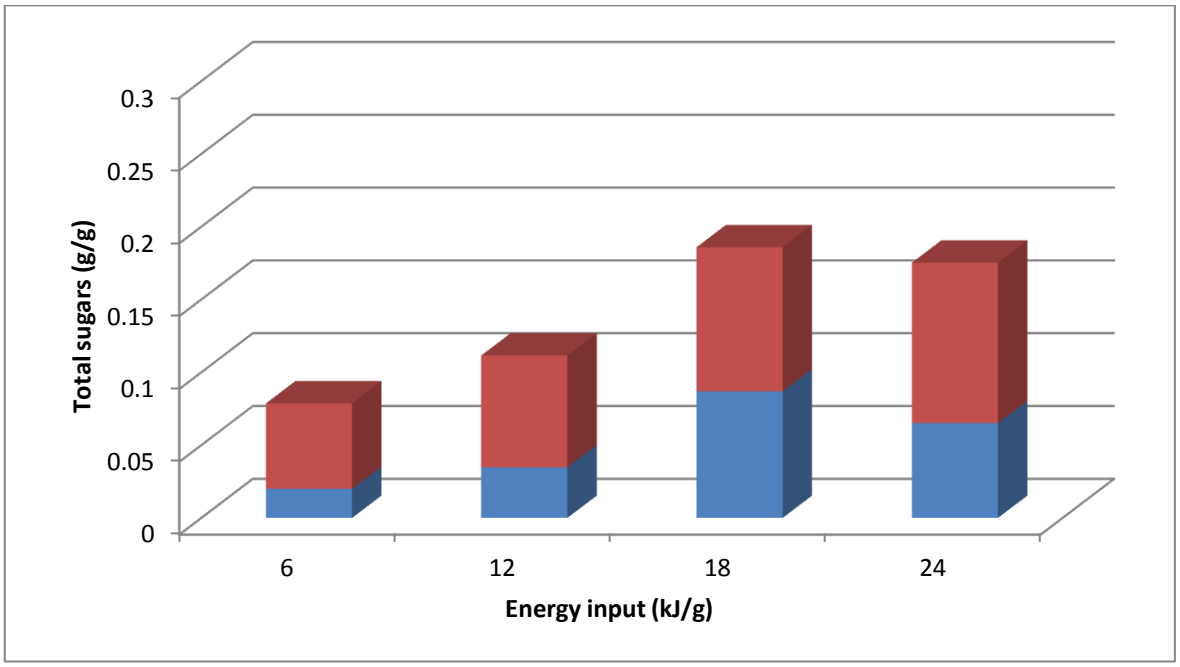

Figure 4.15: The effect of 5% KOH on the type of sugars liberated at 180W (■ pentoses, ■ hexoses).

Figure 4.15 show that KOH pretreatment released more hexose sugars than pentose sugars. The pentoses and hexoses were found to increase with increase in energy input. The highest pentose yield was found to be 0.087 g/g of biomass at an energy input of 18 kJ/g and that of hexoses was found to be 0.11 g/g of biomass at 24 kJ/g. KOH was able to release almost the same amount of pentose (0.087 g/g) as that of hexose (0.099 g/g) sugars when the energy input was 18 kJ/g. Overall, more hexose sugars were released than pentose sugars because hexose sugars are found in both cellulose and hemicellulose while pentose sugars are only from hemicellulose. This shows that KOH was able to disrupt the cellulose chains as well as hemicellulose and this was validated by morphological changes shown on the SEM images where vessel elements that are part of the main structures of cellulose and hemicellulose were exposed and disrupted. These findings were further supported by the results obtained when the biomass before pretreatment (A) and after pretreatment (B) were characterised using FTIR (Figure 4.16).

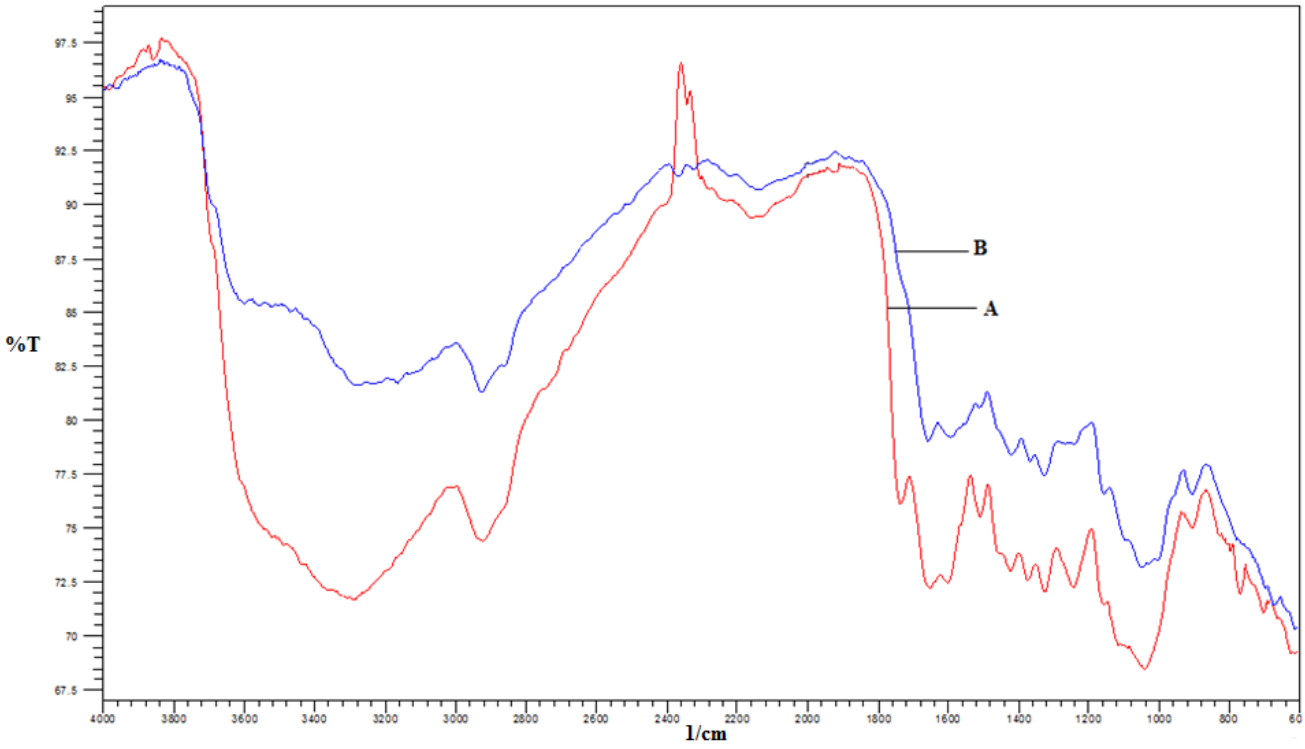

Figure 4.16: FTIR spectra of unpretreated biomass (A) and KOH pretreated amaranth at 180W (B)

A detailed description of the FTIR spectra of unpretreated amaranth biomass (A) has been described in detail in Table 4.2 and only the changes that occurred will be discussed. The two spectra are clearly very distinctive from one another and the changes that occurred are as a result of KOH pretreatment. The broad peak at 3300 cm^{-1} representing OH group is not less broad in the pretreated spectra, this signifies the loss of cellulose, hemicellulose and lignin to the liquor and this caused the preceding following asymmetric stretching of C-H to be sharper. The broad peak at between 2000 cm^{-1} and 2200 cm^{-1} is almost not visible in the pretreated sample which represents the terminal alkyl groups found in hemicellulose. This further proves that KOH pretreatment is able to disrupt hemicellulose. The number of cellulose peaks that are found between 1100 cm^{-1} and 1500 cm^{-1} has also decreased and most are gone and the remaining ones are smaller. The peak at 1600 that represent C=C in the aromatic skeletal of lignin was found have be less sharp in the pretreated sample. The removal of lignin is important during pretreatment because lignin affects enzymatic hydrolysis by binding to the enzymes and it affects fermentation by releasing toxic phenolic compounds. This shows that KOH pretreatment degrades and reduces cellulose crystallinity. These results have demonstrated tremendous effect of KOH pretreatment on the structure of

cellulose and hemicellulose; they also proved that KOH does affect both the release of pentose sugars from hemicellulose and hexose sugars from both cellulose and hemicellulose.

4.6 Summary and comparison of the use of $Ca(OH)_2$, NaOH and KOH on the pretreatment of amaranth lignocellulose

The influence of the different parameters including energy input (microwave power and time), the concentration of the alkaline solution, the type of base, the biomass loading and the 5 and 6-carbon sugar yield were investigated using the three alkaline solutions ($Ca(OH)_2$, NaOH and KOH). In this section, the maximum yields obtained from each alkaline pretreatment will be discussed in relation to one another. The maximum total sugar yields obtained from pretreatment with different alkali solutions is presented in Table 4.3.

Table 4.3: Maximum sugar yields obtained using different alkaline conditions

Alkaline solution	Pretreatment conditions		Total sugar yield (g sugar g^{-1} biomass)	Conversion efficiency (%)
	Concentration (g kg^{-1})	Energy input (kJ/g)		
$Ca(OH)_2$	30	32	0.36	73.7
NaOH	50	32	0.24	49.1
KOH	50	32	0.21	43.0

Table 4.3 shows that pretreatment with $Ca(OH)_2$ gave a higher total sugar yield than either NaOH or KOH. The maximum yield obtained during $Ca(OH)_2$ pretreatment was obtained at lower concentration than both KOH and NaOH because $Ca(OH)_2$ is less soluble in water than the other bases. These results shows that at the same energy input one requires a lower concentration of $Ca(OH)_2$ than that of KOH or NaOH to obtain slightly higher sugar yield. The reason was the higher total sugar yields obtained by pretreatment with $Ca(OH)_2$ is the ability of calcium ions to form linkages with the biomass structure via ionic interactions with negatively charged functional groups present at alkaline conditions resulting in precipitation of lignin and the release of sugars (Keshwani, 2009). NaOH pretreatment gave better results than KOH pretreatment because NaOH is a stronger base, is more electropositive and can therefore ionise more easily in solution and hence react quicker and easier with the biomass. The maximum yield obtained for pretreatment was further used to evaluate the effect of

microwave power on the sugar yield where it was shown that an increase in power input (energy input) resulted in increase in sugar yield and this was the case when either $Ca(OH)_2$, NaOH or KOH was used. When more energy is supplied, more energy will be available to break the strong ester bonds crosslinking lignin and hemicellulose, the hydrogen bonds holding cellulose polymers, the β-(1-4)-glycosidic bonds linking glucose chains in cellulose polymers. The breaking of these bonds results in lignocellulose structural disruption causing the release of more sugars. The use of each base resulted in the release of 5 and 6-carbon sugars in different proportions and Table 4.4 gives the maximum sugars yields obtained.

Table 4.4: Maximum obtained pentose and hexose sugars using 50 g kg^{-1} of alkaline solution

Alkaline solution	Pentose sugar yield (g sugar g^{-1} biomass)	Hexose sugar yield (g sugar g^{-1} biomass)
$Ca(OH)_2$	0.029	0.28
NaOH	0.031	0.17
KOH	0.087	0.11

Table 4.4 shows that amongst all three bases used, KOH released the highest amount of pentose sugars and the least amount of hexose sugars. Pretreatment with KOH resulted in 47% of the total sugars released as pentose (Figure E2.12, Appendix E), whereas when using $Ca(OH)_2$ (Figure E2.4, Appendix E) and NaOH (Figure E2.8, Appendix E) only 11% and 16% respectively of the total sugars were pentoses. Pretreatment with KOH gave the lowest sugar yields of all the bases but it gave the highest pentose concentration. This means that KOH was more effective at breaking the ester bonds crosslinking lignin and hemicellulose, as a result was able to release more hemicellulose sugars than the other bases. These results were validated by characterisation with SEM where the images showed structural disruption and exposure of cellulose. Furthermore, the FTIR was used to analyse the structural changes that occurred during pretreatment. The FTIR spectra clearly showed that KOH interact better with hemicellulose than the other bases by the disappearance of the terminal acetyl groups found in xylan. This shows that the covalent ester bonds crosslinking hemicellulose and lignin has been broken.

4.7. Concluding Remarks

The use of microwave pretreatment has been shown to improve the recovery of fermentable sugars attainable from amaranth lignocellulose in this chapter. Microwave irradiation

coupled with $Ca(OH)_2$, NaOH or KOH promoted the disruption of amaranth lignocellulose structure liberating monomeric sugars. Parameters including concentration of the alkaline solution, the type of alkaline solution and energy input (time and power) were shown to influence the total sugar yield. The use of SEM validated the obtained results by showing morphological changes that occurred during pretreatment while the use of FTIR further supported the obtained results by showing the breaking of bonds in lignocellulose polymers. The liberation of sugars from amaranth lignocellulose has shown its potential as a viable bioethanol production feedstock.

4.8 References

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CHAPTER 5

Hydrolysis and Fermentation

5.1 Overview

The results on the production of ethanol from amaranth lignocellulose will be discussed in this chapter. A general introduction is provided in Section 5.2 followed by the effect of enzymatic hydrolysis in Section 5.3. The results obtained from the fermentation of different hydrolysate with *S. cerevisiae* are discussed in Section 5.4. The concluding remarks and the references are provided in section 5.5 and 5.6 respectively.

5.2. Introduction

Lignocellulose is pretreated inorder to modify the structure of cellulose. This is important for enzymatic hydrolysis of cellulose because enzymes are hindered by cellulose crystallinity, surface area and lignin and hemicellulose content, this causes enzymatic hydrolysis to be slow (Balat *et al.*, 2008). During hydrolysis, the enzymes convert polysaccharides (cellulose and hemicellulose) to sugar monomer, it is these sugar monomers that can be further fermented to bioethanol. The effectiveness of the pretreatment methods used and their effect on enzymatic hydrolysis will be investigated in this section. This will be done by comparing the total sugar yields obtained during pretreatment and those that were obtained during hydrolysis. Furthermore, the fermentation of the obtained sugars will be evaluated.

5.3. Effect of enzymatic hydrolysis on Ca(OH)2, NaOH and KOH pretreated amaranth biomass

Amaranth biomass was pretreated with 50 g kg^{-1} of NaOH and KOH solution in water at 180W (32 kJ/g) and 3 g kg^{-1} of $Ca(OH)_2$ solution in water, using the same energy input. This concentration and energy input was chosen because it gave the highest concentration of sugars during pretreatment. After pretreatment, all samples were enzymatically hydrolysed using Novozyme 188 and Celluclast 1.5L with the addition of Tween 80 as surfactant. The samples were hydrolysed for 48 hours at 50^{o}C in an incubator shaker at 150 rpm. The results that were obtained during hydrolysis of biomass pretreated with NaOH, $Ca(OH)_2$ and KOH are shown in Figure 5.1. The experimental error obtained for the hydrolysis of $Ca(OH)_2$,

NaOH and KOH pretreated samples was calculated to be 3.5%, 3.7% and 4.9% respectively at a 95% confidence level shown in Table B3.2 in Appendix B.

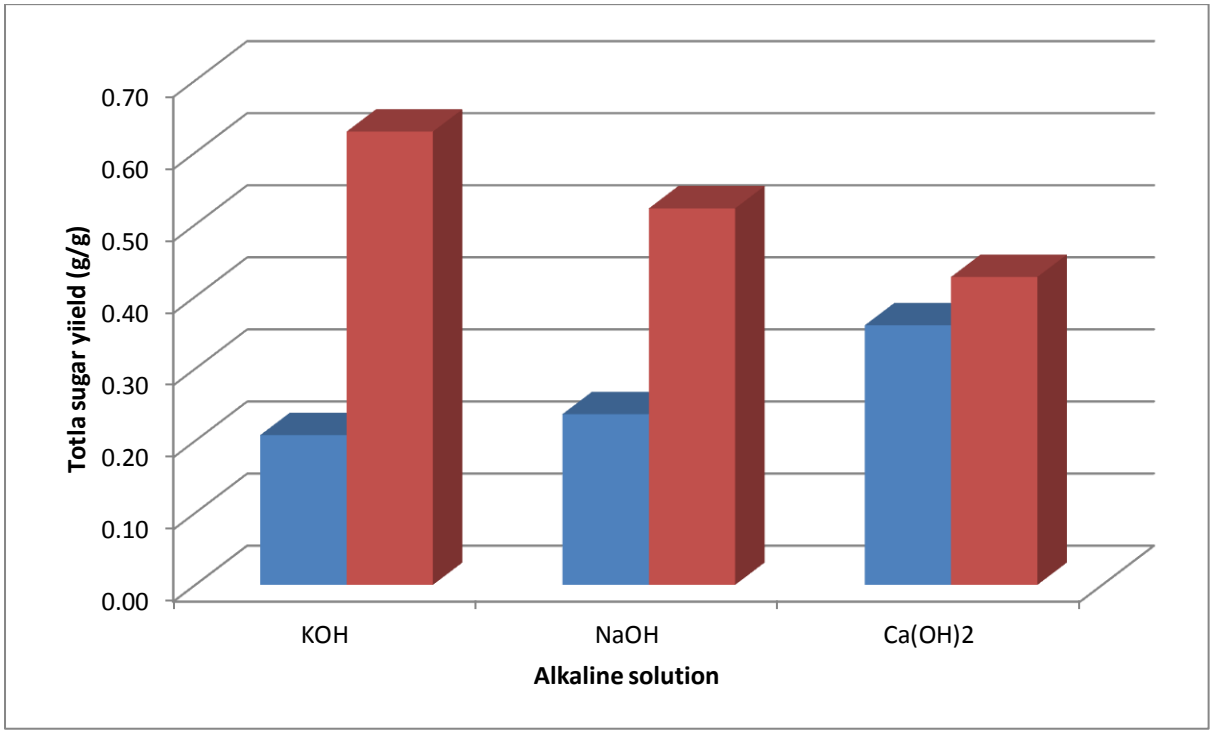

Figure 5.1: The effect of enzymatic hydrolysis of pretreated amaranth biomass pretreated with KOH, NaOH and $Ca(OH)_2$ on total sugar yield (■ pretreatment, ■ hydrolysis).

From Figure 5.1, it can be seen that enzymatic hydrolysis resulted in sugar yields that are significantly higher than those obtained when the samples are only pretreated. Hydrolysis of KOH pretreated sample resulted in the highest yield of 0.63 g sugar g^{-1} biomass amongst all the bases. This corresponds to a conversion efficiency of 100% based on the theoretical yield. Hydrolysis of $Ca(OH)_2$ pretreated sample gave the lowest yield of 0.43 g sugar g^{-1} biomass which is 88% conversion efficiency based on the theoretical yield. Hydrolysis of NaOH pretreated sample resulted in a yield of 0.52 g sugar g^{-1} biomass with a conversion efficiency of 100% based on the theoretical yield. The yield obtained from KOH and NaOH pretreated samples was high because these bases were able to interact better with the ester bonds linking hemicellulose and lignin, therefore they were able to remove lignin from the biomass and render it unreactive as has been shown by structural analysis (FTIR spectra). $Ca(OH)_2$ gave a lower yield than the other bases due to the many interactions that calcium ions has with the biomass including formation of linkages with biomass by ionic interactions with negatively charged functional groups, binding with lignin, interaction with pectin, cellulose and hemicellulose (Keshwani, 2009). This interaction of $Ca(OH)_2$ ions with

biomass inhibits enzyme accessibility. The excess sugars observed maybe from non-structural carbohydrates. A study conducted by Keshwani (2009) also reported that after hydrolysis, microwave pretreatment of switch grass with $Ca(OH)_2$ gave lower total sugars than using NaOH. A study that was conducted by McIntosh and Vancov (2011) using 2% NaOH for pretreatment of wheat straw found similar results to this study that a sugar yield higher than that of the theoretical yield was obtained after hydrolysis. Hu and Wen (2008) reported a sugar yield of 90% (based on theoretical sugars) when 0.1 %wt. of NaOH was used to pretreat switch grass for 120 seconds at 190^{o}C using microwave irradiation. These results are not a good comparison to the ones in this study because they soaked the biomass for 2 hour in the alkali solution before pretreatment. Zhu and co-workers (2013) reported a total sugar yield of 0.26 g g^{-1} of biomass when using 1M KOH for pretreatment of spent mushroom substrate.

5.4 Fermentation

The conversion of sugar monomers to ethanol requires the use of yeast or bacteria that will assimilate and propel the reaction towards production of ethanol. The conversion of sugars obtained during hydrolysis to bioethanol by fermentation using *Saccharomyces cerevisiae* (*S. cerevisiae*) will be investigated in this section. The hydrolysate was fermented using baker's yeast. The hydrolysate used was pretreated with the different bases at the chosen pretreatment conditions prior hydrolysis. These conditions were chosen because they gave the highest yield during pretreatment with minimal volume loss and also a very good sugar yield after hydrolysis. Fermentation was carried out at 30^{o}C in an incubator shaker at 120 rpm. A concentration of 5 g/L of yeast was used and the yeast was revived by dissolution in 10 mL of the hydrozylate shaking at 120 rpm at 30^{o}C for 15 minutes prior to fermentation. The production of bioethanol and consumption of sugars was monitored during fermentation. The optical density of the cells was measured using UV spectrophotometer to monitor cell growth. The results presented in this section are also found in appendix E Section E3 (Figure E4.1, Figure E4.2, Figure E4.3) in units of g/L.

5.4.1. The effect of *S. cerevisiae* on total sugar and ethanol yield for microwave-$Ca(OH)_2$ pretreated amaranth.

The influence of fermentation with *S. cerevisiae* on total sugar yield and ethanol yield was investigated by fermentation of the hydrolysate pretreated with 30 g $kg^{-1}Ca(OH)_2$ in water solution at 180 W for 15 minutes (32.4 kJ/g). The effect of fermentation using *S. cerevisiae*

over different time intervals is shown in figure 5.2. The experimental error obtained was calculated to be 4.5% based on ethanol yield at a 95% confidence level shown in Table B3.3 in Appendix B.

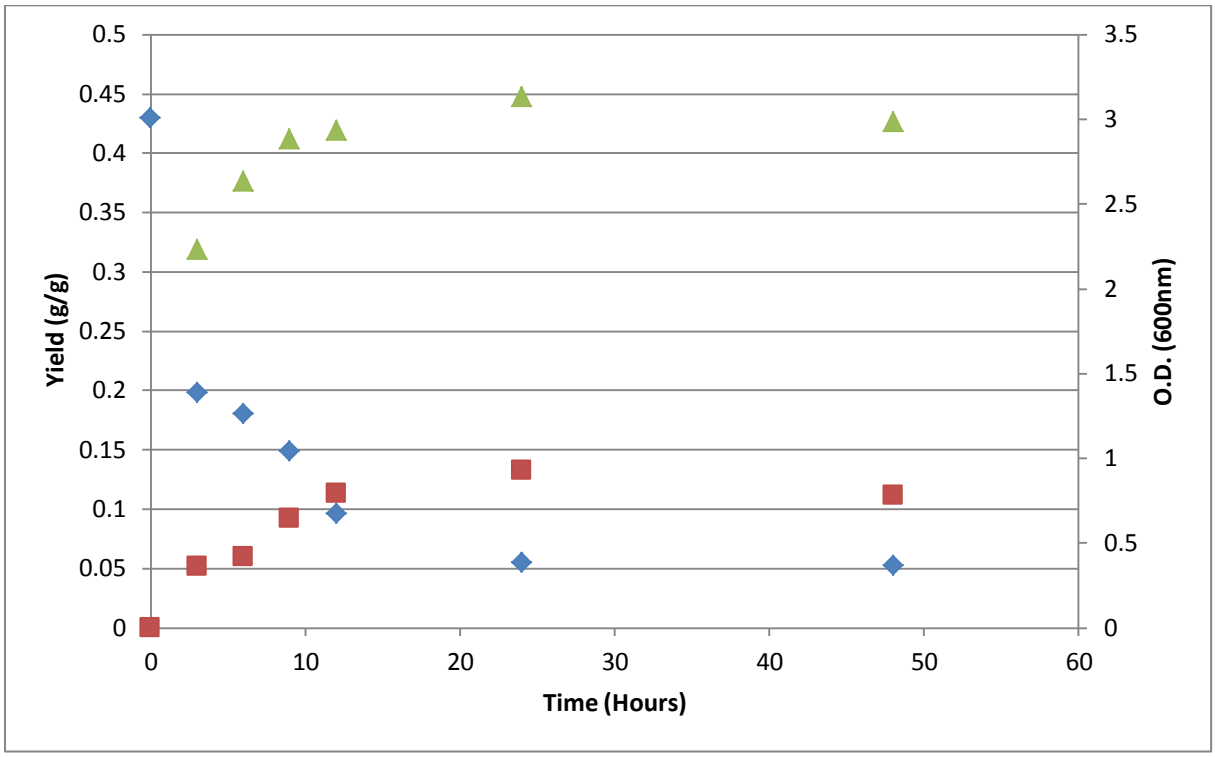

Figure 5.2: Effect of *S. cerevisiae* on total sugar and ethanol yield for amaranth biomass pretreated with 3% $Ca(OH)_2$ at an energy input of 32 kJ/g (■ –ethanol, ♦ total sugars, ▲ O.D.).

Figure 5.2 shows that ethanol started to form within the first three hours of fermentation while an instant drop in total sugar yield occurred. The graph shows that much less ethanol was formed than the consumed sugars. Ethanol yield slowly increased during the first 12 hours of fermentation and the maximum yield was reached within 24 hours. The optical density was found to also increase within the first 12 hours indicating the growth of *S. cerevisiae* cells. The maximum ethanol yield obtained was 0.13 g sugar g^{-1} biomass and based on the theoretical yield of 0.24 g ethanol g^{-1} of dry amaranth biomass; this maximum obtained ethanol yield corresponds to a conversion efficiency of 54%. *S. cerevisiae* only ferments glucose and based on the average glucose yield of 0.20 g g^{-1} of biomass (Table D2.1, Appendix D), the ethanol yield obtained (0.13 g sugar g^{-1} biomass) corresponds to a glucose conversion efficiency of 65%. The productivity was found to be 0.28 g/L/h (calculated as shown in Appendix B Section B3). The production of ethanol is accompanied

by other side reactions that results in the formation of by-products. The by-products formation was observed and these were mainly acetic acid and furfural. Therefore they cannot be neglected as it is responsible for the low ethanol yield obtained.

5.4.2. The effect of *S. cerevisiae* on total sugar and ethanol yield for microwave-NaOH pretreated amaranth.

The hydrolysate pretreated with 50 g kg^{-1} of NaOH solution in water was used to investigate the influence of fermentation using *S. cerevisiae* at 180 W for 15 minutes (32.4 kJ/g) and the results are shown in Figure 5.3. The experimental error obtained was calculated to be 5.2% based on ethanol yield at a 95% confidence level shown in Table B3.3 in Appendix B.

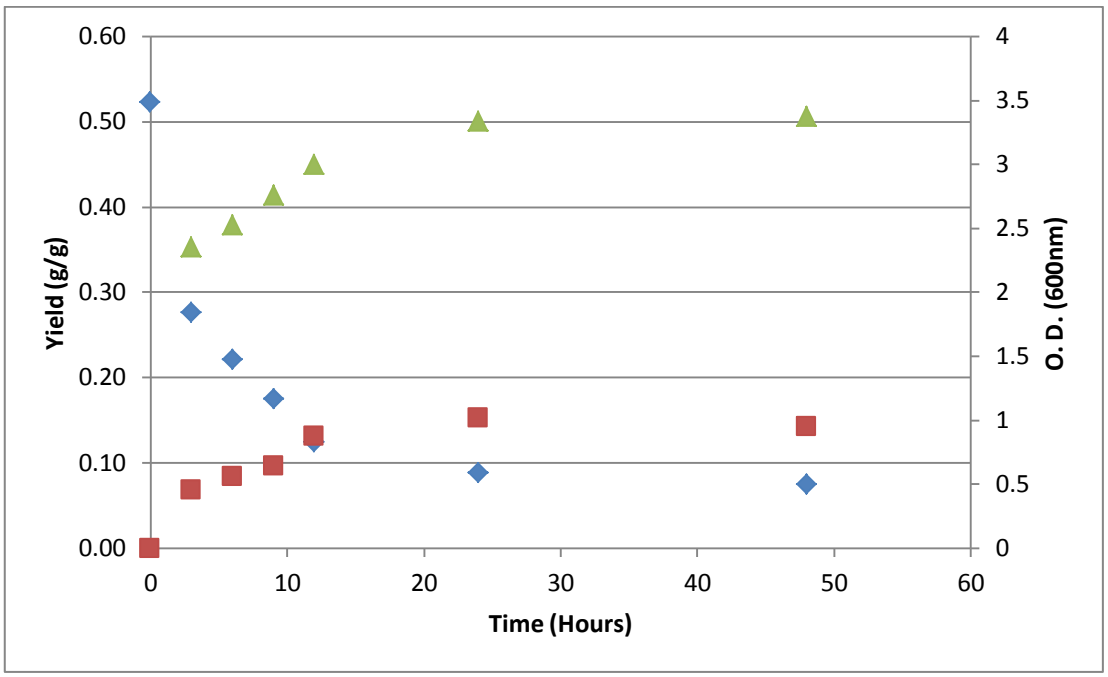

Figure 5.3: Effect of *S. cerevisiae* on total sugar and ethanol yield for amaranth biomass pretreated with 5% NaOH at an energy input of 32 kJ/g (■ –ethanol, ♦ total sugars, ▲ O.D.).

From Figure 5.3, a similar trend to that found during fermentation of $Ca(OH)_2$ pretreated hydrolysate was observed. The formation of ethanol started to occur within the first three hours, increased slowly reaching a maximum ethanol yield in 24 hours. Concurrently, total sugar yield instantly decreased with the first three hours and continued to decrease until a steady state was reached in 24 hours. The optical density was found to increase slowly within the first 12 hours and reached equilibrium in 24 hours. The maximum ethanol yield obtained was found to be 0.15 g ethanol $g^{-1}$of biomass and this corresponds to 62.5% conversion efficiency based on the theoretical ethanol yield. This ethanol yield (0.15 g

ethanol $g^{-1}$of biomass) resulted in a glucose conversion efficiency of 51.7% was obtained based on the average glucose yield of 0.29 g $g^{-1}$of biomass (Table D2.2, Appendix D). The productivity was found to be 0.32 g/L/h calculated as shown in Appendix B Section B4 Table B4.1. The low ethanol yield is due to the formation of inhibitory compounds that inhibits the formation of ethanol by forming acetic acid. Amaranth also contains an unusually large amount of protein. The presence of the large amount of protein in the broth is known to inhibit *S. cerevisiae* to produce ethanol. During fermentation the proteins are converted to volatile fatty acids (Feng *et al.*, 2009); one of the volatile fatty acids detected is acetic acid which is known to inhibit fermentation.

5.4.3. The effect of *S. cerevisiae* on total sugar and ethanol yield for microwave-KOH pretreated amaranth.

The effect of fermentation with *S. cerevisiae* on total sugar yield and ethanol yield was investigated by fermentation of the hydrolysate pretreated with 50 g kg^{-1} KOH in water solution at 180 W for 15 minutes (32.4 kJ/g). The effect of fermentation using *S. cerevisiae* over different time intervals is shown in Figure 5.4. The experimental error obtained was calculated to be 2.9% based on ethanol yield at a 95% confidence level shown in Table B3.3 in Appendix B.

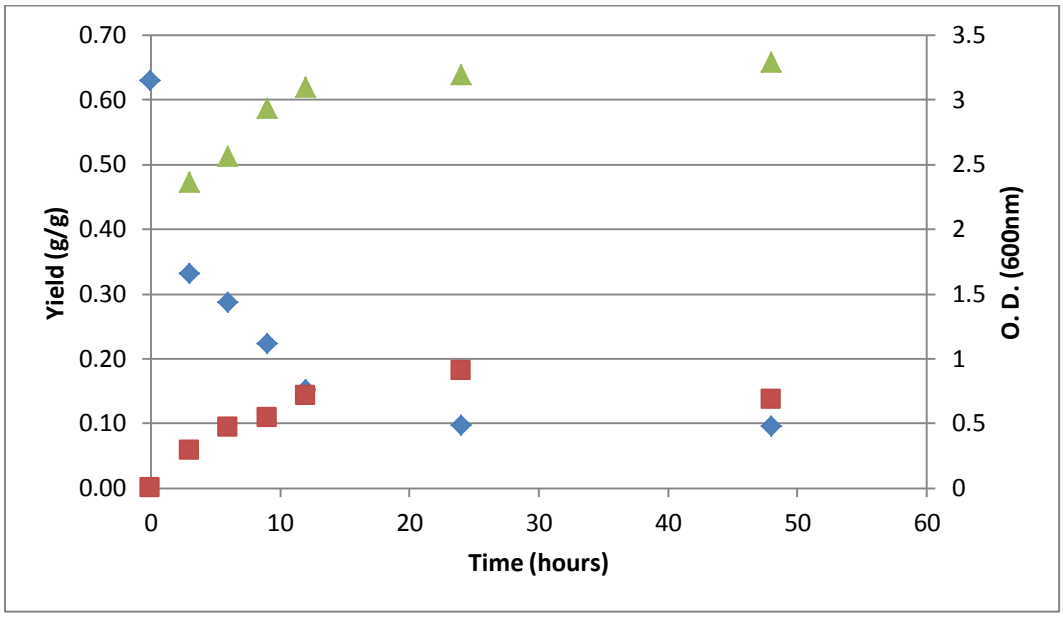

Figure 5.4: Effect of *S. cerevisiae* on total sugar and ethanol yield for amaranth biomass pretreated with 5% KOH at an energy input of 32 kJ/g (■ –ethanol, ♦ total sugars, ▲ O.D.).

Figure 5.4 shows that ethanol formation occurred within the first three hours of fermentation while total sugars instantly decreased. Ethanol yield increase slowly within the first 12 hours and reached a maximum yield in 24 hours while the sugar yield decreased sharply and was constant after 24 hours. The optical density increased within the first 12 hours and was constant after 24 hours. The maximum ethanol yield obtained was found to be 0.18 g ethanol $g^{-1}$of biomass with a productivity of 0.38 g/L/h. This ethanol yield corresponds to a conversion efficiency of 75%. This ethanol yield (0.15 g ethanol $g^{-1}$of biomass) resulted in a glucose conversion efficiency of 62.1% was obtained based on the average glucose yield of 0.29 g $g^{-1}$of biomass (Table D2.3, Appendix D). The consumption of sugars completely stopped after 24 hours resulting in low ethanol yield. The low yields obtained were a result of formation of by products such as furfural, glycerol and acetic acid. The introduction of by-products slows down the formation of ethanol because some of these compounds are toxic to the yeast cells and also they compete with ethanol for the sugars thus reducing the availability of sugars to be converted to ethanol in solution.

5.4.3. Summary and comparison of the effect of *S. cerevisiae* on total sugar and ethanol yield for microwave- $Ca(OH)_2$, NaOH and KOH pretreated amaranth.

The influence of the type of pretreatment used on the total sugar and ethanol yield will be discussed in this section in relation to the type of alkaline solution used in pretreatment. The maximum ethanol yields obtained during fermentation are shown in Table 5.1 below.

Table 5.1: Maximum ethanol yields obtained during fermentation of alkali pretreated hydrozylate.

Hydrolysate Matrix	Ethanol (g/g)	productivity (g/L/h)
Ca(OH)2	0.13	0.28
NaOH	0.15	0.32
KOH	0.18	0.38

From Table 5.1 it can be seen that the maximum ethanol yield was obtained from fermentation of KOH pretreated hydrozylate. This was expected because KOH pretreatment also gave the highest yield during enzymatic hydrolysis. The cell growth occurred steadily because the cells were fist revived in the hydrolysate for 15 minutes to decrease the lag

phase, therefore most of the cells had grown prior to addition of the yeast broth in the rest of the hydrolysate be fermented.

The ethanol yields obtained per gram of biomass were all low. The low ethanol yield obtained was due to the presence of inhibitory compounds that were formed as by products and these are the acetic acid, furfural and glycerol. The presence of proteins also inhibited ethanol formation by forming volatile fatty acids (acetic acid) during fermentation using *S. cerevisiae*. The presence of phenolic compounds that are part of lignin which were released during pretreatment may also have contributed to inhibitory effects, as it has been shown that during pretreatment and proved using FTIR spectra that phenolic associated with lignin were liberated. KOH gave a better yield than the NaOH and $Ca(OH)_2$ because the FTIR spectra showed that KOH has more interaction with lignin and hemicellulose ester bonds, therefore the base was able to interact with lignin more than the other bases. A study that was conducted by El-Zawawy and co-workers (2011) found that less ethanol was produced when corn cob was pretreated with alkali microwave compared to steam explosion and alkaline pulp. Beszedes (2012) and co-workers used *S. cerevisiae* to ferment microwave alkali (NaOH) pretreated sugar beet and obtained a sugar yield of 0.24 g g^{-1} of biomass, this study also reported the presence of phenolic compounds and furfurals.

5.5. Concluding remarks

The effect of using enzymes to enhance the production of sugars monomers that can be fermented to bioethanol has been evaluated in this section. It was shown that enzymes were able to release more sugar monomers than when only pretreatment is used by the conversion of total sugars that was over 80% based on the theoretical yield. The hydrolysate was further fermented using *S. cerevisiae* to produce ethanol. The major challenge experienced was the low ethanol yield that was due to by formation of by inhibitory compounds that drives the reaction towards the formation of acetic acid instead of ethanol. In overall, the potential of amaranth lignocellulose to produce ethanol was demonstrated even though there are some issues that need to be addressed.

5.6. References

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CHAPTER 6

Conclusion and Recommendations

6.1 Overview

This chapter will summarise the results obtained in this study and it will also give recommendations to shed light on the problems that were encountered. Pretreatment of lignocellulose material is important because it changes the structure of the polysaccharides and therefore makes the polymers more susceptible to enzymatic hydrolysis for the production of sugar monomers. The main objectives of this study were to evaluate the effect of parameters such as concentration of bases, type of base, energy input and biomass loading on microwave assisted pretreatment of amaranth lignocellulose by monitoring total sugar yield and ethanol production. The optimum yields and conditions will also be highlighted.

6.2 Conclusion

- The maximum total sugar yield when pretreatment was done using 30 g kg^{-1} $Ca(OH)_2$ solution in water, 50 g kg^{-1} NaOH solution in water and 50 g kg^{-1} KOH solution in water was found to be 0.36 g/g of biomass, 0.24 g/g of biomass and 0.21g/g of biomass respectively when using 32 kJ/g as energy input.
- The sugar yield was found to increase with increase in energy input and reaches a threshold at 32 kJ/g
- The total sugar yield was found to increase with a decrease in biomass loading
- The alkaline bases were found to have an effect to the type of sugars liberated, pretreatment with KOH was found to liberate 47% of the sugars as pentoses, $Ca(OH)_2$ released 11% of the sugars as pentoses and NaOH released 16% of the sugars as pentoses.
- The SEM images showed morphological changes in the biomass pretreated with bases where destruction of cell walls and exposure of vessel elements in pretreated biomass was observed.
- The FTIR spectra were used to track the structural changes that occurred during pretreatment where the breaking of ester bonds and hydrogen bonds in cellulose,

hemicellulose and lignin polymers was observed promoting the release of sugar monomers.

- Enzymatic hydrolysis of the optimum pretreatment parameters was able to liberate more sugars than pretreatment where a total sugar yield of 0.43 g/g, 0.63 g/g and 0.52 g/g of biomass was obtained $Ca(OH)_2$, KOH and NaOH pretreatment respectively.
- The obtained highest ethanol yield was found to be 0.18 g/g of biomass with a productivity of 0.38 g/L/h was obtained for the fermentation of amaranth biomass using S. cerevisiae.
- These results have shown the effect that microwave assisted alkaline pretreatment has on the sugar yield and also it has been shown that amaranth has a potential to be used as a feedstock for bioethanol production.

6.3 Recommendations

- An evaluation needs to be done where closed continuous system microwave is used instead of domestic microwave as this will mitigate the loss of volatile components.
- Evaluation of an increase in temperature rather than power.
- Investigate the use of pentose fermenting micro-organisms.

APPENDIX A

Calibration Data

A1: Introduction

The sugar calibration curves are important as they are used to quantify the sugars and ethanol during pretreatment, hydrolysis and fermentation. Section A2 presents the data used to obtain the sugar and ethanol calibration curves and also the calibration curves are shown.

A2: HPLC Sugar analysis

The calibration curves were obtained by mixing pure known amounts of sugar standards and ethanol with distilled water and this was serially diluted to obtain the a decreasing amount of concentrations. The peak areas obtained on the HPLC chromatogram of each standard are shown in Table A2.2 and Table A2.3. The calibration curves were plotted and the slope (k) of the graph was used to calculate the unknown sugars on the samples. The k value together with the retention times of each sugar are shown in Table A2.1.

Table A2.1: Components obtained and their corresponding symbols used in the calculations

Component	Symbol	Retention time (minutes)	K-value
Glucose	S1	12.4-13.0	23899.37
Sucrose	S2	10.9- 11.1	209495.82
Fructose	S3	17.9- 22-4	268412.59
Xylose	S4	13.3-13.4	217183.91
Mannose	S5	18.3- 20.7	253657.36
Arabinose	S6	16.2-17.0	219377.74
Galactose	S7	14.0-14.5	215848.93
Cellobiose	S8	9.4-10.5	44733.67
Ethanol	S9	11.4-12.1	15896.75

Table A2.2: Peak areas (nRIU.s**)** obtained during HPLC calibration of sugars

Concentration (g/L)	Glucose	Sucrose	Fructose	Xylose	Mannose	Arabinose	Galactose
0.5	9.30E+03	1.42E+04	1.12E+04	8.21E+03	8.11E+03	1.19E+04	1.55E+04
1	2.08E+04	2.22E+04	1.78E+04	2.06E+04	1.95E+04	2.06E+04	2.16E+04
2	4.18E+04	4.51E+04	4.22E+04	4.26E+04	4.46E+04	4.23E+04	4.02E+04
4	8.91E+04	6.60E+04	9.34E+04	8.15E+04	1.02E+05	8.12E+04	8.18E+04
8	1.96E+05	1.75E+05	2.26E+05	1.77E+05	2.05E+05	1.79E+05	1.75E+05

Table A2.3: Peak areas (nRIU.s**)** obtained during HPLC calibration of cellobiose and ethanol

Concentration (g/L)	Cellobiose	ethanol
5.02	2.26E+05	8.60E+04
2.51	1.09E+05	4.01E+04
2.51	1.11E+05	3.89E+04
2.51	1.17E+05	4.26E+04
1.673	6.95E+04	2.33E+04
1.673	7.81E+04	2.47E+04
1.673	7.26E+04	2.02E+04
1.255	5.80E+04	2.05E+04
1.255	5.44E+04	1.69E+04
1.255	5.43E+04	1.57E+04
1.004	4.43E+04	1.16E+04

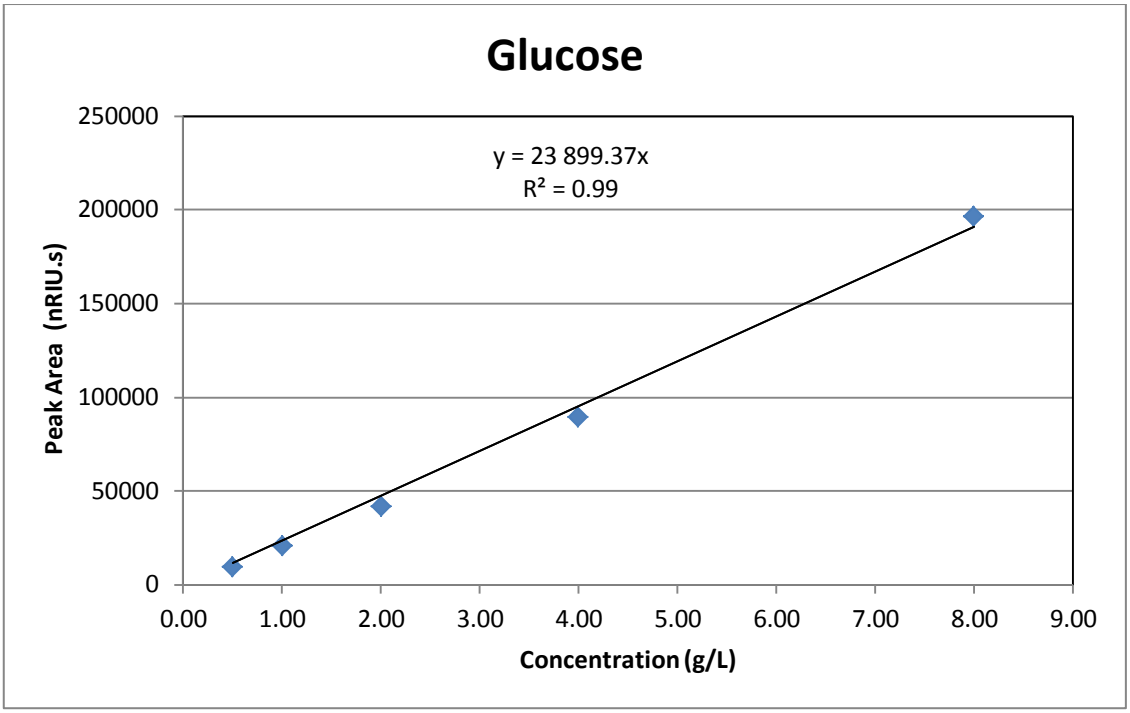

Figure A2.1: Glucose calibration curve

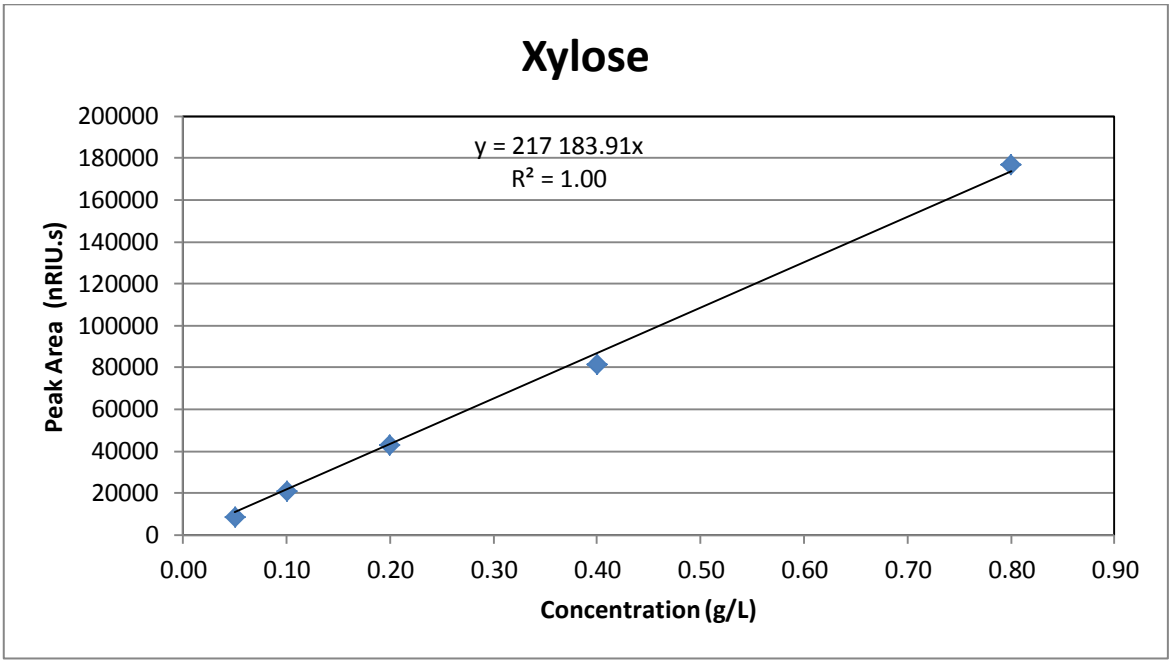

Figure A2.2: Xylose calibration curve

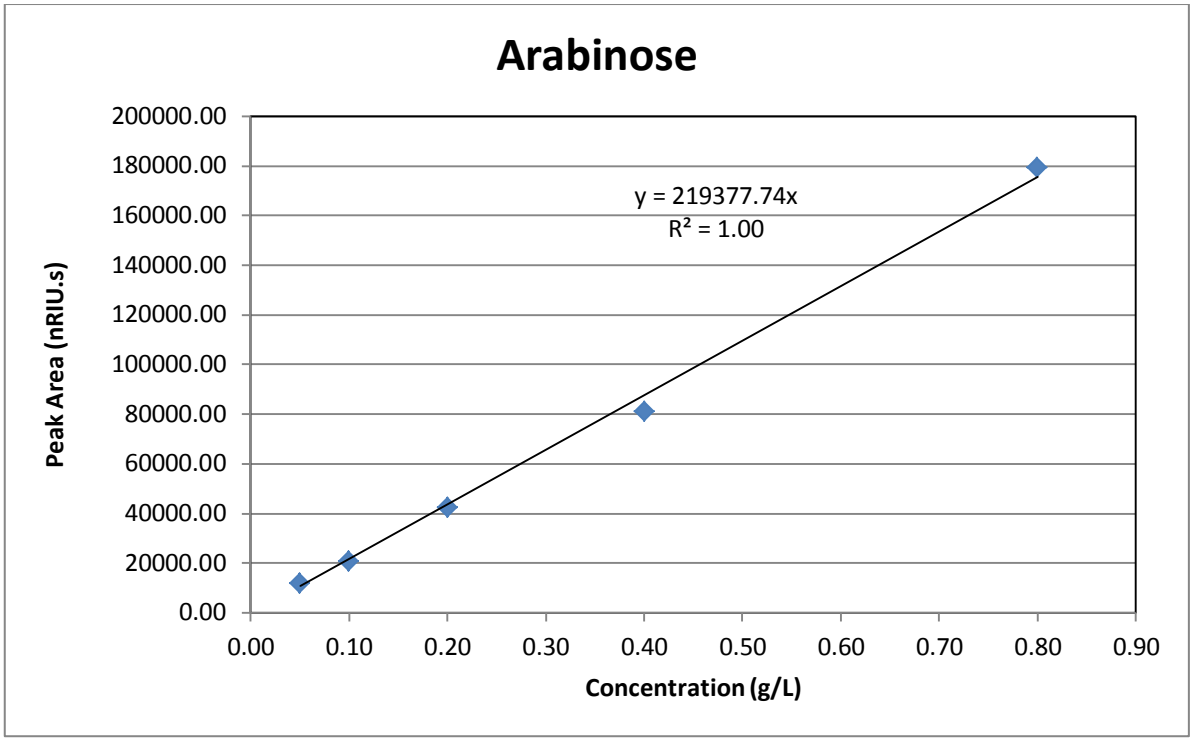

Figure A2.3: Arabinose calibration curve

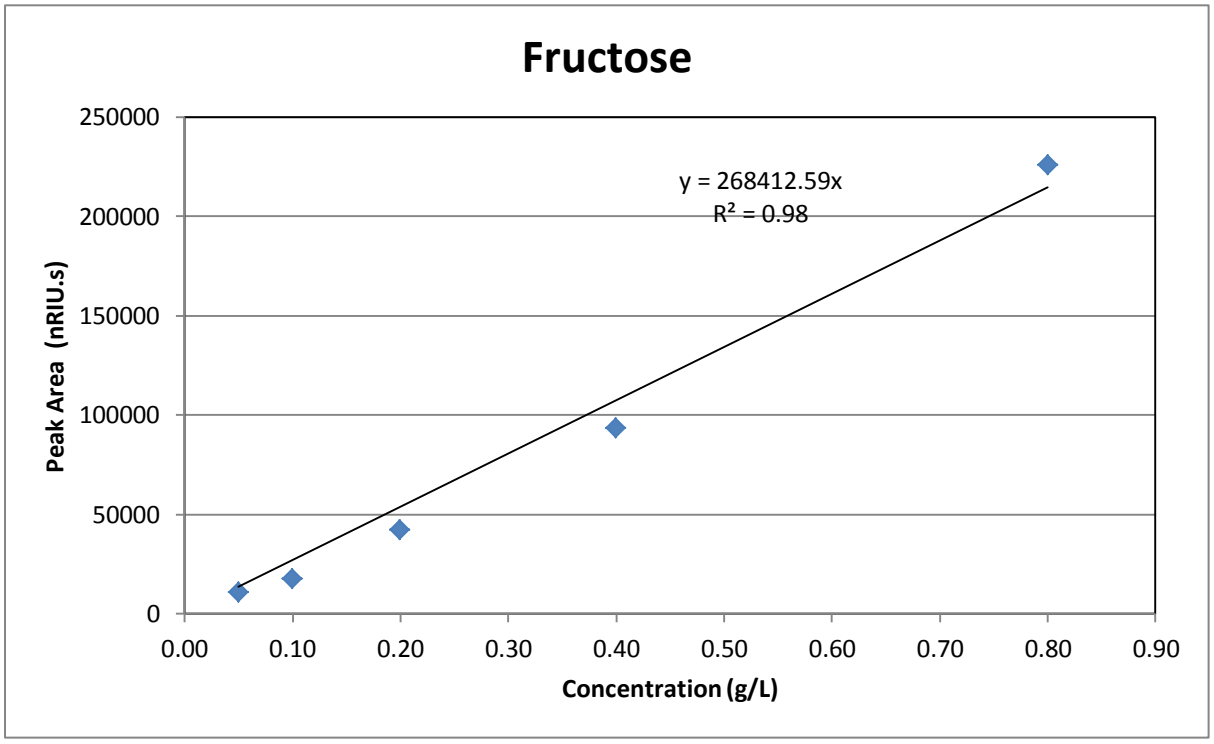

Figure A2.4: Fructose calibration curve

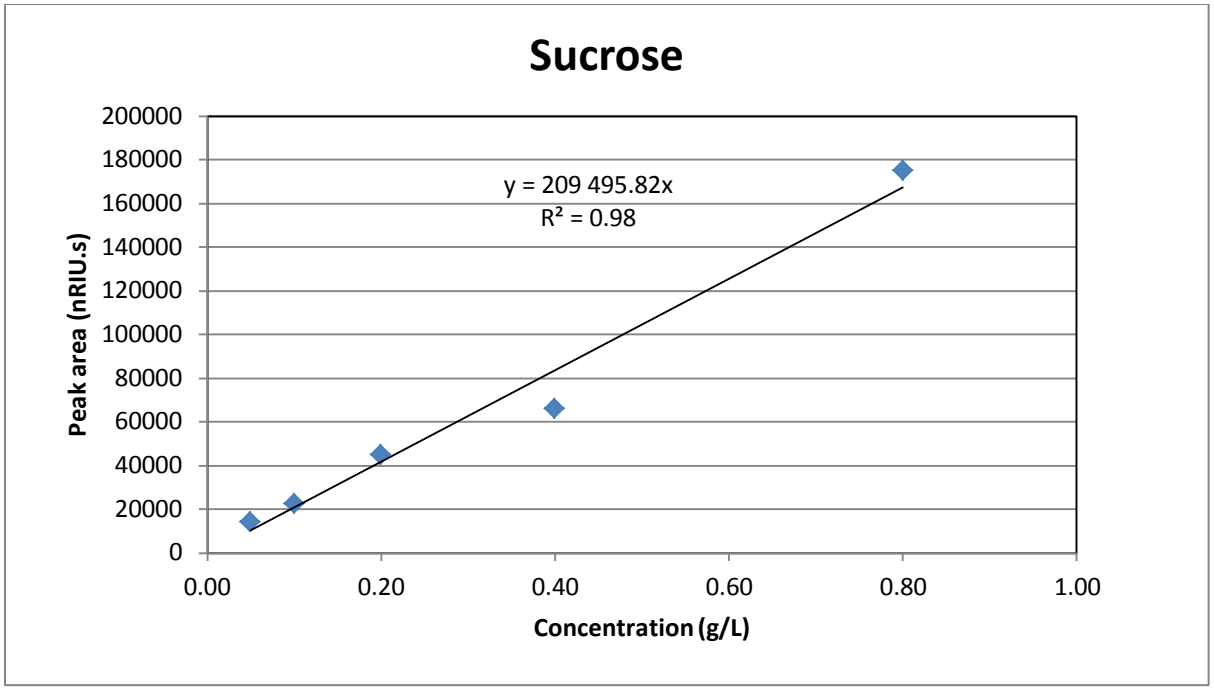

Figure A2.5: Sucrose calibration curve

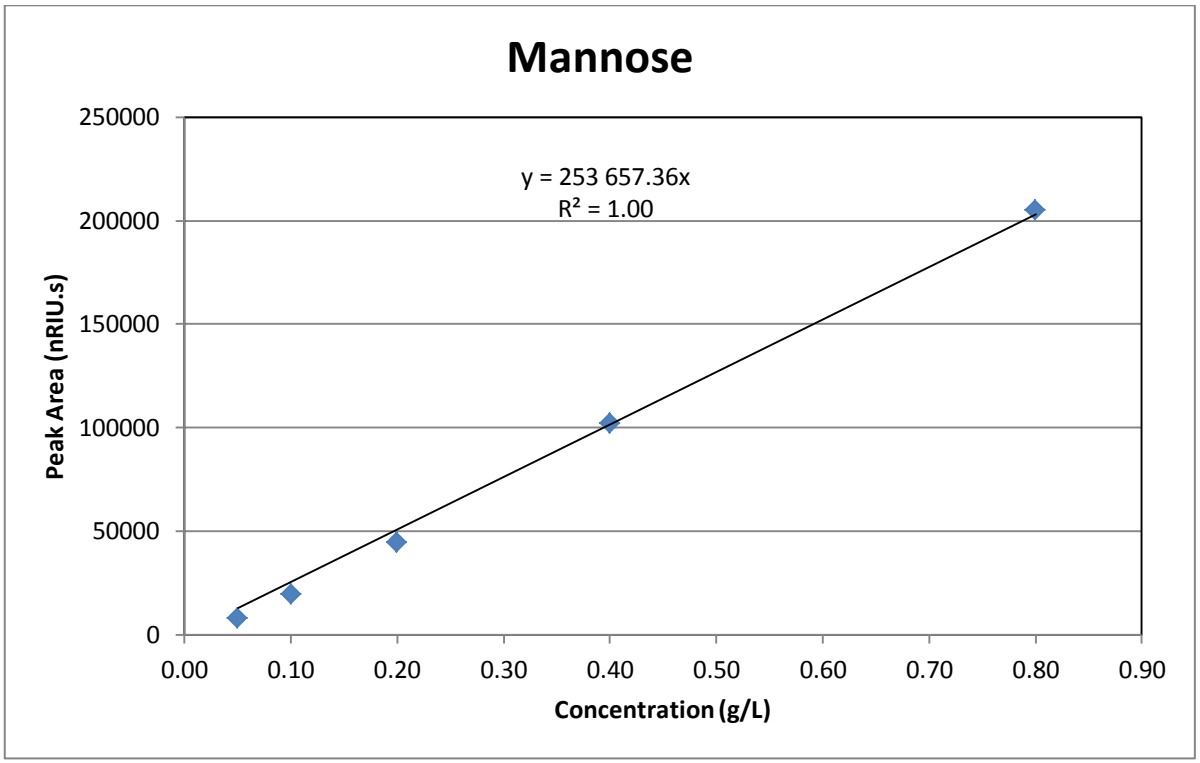

Figure A2.6: Mannose calibration curve

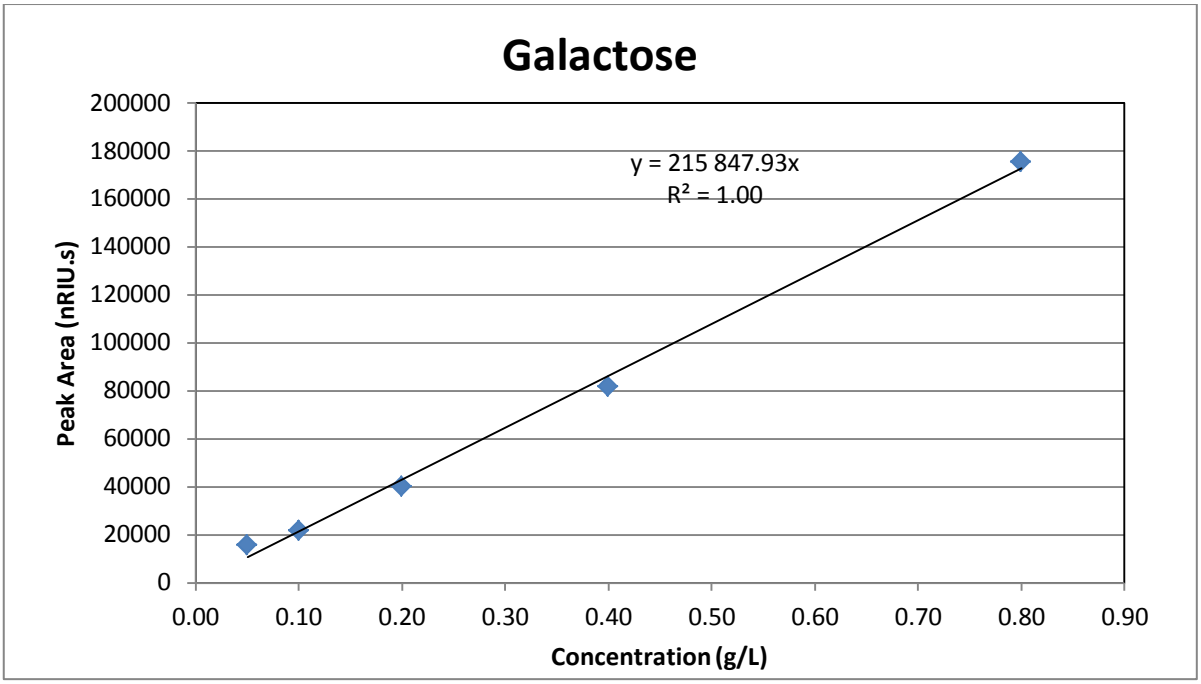

Figure A2.7: Galactose calibration curve

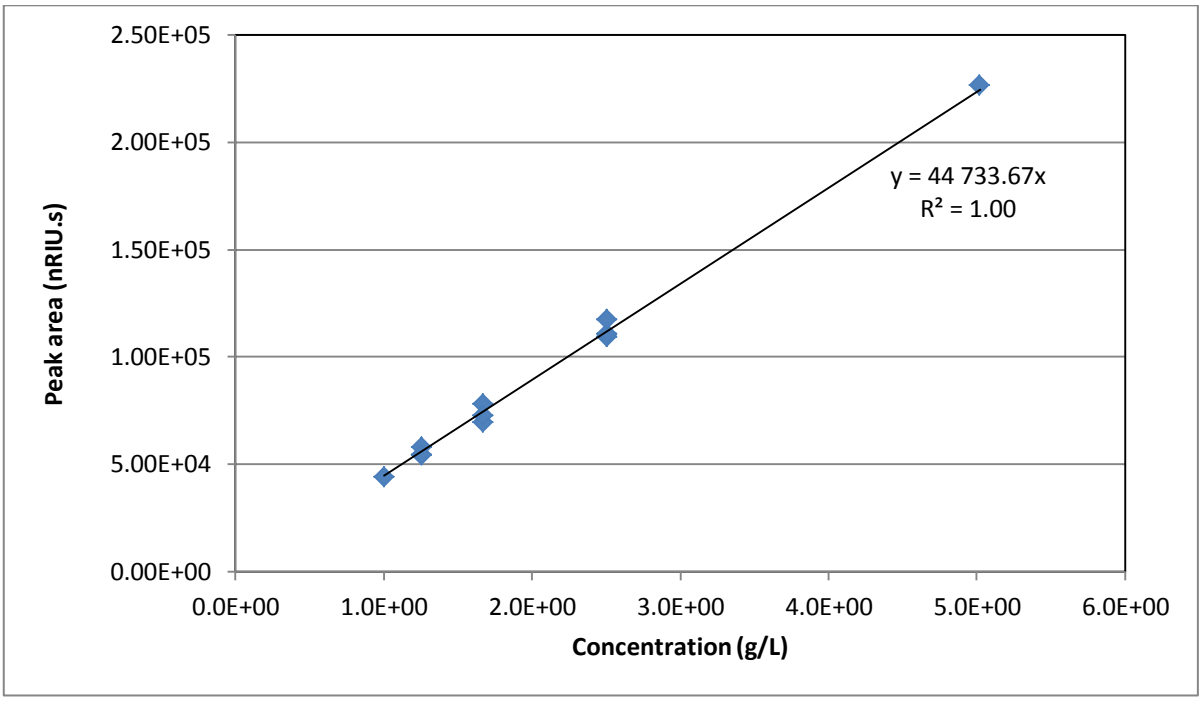

Figure A2.8: Cellobiose calibration curve

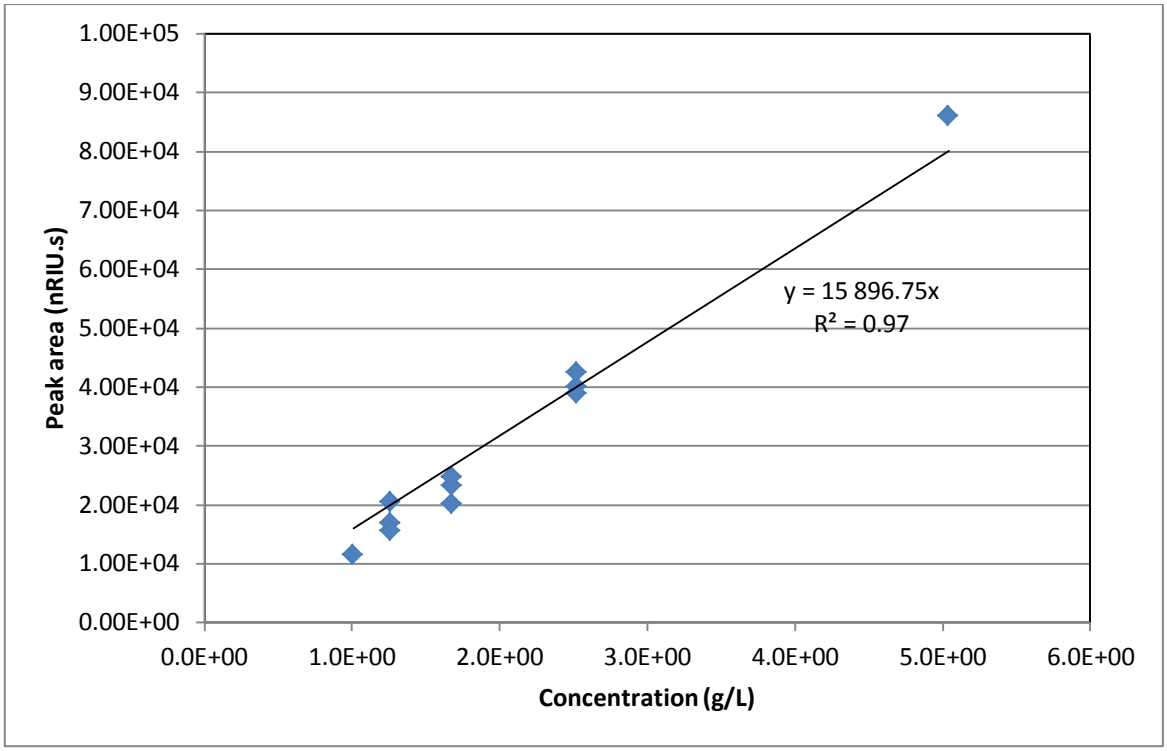

Figure A2.9: Ethanol calibration curve

APPENDIX B

Calculations

B1 Introduction

All the calculations used in this study are shown in this section. The calculations used to quantify the sugars and ethanol are shown in Section B2. The calculations for the energy input are also shown in Section B2. The experimental error calculations are described in Section B3 and the productivity obtained during fermentation is shown in Section B4.

B2. Concentration Calculations

Sugars concentration

$$\left[C_S \frac{g}{L}\right] = \frac{peak\ area}{slope} \times dilution\ factor$$

$$\left[C_S \frac{g}{g}\right] = \frac{\left[glucose \frac{g}{L}\right] \times final\ volume}{Mass\ of\ biomass}$$

Where C_S is the concentration of any of the sugar monomer.

Ethanol Concentration

$$\left[E_s \frac{g}{L}\right] = \frac{peak\ area}{slope} \times dilution\ factor$$

$$\left[E_S \frac{g}{g}\right] = \frac{\left[glucose \frac{g}{L}\right] \times final\ volume}{Mass\ of\ biomass}$$

Where E_S is the concentration of ethanol.

Energy input Calculation

$$Energy\ input\ (\frac{kJ}{g}) = \frac{(power\ \left(\frac{J}{s}\right) \times time\ (s))/1000}{g\ biomass}$$

Table B2.1: Energy input used and corresponding time intervals

Time (minutes)	Energy Input (kJ/g)		
	100W	**180W**	**300W**
5	6	10.8	18
10	12	21.6	36
15	18	32.4	54
20	24	43.2	n/a

B3. Error Calculation

The experimental error was calculated using the following equations:

$$\%Error = \frac{confidence\ limit}{\bar{x}} \times 100$$

Where $\bar{x}$ is the average

The confidence limit was calculated using equation....

$$confidence\ limit = \bar{x} \pm \frac{\sigma}{\sqrt{n}}$$

The standard deviation must be determined to calculate the 95% confidence interval using equation....

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

Where x is the sample mean, $\bar{x}$ is the sample mean and n is the sample size

Table B3.1: Experimental errors associated with pretreatment using KOH, NaOH and $Ca(OH)_2$

Pretreatment	Replicate 1 (g/g)	Replicate 2 (g/g)	Replicate 3 (g/g)	Mean	Standard Deviation	Error (%)
5% KOH	0.208	0.198	0.2	0.202	0.005142	2.5
5% NaOH	0.24	0.23	0.24	0.236	0.005108	2.2
3% $Ca(OH)_2$	0.36	0.36	0.34	0.353	0.011547	3.3

Table B3.2: Experimental errors associated with enzymatic hydrolysis on KOH, NaOH and $Ca(OH)_2$ pretreated samples

Sample	Replicate 1 (g/g)	Replicate 2 (g/g)	Replicate 3 (g/g)	Mean (g/g)	Standard Deviation	Error (%)
5% KOH,	0.60	0.62	0.66	0.63	0.031	4.90
5% NaOH,	0.50	0.52	0.54	0.52	0.019	3.70
3% $Ca(OH)_2$,	0.41	0.43	0.44	0.43	0.015	3.48

Table B3.3: Experimental error obtained during fermentation of $Ca(OH)_2$, NaOH and KOH pretreated amaranth biomass

Analysis	Total sugar yield (g/g)			Ethanol yield (g/g)		
Sample	**Ca(OH)2**	**KOH**	**NaOH**	**Ca(OH)2**	**KOH**	**NaOH**
Experiment 1	0.057	0.097	0.084	0.12	0.17	0.15
Experiment 2	0.054	0.096	0.090	0.12	0.18	0.15
Experiment 3	0.055	0.099	0.089	0.13	0.18	0.14
Mean	0.055	0.10	0.087	0.13	0.18	0.15
Standard deviation	0.0013	0.0014	0.0034	0.0057	0.0052	0.0076
Experimental error (%)	2.34	1.42	3.86	4.49	2.88	5.15

B4. Productivity

$$Productivity = \frac{[Ethanol\ g/L]}{Fermentation\ Time}$$

Table B4.1: Productivity values obtained from fermentation of $Ca(OH)_2$, NaOH and KOH pretreated amaranth biomass

Alkaline solution	Ethanol (g/g)	productivity (g/L/h)
Ca(OH)2	0.13	0.28
NaOH	0.15	0.32
KOH	0.18	0.38

APPENDIX C

Pretreatment Data

C1: Introduction

The data that was obtained during alkaline microwave assisted pretreatment of amaranth is provided in detail in this section. The data obtained when using $Ca(OH)_2$ for pretreatment is shown in Section C2, that obtained for NaOH pretreatment is shown in section C3 and the data obtained for KOH pretreatment is shown in section C4.

C2: Pretreatment using $Ca(OH)_2$

Table C2.1: Pretreatment with 1% $Ca(OH)_2$ at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
5	S1	62.6998	2.62E-02	4.72E-04			
	S2	34395.6	1.64E+00	2.95E-02	0.03	1.83	5.004
	S3	3294.28	1.23E-01	2.21E-03			
	S4	106.308	4.89E-03	8.80E-05			
	S5	604.227	2.38E-02	4.28E-04			
	S6	165.475	7.54E-03	1.36E-04			
10	S1	575.146	2.41E-01	3.83E-03			
	S2	21702.9	1.04E+00	1.65E-02	0.03	1.70	5.021
	S3	5427.23	2.02E-01	3.22E-03			
	S4	3934.96	1.81E-01	2.89E-03			
	S5	38.9782	1.54E-03	2.45E-05			
	S6	883.273	4.03E-02	6.42E-04			
15	S1	451.584	1.89E-01	2.63E-03			
	S2	32336.9	1.54E+00	2.15E-02	0.03	2.32	5.026
	S3	10676.5	3.98E-01	5.54E-03			
	S4	837.952	3.86E-02	5.37E-04			
	S5	12.99672	5.12E-04	7.14E-06			
	S6	3378.6927	1.54E-01	2.15E-03			
20	S1	2366.14	9.90E-01	1.18E-02			
	S2	47212	2.25E+00	2.69E-02	0.05	2.32	5.019
	S3	15073	5.62E-01	6.71E-03			
	S4		0.00E+00	0.00E+00			
	S5	19.4629	7.67E-04	9.17E-06			
	S6		0.00E+00	0.00E+00			

Table C2.2: Pretreatment with 2% $Ca(OH)_2$ at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
5	S1	1558.398	6.52E-01	1.17E-02			
	S2	47516.7	2.27E+00	4.08E-02	0.056	3.13	5.002
	S3	14.9513	5.57E-04	1.00E-05			
	S5	3501.82	1.38E-01	2.48E-03			
	S6	1592.33	7.26E-02	1.31E-03			
10	S1	3606.49	1.51E+00	2.41E-02			
	S2	63915.8	3.05E+00	4.88E-02	0.075	4.66	5.004
	S3	176.363	6.57E-03	1.05E-04			
	S5	2434.1	9.60E-02	1.53E-03			
	S6	53.2267	2.43E-03	3.88E-05			
15	S1	4594.55	1.92E+00	2.68E-02			
	S2	65620	3.13E+00	4.36E-02	0.075	5.37	5.03
	S3	2292.4	8.54E-02	1.19E-03			
	S5	5428.263	2.14E-01	2.98E-03			
	S6	435.166	1.98E-02	2.76E-04			
20	S2	129007.9	6.16E+00	7.36E-02			
	S3	276.53	1.03E-02	1.23E-04	0.089	7.44	5.022
	S5	31969.5	1.26E+00	1.51E-02			
	S6	190.775	8.70E-03	1.04E-04			

Table C2.3: Pretreatment with 3% $Ca(OH)_2$ at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	8912.74	7.46E+00	1.33E-01			
	S2	6011.51807	5.74E-01	1.03E-02	0.149328	8.36	5.036
	S3	115.32336	8.59E-03	1.54E-04			
	S5	47.57061	3.75E-03	6.70E-05			
	S6	3410.44246	3.11E-01	5.56E-03			
10	S1	22584.1	1.89E+01	3.02E-01			
	S2	14871.9	1.42E+00	2.27E-02	0.33123	20.71	5.002
	S3	64.34414	4.79E-03	7.67E-05			
	S5	55.63137	4.39E-03	7.02E-05			
	S6	4185.37301	3.82E-01	6.10E-03			
15	S1	27762.3	2.32E+01	3.24E-01			
	S2	20172.6	1.93E+00	2.69E-02	0.36087	25.85	5.014
	S3	27.10227	2.02E-03	2.82E-05			
	S5	18.81468	1.48E-03	2.07E-05			
	S6	7532.53284	6.87E-01	9.59E-03			
20	S1	22445.08	1.88E+01	2.25E-01			
	S2	14400.4	1.37E+00	1.65E-02	0.25019	20.85	5.001
	S3	66.83692	4.98E-03	5.98E-05			
	S5	61.24464	4.83E-03	5.79E-05			
	S6	7521.86882	6.86E-01	8.23E-03			

Table C2.4: Pretreatment with 5% $Ca(OH)_2$ at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass
5	S1	28648.8	1.20E+01	1.91E-01			
	S3	774.70959	2.89E-02	4.59E-04	0.19	12.03	5.03
	S5	286.45874	1.13E-02	1.80E-04			
	S6	42.02055	1.92E-03	3.05E-05			
10	S1	24683.31	1.03E+01	1.44E-01			
	S3	10763.6	4.01E-01	5.60E-03	0.16	11.19	5.016
	S5	11525.5	4.54E-01	6.34E-03			
	S6	45.03453	2.05E-03	2.86E-05			
15	S1	29622.79	1.24E+01	1.24E-01			
	S3	11316.4	4.22E-01	4.21E-03	0.13	12.87	5.01
	S5	22.65278	8.93E-04	8.91E-06			
	S6	120.72024	5.05E-02	5.04E-04			

Table C2.5: Pretreatment with 5% $Ca(OH)_2$ at 100W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Pentoses (g/g)	Hexoses (g/g)	Mass
5	S1	15788.8	6.61E+00	1.32E-01					
	S6	1245.2	5.68E-02	1.13E-03	0.14	6.91	0.0037	0.13	5.011
	S3	3493.09	1.30E-01	2.60E-03					
	S5	3010.59	1.19E-01	2.37E-03					
10	S1	20534.3	8.59E+00	1.71E-01					
	S6	411.14	1.87E-02	3.74E-04	0.18	8.88	0.0030	0.17	5.011
	S3	3493.09	1.30E-01	2.60E-03					
	S5	3499	1.38E-01	2.75E-03					
15	S1	19220.2	8.04E+00	1.61E-01					
	S6	1746.3	7.96E-02	1.59E-03	0.20	9.82	0.0025	0.19	5.007
	S3	1223.42	4.56E-02	9.10E-04					
	S5	41797.3	1.65E+00	3.29E-02					
20	S1	19403.9	8.12E+00	1.61E-01					
	S6	4232.02	1.93E-01	3.84E-03	0.19	9.35	0.020	0.17	5.028
	S3	21406.8	7.98E-01	1.59E-02					
	S5	6036.97	2.38E-01	0.00473					

Table C2.6: Pretreatment with 3% $Ca(OH)_2$ at 100W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	5302.4	2.218636353	0.044			
	S2	183.013	0.008735871	0.000	0.0731	3.683095239	5.036
	S3	6899.228	0.257038169	0.005			
	S5	14533.89	0.572974134	0.011			
	S6	13726.7	0.625710712	0.012			
10	S1	16788.9	7.024830994	0.1394			
	S2	283.157	0.013516105	0.0003	0.148358112	7.474281663	5.038
	S3	11572.6	0.431149676	0.0086			
	S5	121.372	0.004784887	0.0001			
	S6		0	0.0000			
15	S1	31546.7	13.19980677	0.26363			
	S2	3213.721	0.153402499	0.00306	0.302596159	15.1509897	5.007
	S3	33395	1.24416669	0.02485			
	S5	14042.8	0.553613738	0.01106			
	S6		0	0.00000			
20	S1	29499.2	12.34308945	0.2460			
	S2	4721.63	0.225380437	0.0045	0.290732167	14.58894015	5.018
	S3	18419.3	0.686230858	0.0137			
	S5	19654.68	0.774852655	0.0154			
	S6	12271.7	0.559386753	0.0111			

Table C2.7: Pretreatment with 5% $Ca(OH)_2$ at 300W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Mass (g)
5	S8	36861.5	8.24	0.082	5.009
10	S8	138454.3	30.95	0.31	5.009
15	S8	135694.2	30.33	0.30	5.009

Table C2.8: Effect of biomass loading using 3 g biomass per 100 g $Ca(OH)_2$ solution

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	38174.9	7.99E+00	2.66E-01			
	S4	4019.35	8.41E-01	2.80E-02	0.36	10.81	3.001
	S5	9479.9	1.98E+00	6.61E-02			
10	S1	35217.2	7.37E+00	2.46E-01			
	S4	3889.86	8.14E-01	2.71E-02	0.34	11.68	3.001
	S5	16715.8	3.50E+00	1.17E-01			
15	S1	28082.6	5.88E+00	1.96E-01			
	S4	7485.06	1.57E+00	5.22E-02	0.31	9.36	3.001
	S5	9169.12	1.92E+00	6.39E-02			

C3: Pretreatment using NaOH

Table C3.1: Pretreatment with 1% NaOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	mass
5	S1	673.53	2.82E-01	5.59E-03			
	S2	53.29	2.54E-03	5.05E-05	0.01	0.29	5.04
	S3	21.71	8.09E-04	1.60E-05			
	S4	38.87	1.79E-03	3.55E-05			
	S5	129.71	5.11E-03	1.01E-04			
	S6	15.73	7.17E-04	1.42E-05			
10	S1	862.78	3.61E-01	6.49E-03			
	S2	65.13	3.11E-03	5.59E-05	0.01	0.37	5.003
	S3	42.5	1.58E-03	2.85E-05			
	S4	82.28	3.79E-03	6.82E-05			
	S5	66.05	2.60E-03	4.68E-05			
	S6	23.73	1.08E-03	1.95E-05			
15	S1	2010.37	8.41E-01	1.34E-02			
	S2	23.34	1.11E-03	1.78E-05	0.01	0.85	5.008
	S3	19.17	7.14E-04	1.14E-05			
	S4	101.85	4.69E-03	7.49E-05			
	S5	35.63	1.40E-03	2.24E-05			
	S6	63.79	2.91E-03	4.64E-05			
20	S1	1342.13	5.62E-01	7.78E-03			
	S2	49.22	2.35E-03	3.26E-05	0.01	0.59	5.05
	S3	310.19	1.16E-02	1.60E-04			
	S4	136.88	6.30E-03	8.74E-05			
	S5	42.8	1.69E-03	2.34E-05			
	S6	67.82	3.09E-03	4.29E-05			

Table C3.2: Pretreatment with 2% NaOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	589.37	2.47E-01	4.93E-03			
	S2	72.79	3.47E-03	6.94E-05	0.0053	0.26	5.005
	S3	224.87	8.38E-03	1.67E-04			
	S4	13.21	6.08E-04	1.22E-05			
	S5	106.11	4.18E-03	8.36E-05			
	S6	28.62	1.30E-03	2.61E-05			
10	S1	1691.87	7.08E-01	1.27E-02			
	S2	668.66	3.19E-02	5.74E-04	0.014	0.76	5.007
	S3	89.23	3.32E-03	5.98E-05			
	S4	20.64	9.50E-04	1.71E-05			
	S5	135.27	5.33E-03	9.59E-05			
	S6	156.45	7.13E-03	1.28E-04			
15	S1	3736.9	1.56E+00	2.50E-02			
	S2	105.9811	5.06E-03	8.09E-05	0.025	1.58	5.003
	S3	11.42	4.25E-04	6.80E-06			
	S4	83.74	3.86E-03	6.17E-05			
	S5	31.24	1.23E-03	1.97E-05			
	S6	98.19	4.48E-03	7.16E-05			
20	S1	3304.58	1.38E+00	1.93E-02			
	S2	573.04	2.74E-02	3.82E-04	0.020	1.46	5.006
	S3	29.14	1.09E-03	1.52E-05			
	S4	856.22	3.94E-02	5.51E-04			
	S5	65.46	2.58E-03	3.61E-05			
	S6	143.86	6.56E-03	9.17E-05			

Table C3.3: Pretreatment with 3% NaOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	7604.2	3.18E+00	6.35E-02			
	S2	966.22	4.61E-02	9.21E-04	0.067	3.33	5.008
	S3	438.4	1.63E-02	3.26E-04			
	S4	371.1	1.71E-02	3.41E-04			
	S5	1355.83	5.35E-02	1.07E-03			
	S6	413.5	1.88E-02	3.76E-04			
10	S1	8905.4	3.73E+00	6.70E-02			
	S2	887.77	4.24E-02	7.62E-04	0.073	4.07	5.003
	S3	346.45	1.29E-02	2.32E-04			
	S4	315.76	1.45E-02	2.62E-04			
	S5	213.32	8.41E-03	1.51E-04			
	S6	5906.6	2.69E-01	4.84E-03			
15	S1	1484.5	6.21E-01	9.92E-03			
	S2	775.3	3.70E-02	5.91E-04	0.012	0.74	5.009
	S3	324.95	1.21E-02	1.93E-04			
	S4	361.06	1.66E-02	2.66E-04			
	S5	904.41	3.57E-02	5.69E-04			
	S6	339.6	1.55E-02	2.47E-04			
20	S1	16712.4	6.99E+00	9.78E-02			
	S2	860.24	4.11E-02	5.75E-04	0.10	7.13	5.003
	S3	360.59	1.34E-02	1.88E-04			
	S4	165.6	7.62E-03	1.07E-04			
	S5	1690.94	6.67E-02	9.33E-04			
	S6	251.65	1.15E-02	1.60E-04			

Table C3.4: Pretreatment with 5% NaOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	13297.3	5.56E+00	1.11E-01			
	S2	228.35196	1.09E-02	2.18E-04	0.12	5.99	5.004
	S3	95.7143	3.57E-03	7.13E-05			
	S4	7783.2154	3.58E-01	7.16E-03			
	S5	49.84146	1.96E-03	3.93E-05			
	S6	1100.891	5.02E-02	1.00E-03			
10	S1	24608	1.03E+01	1.84E-01			
	S2	17.99433	8.59E-04	1.54E-05	0.19	10.82	5.034
	S3	89.6398	3.34E-03	5.97E-05			
	S4	9750.9153	4.49E-01	8.03E-03			
	S5	18.24399	7.19E-04	1.29E-05			
	S6	1550.4235	7.07E-02	1.26E-03			
15	S1	33579	1.41E+01	2.24E-01			
	S2	10.08504	4.81E-04	7.68E-06	0.24	14.84	5.013
	S3	71.74016	2.67E-03	4.27E-05			
	S4	15302.7651	7.05E-01	1.12E-02			
	S5	18.2746	7.20E-04	1.15E-05			
	S6	1837.9351	8.38E-02	1.34E-03			
20	S1	34091	1.43E+01	1.99E-01			
	S2	455.80389	2.18E-02	3.04E-04	0.21	15.02	5.018
	S3	105.22809	3.92E-03	5.47E-05			
	S4	14781.6351	6.81E-01	9.49E-03			
	S5	26.41248	1.04E-03	1.45E-05			
	S6	1129.7381	5.15E-02	7.18E-04			

Table C3.5: Pretreatment with 5% NaOH at 100W

Time (minutes)	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Pentoses (g/g)	Hexoses (g/g)	Mass (g)
5	S1	12067	5.05E+00	1.01E-01					
	S6	9.9857	4.55E-04	9.09E-06	0.10	5.14	0.00043	0.10	5.004
	S3	568.11	2.12E-02	4.23E-04					
	S5	1724.58	6.80E-02	1.36E-03					
10	S1	20123	8.42E+00	1.67E-01					
	S6	1276.77	5.82E-02	1.16E-03	0.18	8.85	0.0015	0.17	5.034
	S3	401.63	1.50E-02	2.97E-04					
	S5	8973.36	3.54E-01	7.03E-03					
15	S1	20155	8.43E+00	1.68E-01					
	S6	19860.3	9.05E-01	1.81E-02	0.19	9.61	0.018	0.17	5.018
	S3	360.59	1.34E-02	2.68E-04					
	S5	6445.51	2.54E-01	5.07E-03					
20	S1	22278	9.32E+00	1.86E-01					
	S6	10521.9	4.80E-01	9.59E-03	0.21	10.26	0.014	0.19	5.013
	S3	6255.03	2.33E-01	4.66E-03					
	S5	5706.99	2.25E-01	4.50E-03					

Table C3.6: Pretreatment with 5% NaOH at 300W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	32266.55194	1.35E+01	1.35E-01			
	S8	95259.70901	2.13E+01	2.13E-01	0.35	34.80	5.008
10	S1	35579.33768	1.49E+01	1.49E-01			
	S8	99167.7942	2.22E+01	2.22E-01	0.37	37.06	5.002
15	S1	40110.21732	1.68E+01	1.68E-01			
	S8	105288.9613	2.35E+01	2.35E-01	0.40	40.32	5.002

C4: Pretreatment using KOH

Table C4.1: Pretreatment with 1% KOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
5	S1	984.12	4.12E-01	8.21E-03			
	S2	308.41	1.47E-02	2.93E-04	0.010	0.52	5.016
	S3	104.231	3.88E-03	7.74E-05			
	S4	355.623	1.64E-02	3.26E-04			
	S5	645.23	2.54E-02	5.07E-04			
	S6	951.49	4.34E-02	8.65E-04			
10	S1	1310.3	5.48E-01	9.85E-03			
	S2	490.34	2.34E-02	4.21E-04	0.012	0.68	5.007
	S3	110.534	4.12E-03	7.40E-05			
	S4	671.803	3.09E-02	5.56E-04			
	S5	803.74	3.17E-02	5.70E-04			
	S6	984.6	4.49E-02	8.07E-04			
15	S1	9680.1	4.05E+00	6.46E-02			
	S2	175.94	8.40E-03	1.34E-04	0.067	4.17	5.015
	S3	107.011	3.99E-03	6.36E-05			
	S4	498.532	2.30E-02	3.66E-04			
	S5	824.01	3.25E-02	5.18E-04			
	S6	1168.2	5.33E-02	8.49E-04			
20	S1	1867.7	7.81E-01	1.09E-02			
	S2	258.66	1.23E-02	1.73E-04	0.013	0.94	5.004
	S3	125.792	4.69E-03	6.56E-05			
	S4	714.641	3.29E-02	4.60E-04			
	S5	862.01	3.40E-02	4.75E-04			
	S6	1636.54	7.46E-02	1.04E-03			

Table C4.2: Pretreatment with 2% KOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	2308.8	9.66E-01	1.93E-02			
	S2	275.2	1.31E-02	2.62E-04	0.022	1.11	5.017
	S3	143.8	5.36E-03	1.07E-04			
	S4	754.221	3.47E-02	6.92E-04			
	S5	801.7	3.16E-02	6.30E-04			
	S6	1235.5	5.63E-02	1.12E-03			
10	S1	3199.9	1.34E+00	2.41E-02			
	S2	35.381	1.69E-03	3.04E-05	0.027	1.48	5.003
	S3	31.293	1.17E-03	2.10E-05			
	S4	798.851	3.68E-02	6.62E-04			
	S5	790.1	3.11E-02	5.60E-04			
	S6	1636.6	7.46E-02	1.34E-03			
15	S1	3910.85	1.64E+00	2.61E-02			
	S2	312.774	1.49E-02	2.38E-04	0.029	1.82	5.009
	S3	216.872	8.08E-03	1.29E-04			
	S4	834.123	3.84E-02	6.13E-04			
	S5	821.5	3.24E-02	5.17E-04			
	S6	1905.38	8.69E-02	1.39E-03			
20	S1	3791.562	1.59E+00	2.22E-02			
	S2	244.65	1.17E-02	1.63E-04	0.025	1.77	5.003
	S3	132.29	4.93E-03	6.90E-05			
	S4	873.702	4.02E-02	5.63E-04			
	S5	994.2	3.92E-02	5.48E-04			
	S6	2015.55	9.19E-02	1.29E-03			

Table C4.3: Pretreatment with 3% KOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	1838.16	7.69E-01	1.54E-02			
	S2	289.54	1.38E-02	2.76E-04	0.017	0.83	5.007
	S3	83.34	3.10E-03	6.20E-05			
	S4	750.97	3.46E-02	6.91E-04			
	S5	42.76	1.69E-03	3.37E-05			
	S6	265.42	1.21E-02	2.42E-04			
10	S1	4401.79	1.84E+00	3.31E-02			
	S2	179.39	8.56E-03	1.54E-04	0.034	1.92	5.006
	S3	95.56	3.56E-03	6.40E-05			
	S4	833.91	3.84E-02	6.90E-04			
	S5	106.13	4.18E-03	7.52E-05			
	S6	459.6	2.10E-02	3.77E-04			
15	S1	3067.85	1.28E+00	2.05E-02			
	S2	189.09	9.03E-03	1.44E-04	0.022	1.36	5.004
	S3	107.36	4.00E-03	6.39E-05			
	S4	824.21	3.79E-02	6.07E-04			
	S5	18.22	7.18E-04	1.15E-05			
	S6	460.06	2.10E-02	3.35E-04			
20	S1	6861.68	2.87E+00	4.01E-02			
	S2	130.24	6.22E-03	8.68E-05	0.041	2.95	5.015
	S3	109.02	4.06E-03	5.67E-05			
	S4	926.63	4.27E-02	5.96E-04			
	S5	62.82	2.48E-03	3.46E-05			
	S6	473.01	2.16E-02	3.01E-04			

Table C4.4: Pretreatment with 5% KOH at 180W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	mass (g)
5	S1	11311.14089	4.73E+00	9.43E-02			
	S2	462.05212	2.21E-02	4.39E-04	0.11	5.27	5.02
	S3	178.53372	6.65E-03	1.32E-04			
	S4	8482.33543	3.91E-01	7.78E-03			
	S5	48.03664	1.89E-03	3.77E-05			
	S6	2581.29422	1.18E-01	2.34E-03			
10	S1	18331.83405	7.67E+00	1.38E-01			
	S2	549.19249	2.62E-02	4.72E-04	0.15	8.43	5.003
	S3	133.26245	4.96E-03	8.93E-05			
	S4	12341.54162	5.68E-01	1.02E-02			
	S5	43.90416	1.73E-03	3.11E-05			
	S6	3462.93951	1.58E-01	2.84E-03			
15	S1	28110.94387	1.18E+01	1.87E-01			
	S2	796.74219	3.80E-02	6.04E-04	0.21	13.08	5.037
	S3	425.86225	1.59E-02	2.52E-04			
	S4	19909.3621	9.17E-01	1.46E-02			
	S5	18.07574	7.13E-04	1.13E-05			
	S6	7529.98087	3.43E-01	5.45E-03			
20	S1	25241.02822	1.06E+01	1.47E-01			
	S2	615.08	2.94E-02	4.10E-04	0.16	11.68	5.015
	S3	431.7883	1.61E-02	2.25E-04			
	S4	18195.2147	8.38E-01	1.17E-02			
	S5	128.75458	5.08E-03	7.09E-05			
	S6	5045.9868	2.30E-01	3.21E-03			

Table C4.5: Pretreatment with 5% KOH at 100W

Time (minutes)	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Pentoses (g/g)	Hexoses (g/g)	Mass (g)
5	S1	8204.8	3.43E+00	6.86E-02					
	S6	13072.3	5.96E-01	1.19E-02	0.082	4.11	0.013	0.069	5.008
	S3	2013	7.50E-02	1.50E-03					
	S5	93.9	3.70E-03	7.39E-05					
10	S1	10493.6	4.39E+00	8.72E-02					
	S6	23295.3	1.06E+00	2.11E-02	0.11	5.60	0.024	0.087	5.035
	S3	3693.9	1.38E-01	2.73E-03					
	S5	325.1	1.28E-02	2.55E-04					
15	S1	16227.8	6.79E+00	1.36E-01					
	S6	62808.9	2.86E+00	5.71E-02	0.19	9.72	0.058	0.14	5.01
	S3	1776.9	6.62E-02	1.32E-03					
	S5	50.1003	1.98E-03	3.94E-05					
20	S1	15907	6.66E+00	1.33E-01					
	S6	46138.3	2.10E+00	4.20E-02	0.18	8.83	0.043	0.13	5.002
	S3	1803.1	6.72E-02	1.34E-03					
	S5	17.5	6.91E-04	1.38E-05					

Table C4.6: Pretreatment with 5% KOH at 300W

Time (minutes)	Type of sugar	Area (nRIU.s)	concentration (g/L)	concentration (g/g)	Total sugars (g/g)	total sugars (g/L)	Mass (g)
5	S1	9277.972	3.88E+00	3.88E-02			
	S8	39999.60699	8.94E+00	8.94E-02	0.13	12.82	5.002
10	S1	10757.69858	4.50E+00	4.50E-02			
	S8	60171.91026	1.35E+01	1.34E-01	0.18	17.95	5.004
15	S1	10329.33869	4.32E+00	4.32E-02			
	S8	68712.9688	1.54E+01	1.53E-01	0.20	19.68	5.007

APPENDIX D

Hydrolysis and Fermentation Data

D1: Introduction

All the data used and collected on the work conducted on enzymatic hydrolysis and fermentation will be provided in this section. The calculated sugar yields during hydrolysis will be presented in Section D2. The sugar and ethanol yields obtained during fermentation will be given in Section D3 and were calculated as shown in Appendix B.

D2: Enzymatic Hydrolysis

Table D2.1: Enzymatic hydrolysis of 3% $Ca(OH)_2$ pretreated amaranth biomass

Sample	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
1	S1	17948.7	7.51E+00	1.95E-01			
	S4	739.814	3.41E-02	8.85E-04	0.41	15.81	5.002
	S5	2760.59	1.09E-01	2.83E-03			
	S6	4851.73	2.21E-01	5.75E-03			
	S8	35495.9	7.93E+00	2.06E-01			
2	S1	17829.3	7.46E+00	1.94E-01			
	S4	687.148	3.16E-02	8.22E-04	0.43	16.57	5.001
	S5	2593.88	1.02E-01	2.66E-03			
	S6	4915.36	2.24E-01	5.82E-03			
	S8	39133.7	8.75E+00	2.27E-01			
3	S1	19943.7	8.34E+00	2.17E-01			
	S4	842.98	3.88E-02	1.01E-03	0.44	16.93	5.004
	S5	2930.12	1.16E-01	3.00E-03			
	S6	4641.51	2.12E-01	5.50E-03			
	S8	36787.8	8.22E+00	2.14E-01			

Table D2.2: Enzymatic hydrolysis of 5% NaOH pretreated amaranth biomass

Sample	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
1	S1	25196.4	1.05E+01	2.74E-01			
	S4	1143.795	5.27E-02	1.37E-03	0.50	19.33	5.004
	S5	3609.33	1.42E-01	3.70E-03			
	S6	6327.99	2.88E-01	7.49E-03			
	S8	37154.5	8.31E+00	2.16E-01			
2	S1	26398.1	1.10E+01	2.87E-01			
	S4	1457.635	6.71E-02	1.74E-03	0.52	20.15	5.004
	S5	3815.07	1.50E-01	3.91E-03			
	S6	6831.82	3.11E-01	8.09E-03			
	S8	38353.9	8.57E+00	2.23E-01			
3	S1	27042.6	1.13E+01	2.94E-01			
	S4	1270.792	5.85E-02	1.52E-03	0.54	20.81	5.003
	S5	3519.66	1.39E-01	3.61E-03			
	S6	6458.01	2.94E-01	7.65E-03			
	S8	40277.1	9.00E+00	2.34E-01			

Table D2.3: Enzymatic hydrolysis of 5% KOH pretreated amaranth biomass

Sample	Type of sugar	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
1	S1	25196.4	1.05E+01	2.74E-01			
	S4	6773.959	3.12E-01	8.09E-03	0.60	23.28	5.009
	S5	2805.04	1.11E-01	2.87E-03			
	S6	7473.11	3.41E-01	8.84E-03			
	S8	53553.1	1.20E+01	3.11E-01			
2	S1	26008.5	1.09E+01	2.82E-01			
	S4	6942.013	3.20E-01	8.30E-03	0.62	23.77	5.008
	S5	2674.67	1.05E-01	2.74E-03			
	S6	7941.33	3.62E-01	9.40E-03			
	S8	54134.7	1.21E+01	3.14E-01			
3	S1	29483.2	1.23E+01	3.20E-01			
	S4	6529.881	3.01E-01	7.80E-03	0.66	25.54	5.009
	S5	3027.23	1.19E-01	3.10E-03			
	S6	8106.94	3.70E-01	9.59E-03			
	S8	55515.5	1.24E+01	3.22E-01			

D3. Fermentation

Table D3.1: Sugar obtained during fermentation of $Ca(OH)_2$ pretreated amaranth biomass

Sample (hours)	Sugars	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)
3	S1	11165.4	4.67E+00	9.34E-02		
	S4	678.481	3.12E-02	6.25E-04	0.20	9.91
	S5	2671.95	1.05E-01	2.11E-03		
	S6	4518.43	2.06E-01	4.12E-03		
	S8	21922	4.90E+00	9.80E-02		
6	S1	9761.9	4.08E+00	8.17E-02		
	S4	667.768	3.07E-02	6.15E-04	0.18	9.02
	S5	2545.61	1.00E-01	2.01E-03		
	S6	4682.09	2.13E-01	4.27E-03		
	S8	20532.7	4.59E+00	9.18E-02		
9	S1	8021.5	3.36E+00	6.71E-02		
	S4	659.11	3.03E-02	6.07E-04	0.15	7.44
	S5	2794.21	1.10E-01	2.20E-03		
	S6	4563.03	2.08E-01	4.16E-03		
	S8	16729.2	3.74E+00	7.48E-02		
12	S1	7673	3.21E+00	6.42E-02		
	S4	622.999	2.87E-02	5.73E-04	0.10	4.82
	S5	2719.33	1.07E-01	2.14E-03		
	S6	4541.78	2.07E-01	4.14E-03		
	S8	5674.4	1.27E+00	2.54E-02		
24	S1	3355.6	1.40E+00	2.81E-02		
	S4	725.834	3.34E-02	6.68E-04	0.055	2.77
	S5	2486.01	9.80E-02	1.96E-03		
	S6	4891.15	2.23E-01	4.46E-03		

	S8	4530.3	1.01E+00	2.02E-02		
48	S1	3232.18	1.35E+00	2.70E-02		
	S4	664.123	3.06E-02	6.11E-04	0.053	2.66
	S5	2531.89	9.98E-02	2.00E-03		
	S6	4647.9	2.12E-01	4.24E-03		
	S8	4297.88	9.61E-01	1.92E-02		

Table D3.2: Sugars obtained during fermentation of $Ca(OH)_2$ pretreated amaranth biomass (replicates used in experimental error)

Sample (hours)	Sugars	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
24	S1	3492.7	1.46E+00	2.92E-02			
	S4	683.905	3.15E-02	6.30E-04	0.057	2.83	5.001
	S5	2503.99	9.87E-02	1.97E-03			
	S6	4671.63	2.13E-01	4.26E-03			
	S8	4604.1	1.03E+00	2.06E-02			
24	S1	3318.35	1.39E+00	2.78E-02			
	S4	795.203	3.66E-02	7.32E-04	0.054	2.71	5.003
	S5	2371.77	9.35E-02	1.87E-03			
	S6	4578.8	2.09E-01	4.17E-03			
	S8	4374.01	9.78E-01	1.95E-02			

Table D3.3: Ethanol obtained during fermentation of $Ca(OH)_2$ pretreated amaranth biomass

Sample (hours)	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)
3	4114.01	2.59	0.052
6	4752.49	2.99	0.060
9	7360.15	4.63	0.093
12	9030.02	5.68	0.11
24	10532.2	6.63	0.13
24	9816.8	6.18	0.12
24	9704.1	6.10	0.12
48	8867.8	5.58	0.11

Table D3.4: Sugars obtained during fermentation of NaOH pretreated amaranth biomass

Sample (hours)	component	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)
3	S1	17576.8	7.35E+00	1.47E-01		
	S4	1345.163	6.19E-02	1.24E-03	0.28	13.82
	S5	2784.11	1.10E-01	2.19E-03		
	S6	5347.05	2.44E-01	4.87E-03		
	S8	27045.9	6.05E+00	1.21E-01		
6	S1	13918.7	5.82E+00	1.16E-01		
	S4	1577.536	7.26E-02	1.45E-03	0.22	11.10
	S5	3245.66	1.28E-01	2.56E-03		
	S6	5542.84	2.53E-01	5.05E-03		
	S8	21567.1	4.82E+00	9.63E-02		
9	S1	10052.9	4.21E+00	8.41E-02		
	S4	1269.924	5.85E-02	1.17E-03	0.17	8.74
	S5	3114.78	1.23E-01	2.45E-03		
	S6	5754.11	2.62E-01	5.24E-03		
	S8	18287.6	4.09E+00	8.17E-02		
12	S1	8552.3	3.58E+00	7.15E-02		
	S4	1496.774	6.89E-02	1.38E-03	0.12	6.23
	S5	3444.93	1.36E-01	2.71E-03		
	S6	5692.04	2.59E-01	5.19E-03		
	S8	9777.5	2.19E+00	4.37E-02		
24	S1	5295.1	2.22E+00	4.43E-02		
	S4	1384.512	6.37E-02	1.27E-03	0.089	4.44
	S5	2813.17	1.11E-01	2.22E-03		
	S6	5404.88	2.46E-01	4.92E-03		
	S8	8076.1	1.81E+00	3.61E-02		
48	S1	4975.12	2.08E+00	4.16E-02		

	S4	1049.007	4.83E-02	9.65E-04	0.075	3.77
	S5	2419.36	9.54E-02	1.91E-03		
	S6	5569.15	2.54E-01	5.07E-03		
	S8	5795.3	1.30E+00	2.59E-02		

Table D3.5: Sugars obtained during fermentation of NaOH pretreated amaranth biomass (replicates used in experimental error)

Sample (hours)	Sugars	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
24	S1	5213.9	2.18E+00	4.36E-02			
	S4	1486.687	6.85E-02	1.37E-03	0.084	4.18	5.002
	S5	2532.76	9.98E-02	2.00E-03			
	S6	5079.12	2.32E-01	4.63E-03			
	S8	7161.4	1.60E+00	3.20E-02			
24	S1	5188.3	2.17E+00	4.34E-02			
	S4	1415.902	6.52E-02	1.30E-03	0.090	4.50	5.005
	S5	2711.05	1.07E-01	2.14E-03			
	S6	5298.64	2.42E-01	4.83E-03			
	S8	8583.2	1.92E+00	3.83E-02			

Table D3.6: Ethanol obtained during fermentation of NaOH pretreated amaranth biomass

Sample (hours)	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)
3	5394.55	3.39	0.068
6	6659.87	4.19	0.084
9	7698.53	4.84	0.097
12	10443.33	6.57	0.13
24	12130.8	7.63	0.15
24	12072.59	7.59	0.15
24	11059.01	6.96	0.14
48	11352.07	7.14	0.14

Table D3.7: Sugars obtained during fermentation of KOH pretreated amaranth biomass

Sample (hours)	component	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)
3	S1	17187.8	7.19E+00	1.44E-01		
	S4	2974.55	1.37E-01	2.74E-03	0.33	16.58
	S5	4891.02	1.93E-01	3.86E-03		
	S6	6481.74	2.95E-01	5.91E-03		
	S8	39212.6	8.77E+00	1.75E-01		
6	S1	12785.3	5.35E+00	1.07E-01		
	S4	2717.99	1.25E-01	2.50E-03	0.29	14.31
	S5	4532.18	1.79E-01	3.57E-03		
	S6	6379.62	2.91E-01	5.81E-03		
	S8	37404.5	8.36E+00	1.67E-01		
9	S1	10358.6	4.33E+00	8.67E-02		
	S4	2566.87	1.18E-01	2.36E-03	0.22	11.11
	S5	4229.04	1.67E-01	3.33E-03		
	S6	6098.31	2.78E-01	5.56E-03		
	S8	27789.4	6.21E+00	1.24E-01		
12	S1	9551.1	4.00E+00	7.99E-02		
	S4	2374.56	1.09E-01	2.19E-03	0.15	7.61
	S5	3851.07	1.52E-01	3.04E-03		
	S6	5309.13	2.42E-01	4.84E-03		
	S8	13897.2	3.11E+00	6.21E-02		
24	S1	5053.61	2.11E+00	4.23E-02		
	S4	8462.44	3.90E-01	7.79E-03	0.097	4.86
	S5	3556.89	1.40E-01	2.80E-03		
	S6	5001.46	2.28E-01	4.56E-03		
	S8	8894.34	1.99E+00	3.98E-02		
48	S1	5621.5	2.35E+00	4.70E-02		

	S4	2106.01	9.70E-02	1.94E-03	0.095	4.73
	S5	3020.92	1.19E-01	2.38E-03		
	S6	5123.77	2.34E-01	4.67E-03		
	S8	8645.25	1.93E+00	3.86E-02		

Table D3.8: Sugars obtained during fermentation of KOH pretreated amaranth biomass (replicates used in experimental error)

Sample (hours)	component	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)	Total sugars (g/g)	Total sugars (g/L)	Mass (g)
24	S1	4902.19	2.05E+00	4.10E-02			
	S4	8541.97	3.93E-01	7.86E-03	0.096	4.82	5.002
	S5	3655.72	1.44E-01	2.88E-03			
	S6	5023.88	2.29E-01	4.58E-03			
	S8	8972.36	2.01E+00	4.01E-02			
24	S1	5309.3	2.22E+00	4.44E-02			
	S4	8289.01	3.82E-01	7.63E-03	0.099	4.96	5.003
	S5	3778.54	1.49E-01	2.98E-03			
	S6	5234.65	2.39E-01	4.77E-03			
	S8	8805.75	1.97E+00	3.93E-02			

Table D3.9: Ethanol obtained during fermentation of NaOH pretreated amaranth biomass

Sample (hours)	Area (nRIU.s)	Concentration (g/L)	Concentration (g/g)
3	4632.89	2.91	0.06
6	7485.36	4.71	0.09
9	8673.25	5.46	0.11
12	11321.1	7.12	0.14
24	13793.4	8.68	0.17
24	14578.69	9.17	0.18
24	14392.22	9.05	0.18
48	10891.45	6.85	0.14

APPENDIX E

Additional data

E1: Introduction

Supporting data to the results presented in chapter 4 and 5 in units of g/L is presented in this section. The Results obtained during pretreatment is presented in Section E2. Enzymatic hydrolysis results are shown in section E2. The fermentation results are shown in Section E3.

E2: Pretreatment

Pretreatment with Calcium hydroxide ($Ca(OH)_2$)

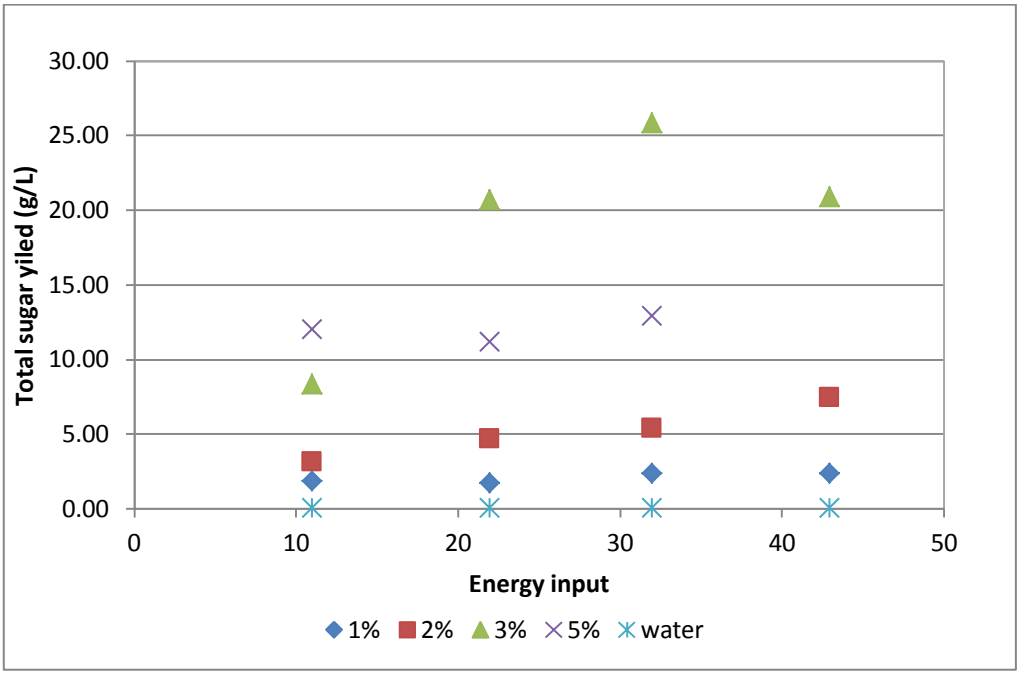

Figure E2.1: Total sugars (g/L) of microwave assisted pretreatment of $Ca(OH)_2$ at different concentrations at 180 W (× 5% $Ca(OH)_2$, ▲ 3% $Ca(OH)_2$, ■ 2% $Ca(OH)_2$, ♦ 1% $Ca(OH)_2$, Ж- Control).

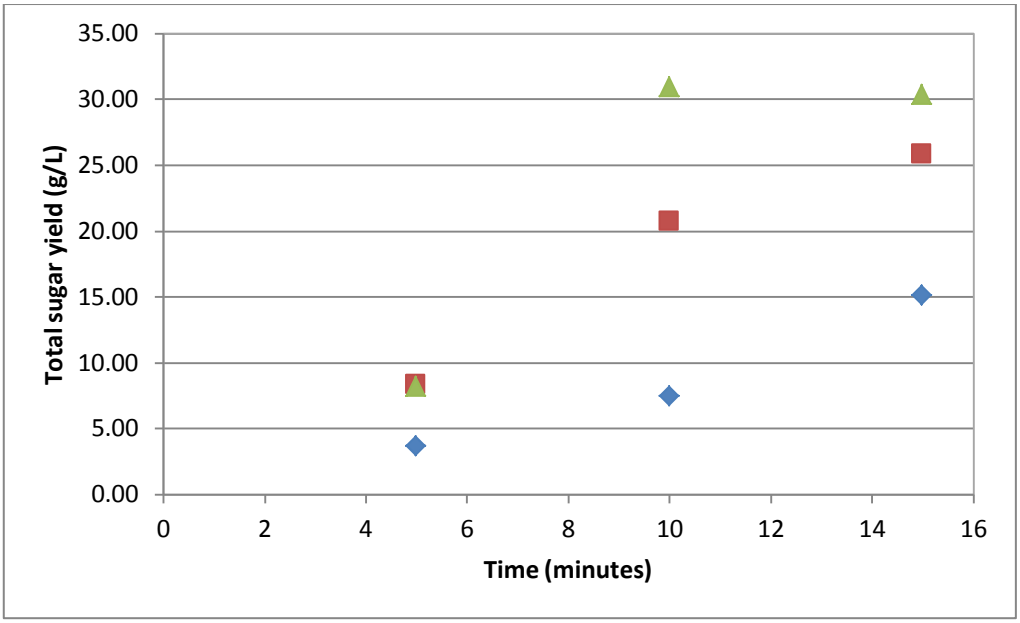

Figure E2.2: The effect of microwave irradiation power on total sugar yield (g/L) (♦-18 kJ.g^{-1}, ■-32.4 kJ.g^{-1}, ▲-54 kJ.g^{-1})

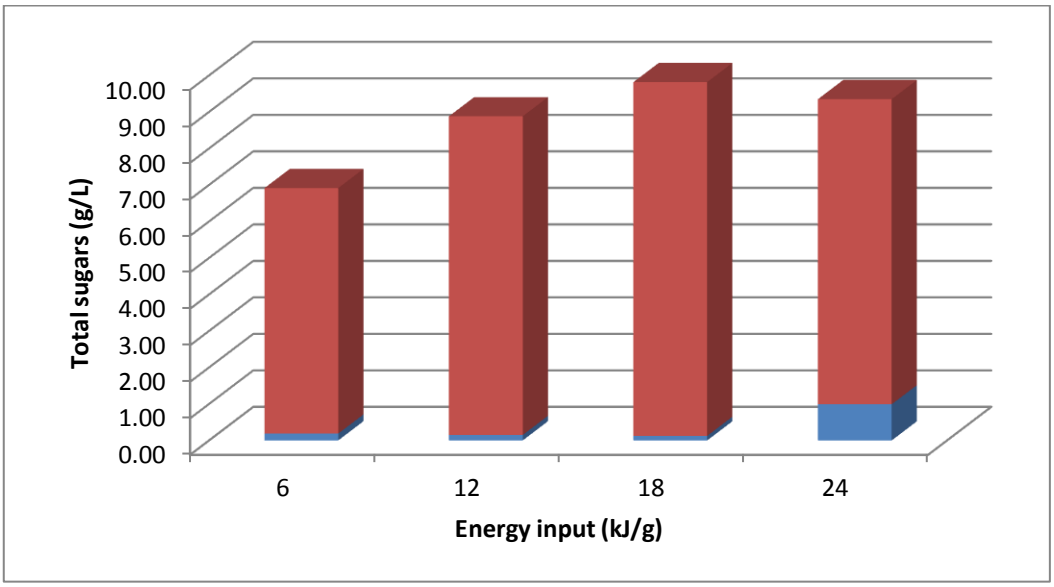

Figure E2.3: The effect of 5% $Ca(OH)_2$ on the type of sugars (g/L) liberated at 100W (■ pentoses, ■ hexoses).

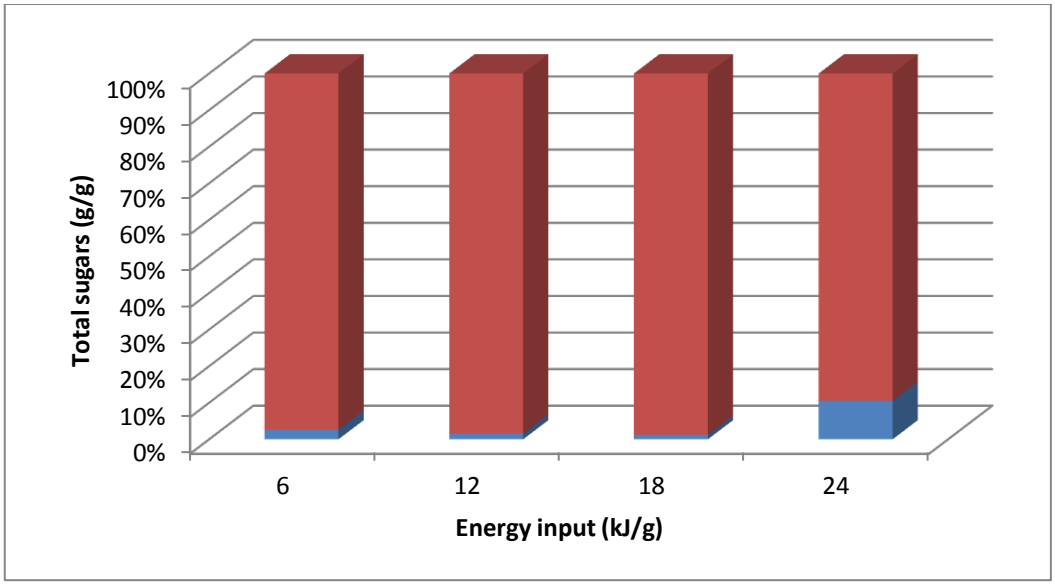

Figure E2.4: The effect of 5% $Ca(OH)_2$ on the type of sugars (%) liberated at 100W (■ pentoses, ■ hexoses).

Pretreatment with Sodium hydroxide (NaOH)

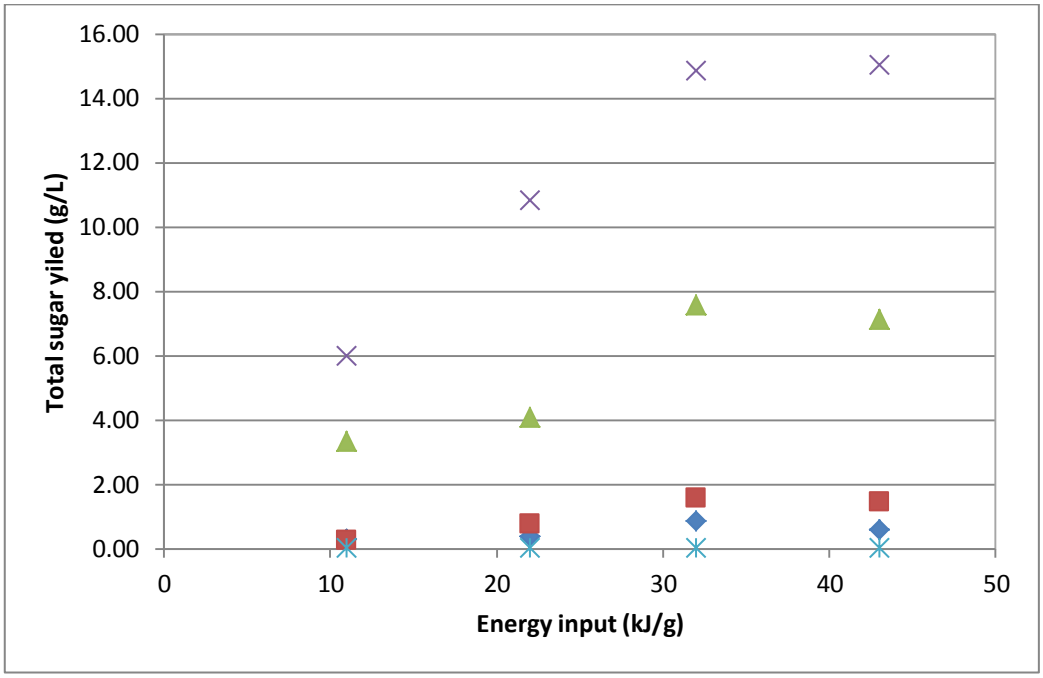

Figure E2.5: Total sugars (g/L) of microwave assisted pretreatment of NaOH at different concentrations at 180 W (× 5% NaOH, ▲ 3% NaOH, ■ 2% NaOH, ♦ 1% NaOH, Ж- Control).

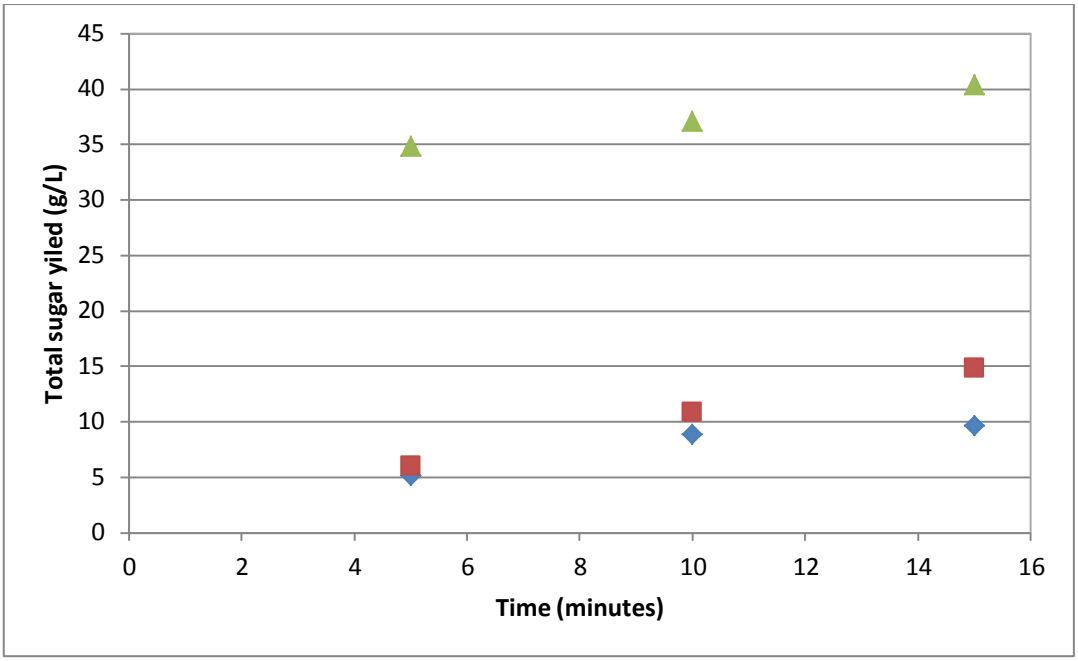

Figure E2.6: The effect of microwave irradiation power on total sugar yield (g/L) (♦-18 kJ.g^{-1}, ■-32.4 kJ.g^{-1}, ▲-54 kJ.g^{-1})

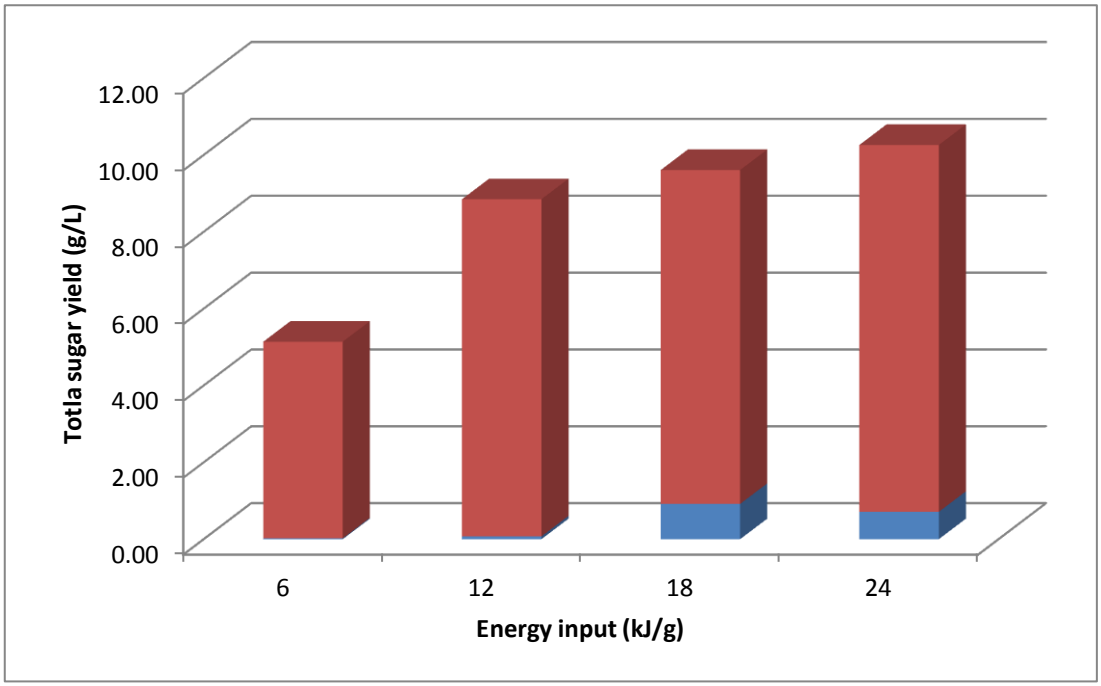

Figure E2.7: The effect of 5% NaOH on the type of sugars (g/L) liberated at 100W (■ pentoses, ■ hexoses).

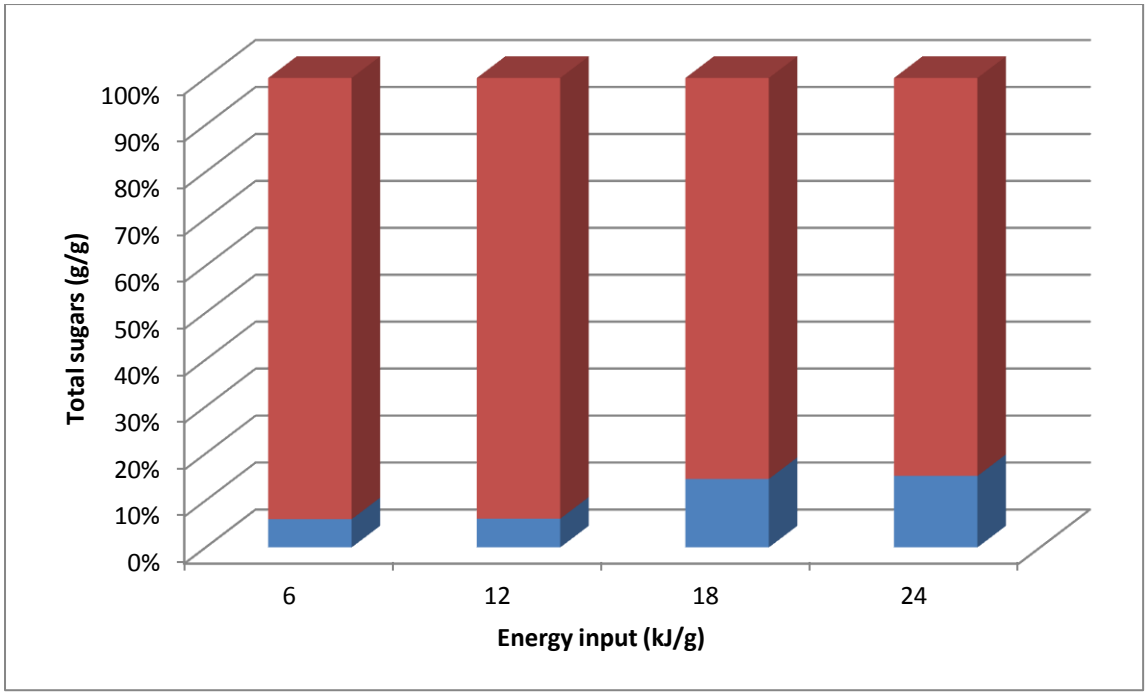

Figure E2.8: The effect of 5% NaOH on the type of sugars (%) liberated at 100W (■ pentoses, ■ hexoses).

Pretreatment with Potassium hydroxide (KOH)

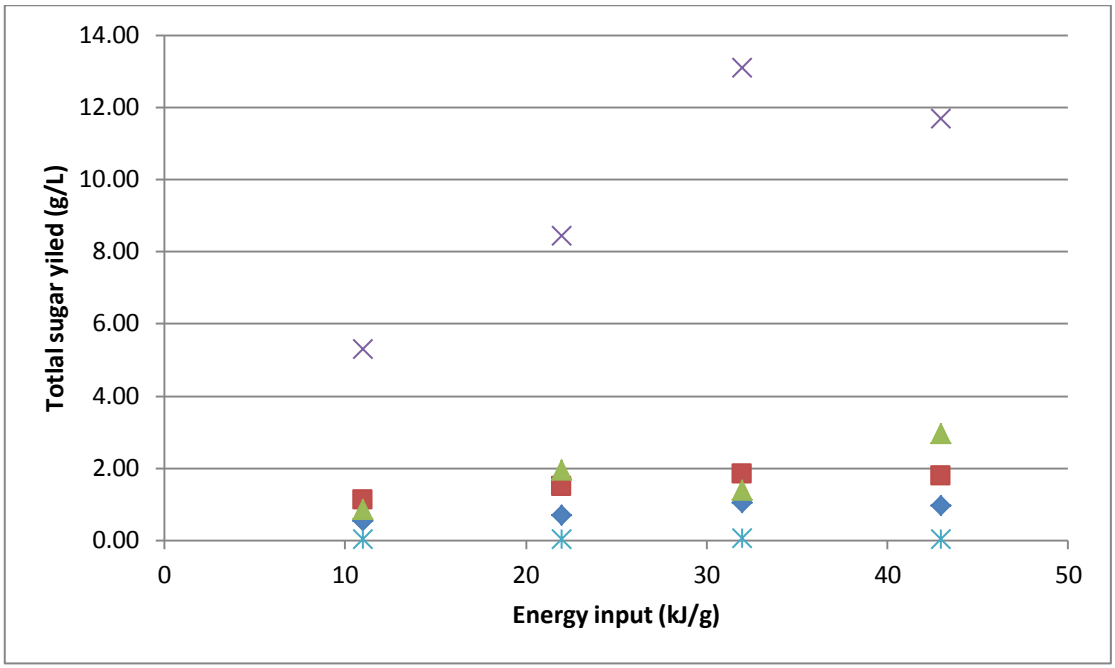

Figure E2.9: Total sugars (g/L) of microwave assisted pretreatment of KOH at different concentrations at 180 W (× 5% KOH, ▲ 3% KOH, ■ 2% KOH, ♦ 1% KOH, Ж- Control).

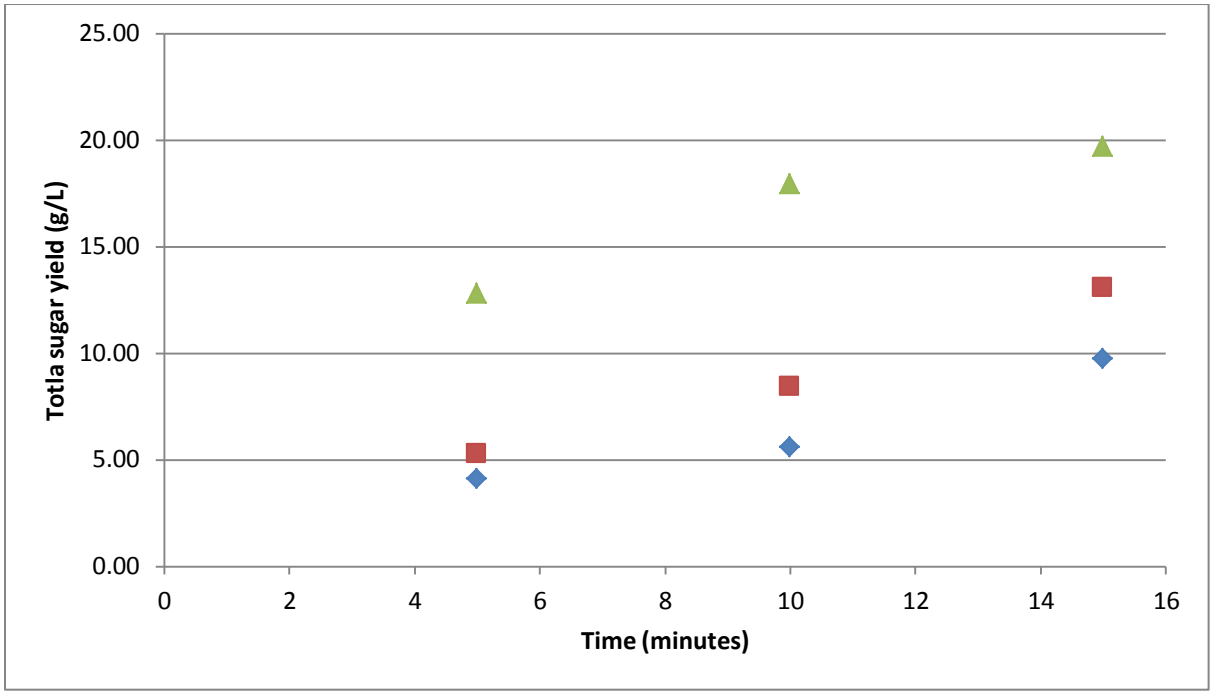

Figure E2.10: The effect of microwave irradiation power on total sugar yield (g/L) (♦-18 kJ.g^{-1}, ■-32.4 kJ.g^{-1}, ▲-54 kJ.g^{-1})

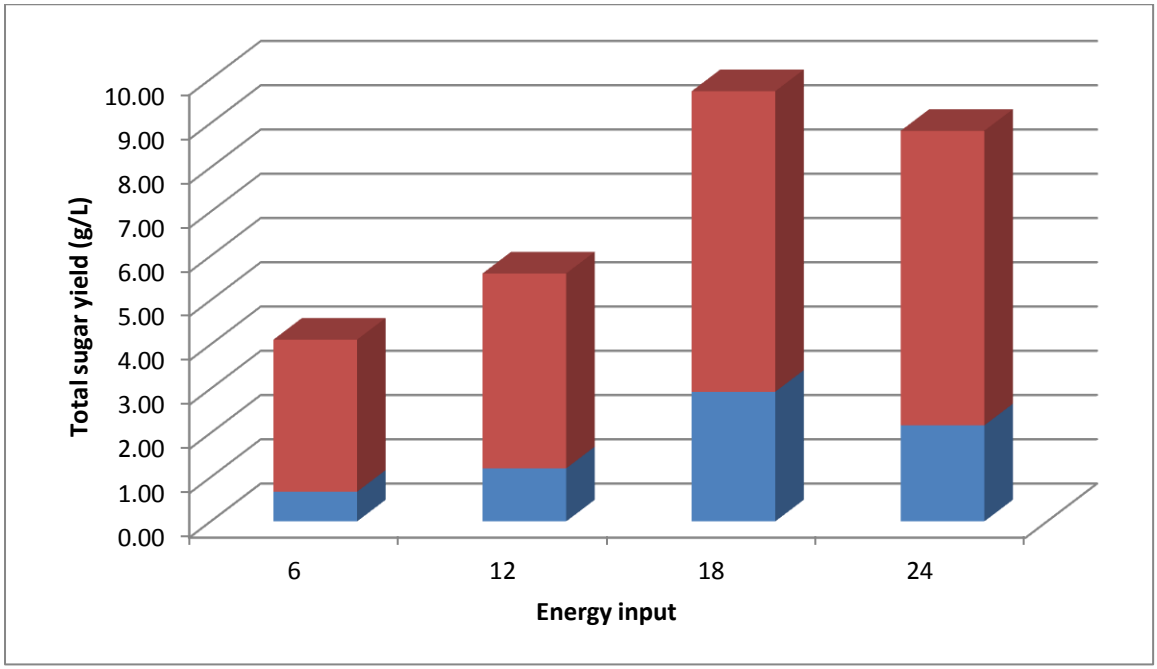

Figure E2.11: The effect of 5% KOH on the type of sugars (g/L) liberated at 100W (■ pentoses, ■ hexoses).

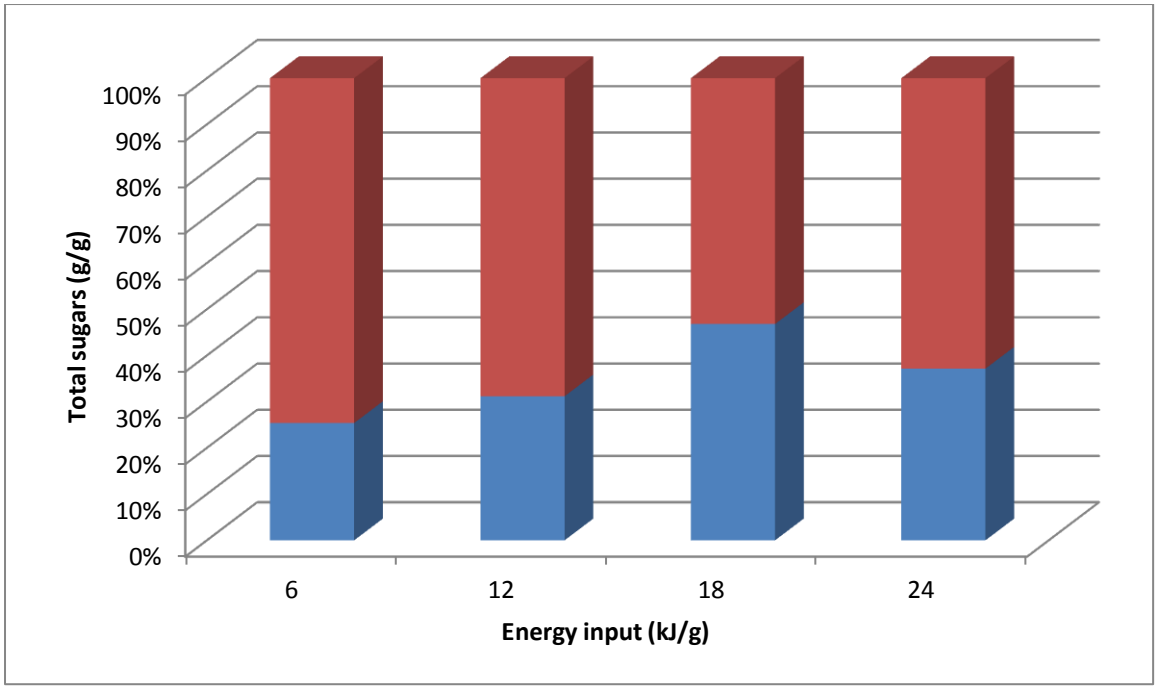

Figure E2.12: The effect of 5% KOH on the type of sugars (%) liberated at 100W (■ pentoses, ■ hexoses).

E3: Enzymatic Hydrolysis

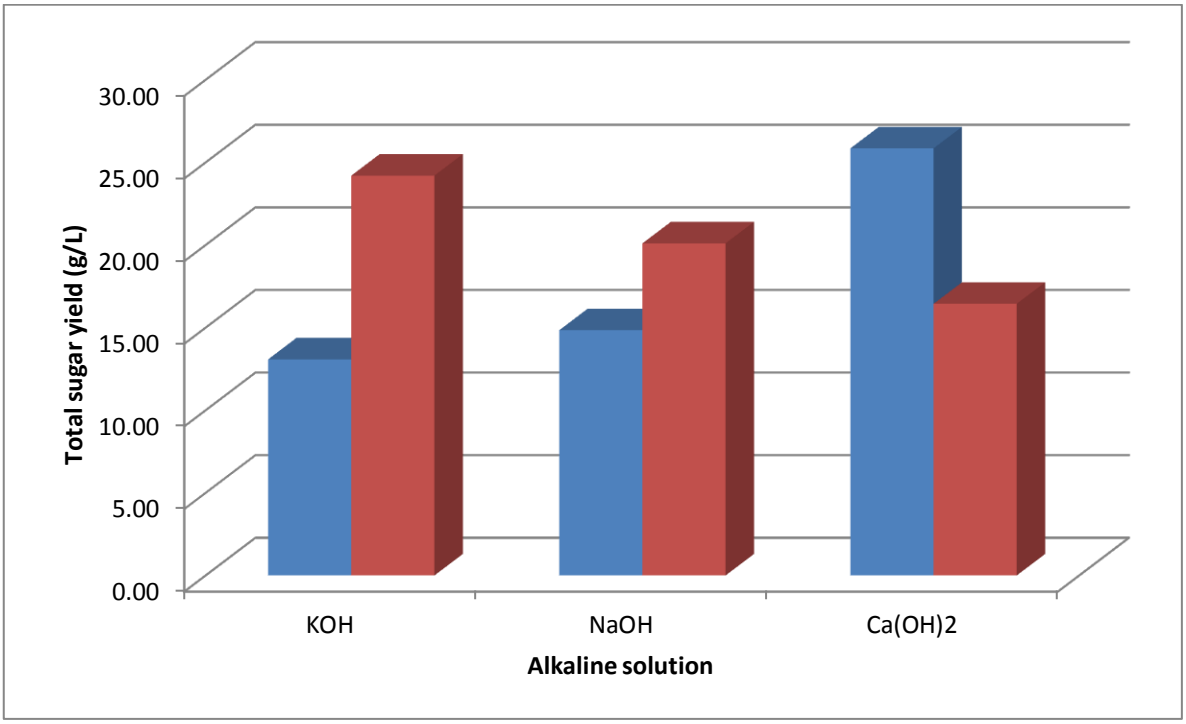

Figure E3.1: The effect of enzymatic hydrolysis of pretreated amaranth biomass pretreated with KOH, NaOH and $Ca(OH)_2$ on total sugar yield (■ pretreatment, ■ hydrolysis).

E4 Fermentation

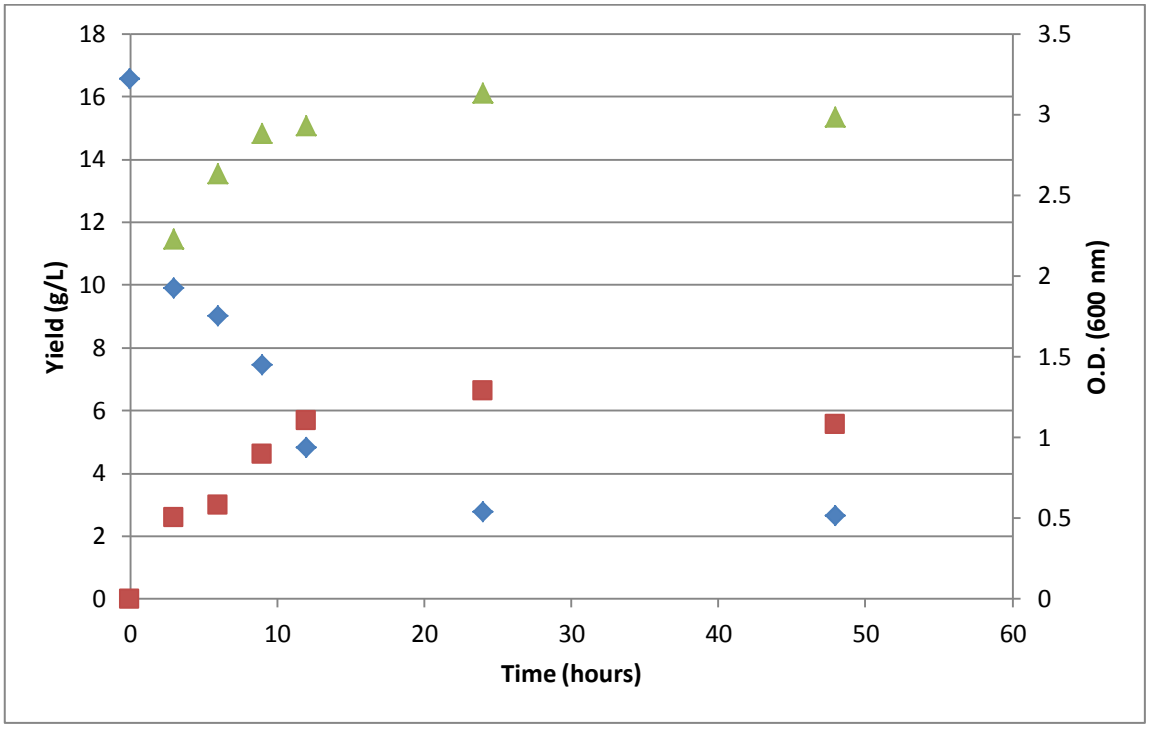

Figure E4.1: Effect of *S. cerevisiae* on concentration of ethanol (g/L) for amaranth biomass pretreated with 3% $Ca(OH)_2$ at 180W (■ –ethanol, ♦ total sugars, ▲ O.D.).

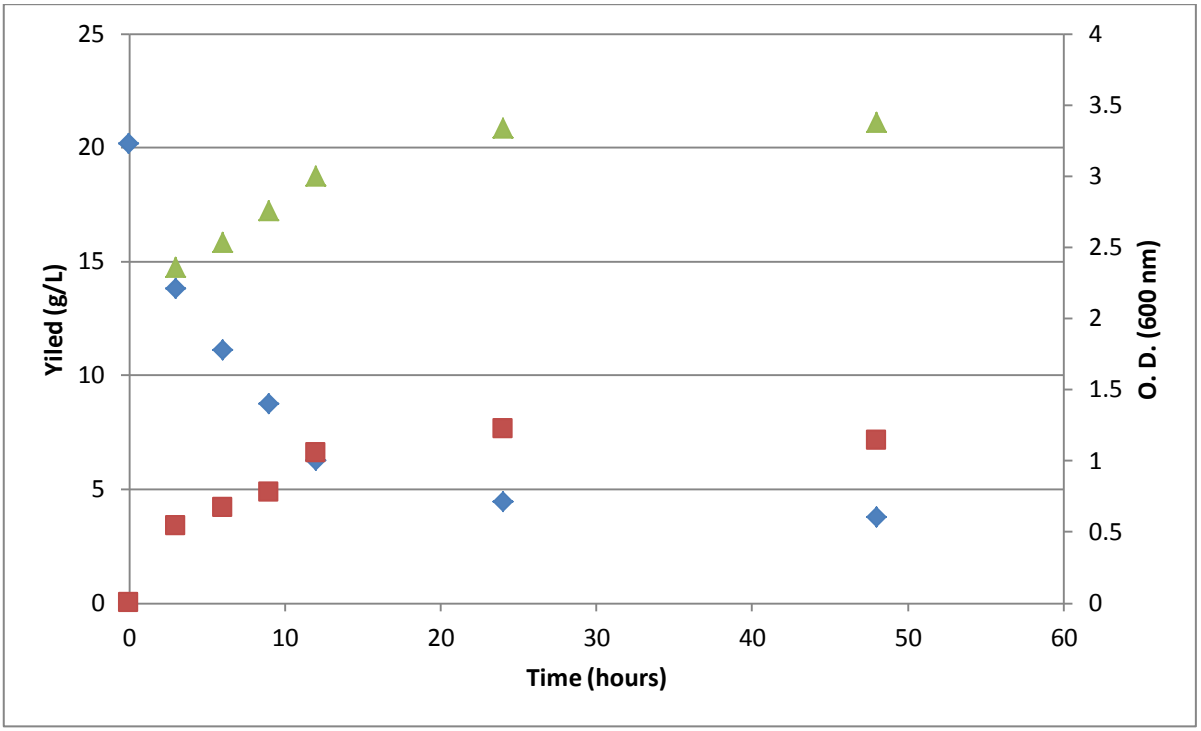

Figure E4.2: Effect of *S. cerevisiae* on concentration of ethanol (g/L) for amaranth biomass pretreated with 5% NaOH at 180W (■ –ethanol, ♦ total sugars, ▲ O.D.).

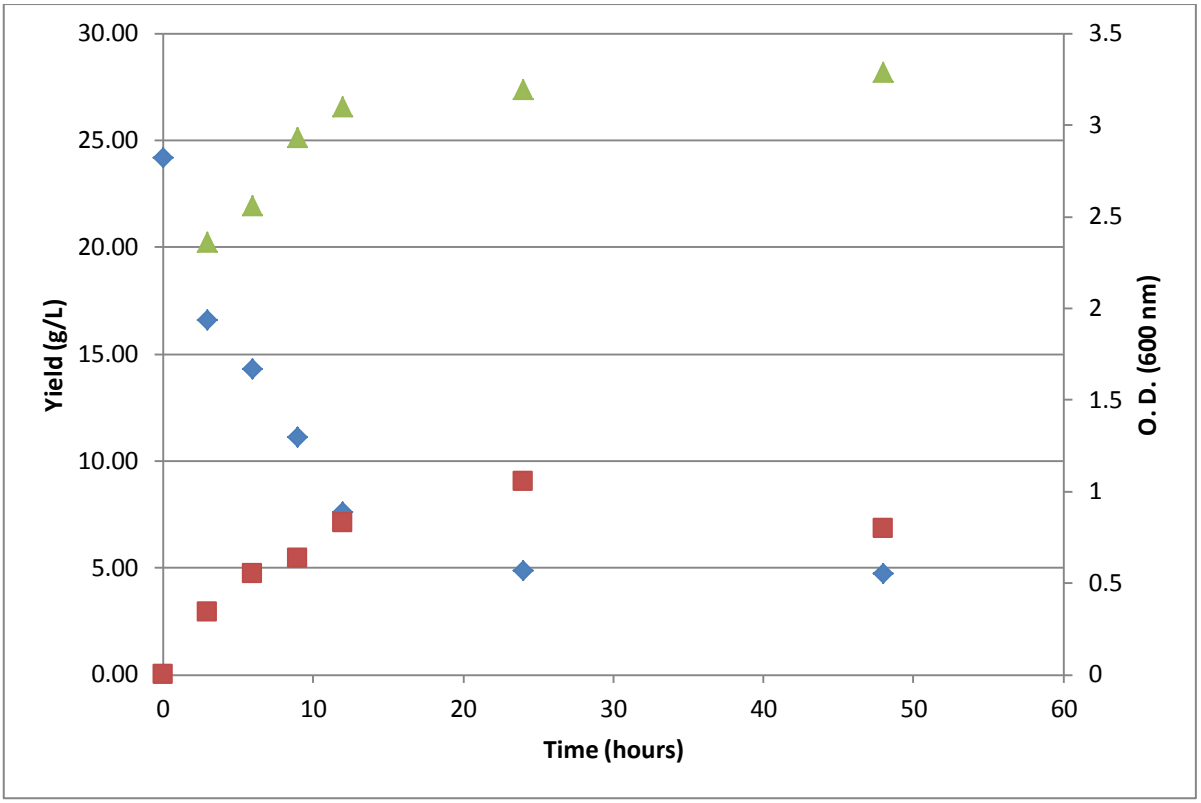

Figure E4.3: Effect of *S. cerevisiae* on concentration of ethanol (g/L) for amaranth biomass pretreated with 5% KOH at 180W (■ –ethanol, ♦ total sugars, ▲ O.D.).