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UNIVERSITY OF THE WITWATERSRAND

SCHOOL OF MOLECULAR AND CELL BIOLOGY

THE UTILISATION OF CELLULOSIC BIOMASS IN THE TREATMENT OF ACID MINE DRAINAGE AND THE SUBSEQUENT PRODUCTION OF FERMENTABLE SUGARS FOR BIOPROCESSING.

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment for the degree of Masters of Science.

BY

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2014

UNIVERSITY OF THE WITWATERSRAND

SCHOOL OF MOLECULAR AND CELL BIOLOGY

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Table of contents

Table of contents……iii

List of figures……viii

List of tables……ix

List of abbreviations……xi

Acknowledgements……xiii

Abstract……1

1.0 Introduction……2

1.1 Acid mine drainage……3

1.2 Sources of AMD……4

1.3.0 Formation AMD……5

1.3.1 Role of bacteria in acid mine drainage formation……6

1.4 Effect of AMD on aquatic resources……7

1.5 Major environmental incidents caused by AMD in South Africa……9

1.6 Control of AMD……13

1.7 Treatment of AMD……15

1.7.1 Active AMD treatment……16

1.7.1.1 Other active treatment methods……19

1.7.1.1.1 Iron exchange……19

1.7.1.1.2 Crystallisation……20

1.7.1.1.3 Adsorption……20

1.7.1.1.4 Reverse osmosis……..20

1.7.1.1.5 Electrochemical technology……21

1.7.1.2.1 Advantages of active treatment……21

1.7.1.2.2 Disadvantages of active treatment……21

1.7.2 Passive Treatment Systems……22

1.7.2.1 Aerobic wetlands……22

1.7.2.2 Anaerobic wetlands……23

1.7.2.3 Anoxic limestone drains……24

1.7.2.4 Limestone ponds……25

1.7.2.5 Bioreactors……25

1.7.3.1 Advantages of the passive treatment systems……27

1.7.3.2 Disadvantages of the passive treatment systems……27

1.7.3.3 Lignocellulose in AMD treatment……27

1.7.3.4 Lignocellulose filter systems in treating AMD……28

1.7.3.5 AMD treatment and sugar generation……29

2.0 Biomass and biofuels……30

2.1 Lignocellulose……31

2.2 Chemical composition and physical structure of lignocellulose……33

2.2.1 Cellulose……34

2.2.2 Hemicellulose……35

2.2.3 Lignin ……36

2.3 Cellulosic Bio-processes ……39

2.3.1 Physical pretreatment……43

2.3.1.1 Milling……43

2.3.1.2 Steam explosion ……43

2.3.1.3 Irradiation……44

2.3.2 Chemical pretreatment……44

2.3.2.1 Dilute acid treatment……45

2.3.2.2 Concentrated acid hydrolysis……46

2.3.2.3 Pretreatment of biomass under alkaline conditions……46

2.3.2.4 Substrate cleaning……47

2.3.3 Biological pretreatment……48

2.4 Enzymatic hydrolysis……49

2.4.1 Lignocellulose degrading enzymes ……50

2.4.2 Sources of lignocellulose degrading enzymes……52

2.5 Overview of ethanol fermentation……53

2.5.1 Ethanol fermentation from lignocellulosic biomass……55

2.5.1.1 Sequential hydrolysis and fermentation ……55

2.5.1.2 Direct microbial conversion……55

2.5.1.3 Simultaneous saccharification and fermentation……56

2.5.1.4 Simultaneous saccharification and co-fermentation……57

2.5.1.5 Ethanol from pentose (C5) sugars……57

2.5.1.6 Ethanol purification……59

2.5.1.7 Bioethanol application……59

2.6 Other products from lignocellulose metabolism……60

2.6.1 Lactic acid……61

2.6.2 Citric acid……63

2.6.3 Itaconic acid……64

2.7 The advantages of using lignocellulosic biomass as a passive AMD treatment system ……65

2.8 Benefits of biofuels……65

3.0 Linking bioremediation to bioprocessing……67

3.1 AIM……68

3.2 Hypotheses and questions……68

4.0 Research design and methods……69

4.1 Materials……69

4.1.1 Biomass……69

4.1.2 Enzymes……69

4.1.3 Equipment……70

4.2 Biomass pre-treatment and lignocelluloses digestion……70

4.2.1 Pre-treatment experiments and enzyme hydrolysis……70

5. Results……74

5.1 Dissolved iron and pH changes during treatment of ADM……74

5.2 The release of glucose during the treatment of AMD……79

5.3 Scanning electron microscope (SEM) analysis of water treated and AMD treated switch grass before and after enzymatic hydrolysis……..81

5.4 Enzymatic hydrolysis of AMD treated a switch grass……..82

5.5 Summary of results……85

6.0Discussion……..86

7.0 Conclusions……..89

References……91

Appendix……106

List of figures

Figure1.1: Fish kills like the one pictured above are typical of mine spills……..……9

Figure 1.2: Robinson Lake in Randfontein, the source of the Tweelospruit……..12

Figure 1.3: Sulfate salts encrusting the rocks and soil adjacent to Tweelospruit ……..12

Figure 1.4: Acid mine drainage water from abandoned mine. ……..13

Figure 1.5: active treatment, oxidation, dosing and sedimentation……..18

Figure 1.6: Aerobic wetlands for the passive treatment of mine waters ……..……24

Figure 2.1: Structure of single cellulose molecule. ……34

Figure 2.2: A schematic representation of hemicellulose……35

Figure 2.3: P-comaryl, coniferyl and sinapyl alcohol: dominant building block of the 3-dimensional……..36

Figure 2.4:lignin from gymnosperm showing the different linkages between phenyl-propane units……..37

Figure 2.5: The arrangement of lignin, cellulose and hemicellulose to form a micro fibril……..38

Figure 2.6: Schematic representation of effects of pretreatment on lignocellulosic biomass……41

Figure 2.7: Reaction pathway from cellulose to glucose……..52

Figure 2.8: Schematic representation of the fermentation process……..54

Figure 2.9a Heterolactic fermentation of pentoses ……..62

Figure 2.9b: Homolactic fermentation of glucose ……63

Figure 3.0: formula of itaconic acid ……65

Figure 4.1: Dried and milled switch grass……69

Figure 4.2: Dried switch grass cut into 2-5cm particle size……69

Figure 4.4: Switch grass at day 5 of treatment……71

Figure 4.5: Switch grass at 20 days of treatment……71

Figure 5.1: Changes in pH and iron concentration as effected by milled switch grass……74

Figure 5.2: Changes in pH and iron concentration as effected by switch grass (2-5cm)……75

Figure 5.3: Changes in pH and iron concentration as effected by sugar cane bagasse……75

Figure 5.4: Changes in pH in the control reactor……76

Figure 5.5: The release of glucose during the pretreatment of milled switch grass using AMD……..80

Figure 5.6: Scanning electron microscope images of switch grass……81

List of tables.

Table1.1: Chemical composition of AMD from an abandoned mine in Witbank coalfield. South Africa. ……10

Table 1.2. Chemical, physical and biological mechanisms for the treatment of AMD. Depending on site specific conditions, one or a combinations of these mechanisms may be suitable for AMD treatment ……16

Table 1.3. Neutralisation materials that can be used for treatment of AMD ……19

Table 1.4. Methods for passive treatment of AMD……26

Table 2.0: Main chemical compositions of several typical grass biomass feedstocks……33

Table 2.1: Effects of chemical compositions and physical structures on enzymatic digestibility (ED) of lignocellulosic biomass ……42

Table 4: Characteristics of simulated AMD……71

Table 5.1: The release of glucose and xylose obtained from the enzymatic digestion of water treated switch grass……83

Table 5.2: The release of glucose and xylose obtained from different enzyme concentrations on AMD treated biomass**……..**……84

Table 5.3: Summary of pH, %Fe and sugar production during pretreatment and after enzyme hydrolysis……..85

List of abbreviations.

AL	Aluminium
ALD	Anoxic limestone drains
AMD	Acid mine drainage
APS	Alkalinity producing systems
ASA	Accessible surface area
Co	Company
Cu	Copper
CM	Centimetre
CRI	Crystalline index
DMC	Direct microbial conversion
DSR	Dissimilatory sulfate reduction
DP	Degree of polymerisation
ED	Enzymatic digestion
EMP	Embden Meyerhof-Panas Pathway
FeS_2	Iron sulfide
Fe^{2+}	Iron (11) ions
Fe^{3+}	Iron (111) ions
$Fe(OH)_3$	Iron hydroxide
$FeSO_4.7H_2O$	Iron sulfate heptahydrate
G	gram
H^+	Hydrogen iron
HCl	Hydrochloric acid
H2O	Water
H_2SO_4	Sulfuric acid
HMF	Hydroxymethyl-furfural
HPLC	High performance liquid chromatography
Ind.	Industry
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO4	Dipotassium hydrogen phosphate
L	Litre
LDW	Limestone diversion wells
M	Molar
MCB	Molecular and Cell Biology
Mg	Milligram
Ml	Millilitre
MRS	Microbial reactor systems
NaOH	Sodium hydroxide
O_2	Oxygen
OLD	Oxic limestone drains
PJ	Peta joules
PK	Phosphokinase pathway
PRD	permeable reactive barriers
PSI	Pascals per square inch
RID	Refractive index

RPM	Revolutions per minute
SAMD	Synthetic acid mine drainage
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SHF	Simultaneous hydrolysis and fermentation
SLD	Slag leach beds
SG	Switch grass
SO_4^{2-}	Sulfate ion
Soln	Solution
SRB	Sulfate Reducing Bacteria
SSA	Specific surface area
SSCF	Simultaneous saccharification and co-fermentation
SSF	Simultaneous saccharification and fermentation
TDS	Total Dissolved Solids
USA	United States of America
UV-VIS	Ultraviolet-Visible
Vol	Volume

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Abstract.
Sugar cane bagasse and switch grass were used to investigate their potential in the remediation (decreasing metal ion concentration and increasing pH) of Acid Mine Drainage (AMD) and the possibility that the AMD residue sludge containing cellulose could be further hydrolysed using a commercial cellulase enzyme system to produce glucose for bioprocesses. In general both the feedstocks series appeared to increase pH and reduce dissolved iron concentration after being incubated with AMD for a period of 14 weeks at room temperature. The milled switch grass was shown to have a greater remediating effect on AMD, raising the pH from 2.11 to 5.46, and decreasing iron concentration from 500mg/l to 174mg/l, a decrease of 62%. The sugar cane bagasse was shown to have the least remedial effect, increasing pH from 2.11 to 2.38, and only reducing iron concentration by 30%. The 2-5cm switch grass raised the pH from 2.11 to 3.86, and the iron concentration was reduced from 500mg/l to 283mg/l, a 42% reduction. The milled grass series was chosen for further enzymatic hydrolysis. The milling reduced the size of the switch grass and destroyed the cell structure making it more accessible to AMD treatment. This also allowed the enzyme in the hydrolysis to penetrate to the fibres and reach the sugar oligomers. The sludge of the AMD treated switch grass was incubated with cellulases enzymes for 24 hours at 50°C, producing glucose concentration of up to 4,86mg/ml.

Literature review

1.0 Introduction

Mining activities tends to make a notable impact on the environment, the impacts varying depending on wether the mine is working or abandoned, the mining methods used and the geological conditions (Bell *et al*., 2001). To some extent climate conditions also play an important role, particularly temperature, rainfall and wind velocity (Johnson and Hallberg, 2005). Mining activity is one of the many causes of water pollution and creates a condition of imbalance between the land and water regime, in both quantitative and qualitative manners (Saharan *et al*., 1995).

Mining activities generate acid mine drainage (AMD) which is generally characterized by high concentration of dissolved metals, sulfate and low pH. Acidic drainage is not exclusively limited to mining activities, it can happen whenever the sulfide minerals are exposed to oxygen, producing acidic sulfate rich water. Such water pose an additional risk to the environment because it contain elevated amounts of metals (iron, aluminium, manganese and other heavy metals) and metalloids (such as arsenic). Releasing the AMD into rivers and dams will negatively affect aquatic life (Greben *et al*., 2009). AMD is also responsible for the degradation of soil quality and leaching of metals into ground water (Roman, 2004).

In South Africa, mining is a key economic driver. The existing and abandoned mining operations have led to the formation of AMD, which contaminates surrounding water. In South Africa AMD has been observed in various mining regions, including the Witwatersrand Gold fields, the Mpumalanga and the Kwazulu Natal Coalfields. Of concern is the Western basin, Eastern and Central basin in Gauteng were the situation is critical (Ramla, 2010).

The nature of the impact of AMD experienced in South Africa is illustrated by a case study of the Middleburg colliery near Witbank (Bell *et al*., 2001). The mine was decommissioned in 1947, and in 1996 was still discharging water with sulfate levels in excess of 1000mg/l and a pH<3. The water entered the

Blesbokspruit river and resulted in reducing the upstream from pH>7 to a pH of approximately 3.2 and sulfate concentration between 128 and 2250mg/l. Vegetation in the surrounding areas of the mine showed adverse effects. To prevent these adverse environmental effects of AMD on the environment, the AMD should be treated before being discharged into the water.

A number of treatment techniques have been developed to reduce acidity, and dissolved metals and sulfate concentration. Both physico-chemical and biological methods of treatment have been investigated (Roman, 2004). Some of the techniques are costly and difficult to implement, as a result successful implementation and treatment is not achieved (Ramla, 2012; Munali and Riwandi, 2010).

This study seeks to use locally available indigenous grass (switch grass) and sugarcane bagasse for the treatment of AMD, and the possibility that the lignocellulosic residue left after the treatment can be hydrolysed by enzymes to produce sugars for bioprocessing (Sheridan *et al*., 2013). The hypothesis put forward in this research is that the sulfuric acid in the AMD will react with the lignocellulose. The sulfuric acid will alter the morphological structure of the lignocellulosic material by hydrolysing the hemicellulose component making the lignocellulosic material amenable to enzymatic hydrolysis.

1.1 Acid Mine Drainage

Acid mine drainage refers to the outflow of acidic water from (usually abandoned) metal and coal mines. It is characterized by low pH and high concentrations of sulfate and heavy metals (Acid mine Drainage (AMD) South Africa, 2011). It is considered the most important mining industry related pollution problem (Rob and Robson, 1995; Johnson and Hallberg, 2005; Kuyacak *et al*, 2011). AMD occurs when sulfide ores are exposed to the atmosphere, which can be magnified through mining and milling activities, where oxidation reactions are initiated (Jennings *et al*., 2008; Zipper *et al.*, 2011; McCathy, 2011; Johnson and Hallberg, 2005). Mining increases the exposed surface of the ore-bearing rocks allowing acid generation beyond the natural buffering capacity of the host rock and water resources.

Since large masses of sulfide rocks are exposed during mining and milling, the surrounding environment can often not attenuate the resulting low pH. These conditions allow metals to be solubilised, the concentration of common metals such as Zn, Cu, Al and manganese increase in water with low pH (Jennings *et al*., 2008). The major sources of AMD include drainage from underground mine shafts, runoff from open mine pits and mine dumps and other places were earth has been disturbed such as construction sites. Acid mine drainage is generated through a combination of chemical and biological processes by which pyrite is converted to sulfates and iron oxyhydroxides (Smith, 1997). Iron sulfides are most common but other minerals may also produce AMD upon exposure to oxidizing conditions. The sulfide minerals oxidize in the presence of water and oxygen to form acidic sulfate rich drainage (Akcil and Koldas, 2006; Hanna *et al*., 1963).The oxidation of sulfide minerals and the subsequent acidity occurs in several reactions. The primary ingredients for acid generation are as follows: (1) Sulfide minerals, (2) water or humid atmosphere, and (3) an oxidant particularly oxygen from the atmosphere or from chemical sources. The metal content of the AMD is a result of the type and composition of material found in the mineral being oxidized (Akcil and Koldas, 2006; Kuyacak, 1999).

1.2 Sources of AMD

Burgers (2002), has listed the sources of AMD as follows:

a) Underground workings and exposed mining faces can result in a high concentration of salts, metals and acidity being released as a result of fluctuating water tables that cause the accumulation of salts during law water table and the mobilisation of those salts during high water table.
b) Open pits expose very large areas to the atmosphere and moisture. A fluctuating water table ensures that oxidised sulfate material is dissolved and the pyrite surface is continuously renewed and exposed to undergo further oxidation.

c) Seepage and runoff from waste rock poses a large problem since these piles are generally made up of coarse material, which results in high permeability for water and oxygen. These dumps have a tendency to build up oxidation products through evaporation and supersaturation processes, these oxidation products are released in large quantities during the wet period.

d) Process tailings stored as dumps are of finer particle size, which can restrict oxygen and moisture movement but may mean that they have a higher sulfate content. The larger surface area and greater reactivity as a result of smaller particle size, is offset by lower permeability. Tailings tend to produce AMD at a much slower rate than waste rock.

1.3. FORMATION OF AMD

Although a host of chemical processes contribute to AMD, Pyrite oxidation is by far the greatest contributor. The oxidation of iron sulfide, pyrite is the primary mechanism by which the acid is released into mine drainage (Ackil and Kodas, 2006; Neculita *et al.*, 2007; Sangita *et al.*, 2010). The process is initiated by the breakdown of pyrite in the presence of oxygen and water to yield ferrous iron sulfate and acidity (Sangita *et al*., 2010; Pradhan and Deshmukh, 2008). . A general equation for this process is (Equation 1):

$$2FeS_2(s) + 7O_2\,(g) + 2H_2O\,(l) = 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \qquad (1)$$

The oxidation of sulfide to sulfate solubilises the ferrous ion (Fe^{2+}), which is subsequently oxidised to ferric ion (Fe^{3+}), this occurs when there is sufficient oxygen dissolved in the water (Equation 2). This step is the rate determining step for the overall sequence. This reaction is greatly accelerated by a species of bacteria, *Thiobacillus ferroxidants* (Sangita *et al*., 2010)

$$4Fe^{2+}\,(aq) + O_2\,(g) + 4H^+ = 4Fe^{3+}\,(aq) + 2H_2O\,(l). \qquad (2)$$

Either of these reactions can occur spontaneously or can be catalysed by microorganisms that derive their energy from the oxidation reaction. Ferric ions produced can also oxidise additional pyrite into ferrous ions (Equation3):

FeS_2 (s) + $14Fe^{3+}$ (aq) + $8H_2O$ (l) = $15Fe^{2+}$ (aq) + $2SO_4^{2-}$ (aq) + $16H^+$ (aq). (3)

The net effect of these reactions is to release H^+, generating acidity, which lowers pH and maintains the solubility of the ferric ions (Blodau, 2006). The rates of these reactions are determined by several factors which include: pH, temperature, oxygen content of the gas phase, if saturation is less than 100%, oxygen concentration in the water phase, degree of saturation with water, chemical activity of Fe^{3+}, surface area of exposed metal sulfide, chemical activation energy required to initiate acid generation, and bacterial activity (Akcil and Kodas, 2006).

It is apparent that formation of AMD is complicated due to the number of factors that influence its production and hence it would be highly probable that the constituents of AMD vary from region to region. Since factors like number of microorganisms, weather, temperature and type of minerals vary from place to place, thus influencing the quality (pH and metal content) of AMD (Motsi, 2010).

1.3.1 Role of bacteria in acid mine drainage formation

Interaction of bacteria with sulfide minerals is a significant factor in the formation of AMD. *Thiobacillus ferroxidants* and *Thiobacillus thiooxidants* are ubiquitously present in sulfide bearing ore deposits, mine tailings and abandoned mines (Sangita *et al*., 2010). The *thiobacillus* species, except *Theobacillus ferroxidants*, are unable to oxidise iron but can oxidise sulfur compounds to sulfuric acid (H_2SO_4). *T. ferroxidants* has been the most documented microorganism having a catalytic role in the production of AMD (KuyuCak, 2002). According to Sangita *et al*., (2010) the bio-oxidation of sulfide minerals is

explained by direct and indirect mechanism. The direct mechanism occurs due to direct contact between bacteria and sulfide minerals by the following reaction:

$$FeS_2 + H_2O + 7/2O_2 \xrightarrow{\text{bacteria}} Fe^{2+} + 2SO_4^{2-} + 2H+ \quad (4)$$

The indirect mechanism takes place by the action of ferric iron produced by bacteria oxidation by the following reaction:

$$FeS_2 + 8H_2O + 14Fe^{3+} \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (5)$$

$$14Fe^{2+} + 3.5\ O_2 + 14H^+ \xrightarrow{\text{bacteria}} 14Fe^{3+} + 7H_2O \quad (6)$$

The overall reaction can be written as:

$$2FeS_2 + 15/2\ O_2 + H_2O \xrightarrow{\textit{T. ferroxidants}} Fe_2(SO_4)_3 + H_2SO_4 \quad (7)$$

This cycle is maintained as the ferrous ions are again biologically oxidised to ferric irons. *T. ferroxidants* is an acidophilic chemolithotroph that oxidises ferrous iron as its primary energy source. Since very little energy is generated in the oxidation of ferrous iron, these bacteria needs large amounts of iron to grow. Even small amounts of organisms are responsible for precipitating large amounts of iron (Taylor, 1996).

1.4 EFFECT OF AMD ON AQUATIC RESOURCES.

The creation of AMD results in release of metals into the surrounding environment. The metals will therefore become available to biological organisms. The metals thus enter the food chain through the aquatic system. In water, for example, when fish are exposed directly to metals through gills, impaired respiration may result from chronic and acute toxicity (Jennings *et al*., 2008). Dissolved metals also act as metabolic poisons, leading to the death of aquatic creatures (Figure 1.1). A common weathering product of sulfide oxidation is the formation of iron hydroxide ($Fe(OH)_3$), a red/orange colored precipitate also called yellow boy found in streams affected with AMD (Figure1.4). Iron hydroxides and

oxyhydroxides physically coat the surface of stream sediments and streambeds destroying habitat, diminishing availability of clean gravel for spawning, and reducing fish food such as benthic microinvertabrates. The precipitation of metals consumes oxygen, thereby reducing the oxygen content available to aquatic life (Motsi, 2010). The increased acidity caused by AMD has a range of negative effects depending on the severity of the pH change, many river systems and former mine sites are inhospitable to aquatic life with the exception of extremophilic bacteria. Heightened acidity reduces the ability of steams to buffer further chemical change (Jennings *et al*., 2008). If the pH falls below the tolerance range, death may occur due to respiratory or osmo-regulatory failure (Kimmel, 1983). Hydrogen ions maybe absorbed by the body cells displacing vital sodium ions (Morris *et al*., 1989), which are important for normal body operation. Sodium is important in nerve and muscle function and in regulating body fluids. The overall ecological effect is a reduction of habitat, an accumulation of toxic elements in the food chain and a food chain breakdown (Kimmel, 1983; Sangita *et al*, 2010). These combined effects may lead receiving streams to be devoid of living creatures (Kimmel, 1983; Burgers 2002).



Figure1.1: Fish kills like the one pictured above are typical of mine spills (image from Motsi, 2010)

1.5 Major environmental incidents caused by AMD in South Africa.

South Africa is blessed with the occurrence of many minerals often in large quantities, most notably gold as well as large reserves of coal. As such mining of these resources is one of the largest industries in the country. The current production of AMD is primarily as a result of current and historic coal and gold mine operations (Potgieter *et al*., 2006; McCarthy, 2011). The leaching and extraction of minerals from mines whether open pit or shaft often results in waste water and effluent (Akcil and Koldas, 2006), further because of high cost of treating AMD, a trend has developed in South Africa in which companies submit to closure of an AMD affected mine rather than incur costs associated with AMD treatment (Labuschagne, *et al*., 2008). Most of the mines on the East Rand, Central Rand and West Rand have been closed down. The AMD affected mine effluent emanating from mines on the East Rand drains into rivers such as the Blesbokspruit, Rietspruit and Natalspruit flowing into the Vaal River (Scott, 1995).

The West Rand and the Central Rand mines were built on the continental divide. The effluent emanating from these mines affect both the Vaal River to the south and the Limpopo River to the North (Coetzee *et al*., 2006). The AMD in the West Rand started to decant from defunct flooded underground mine workings in 2002 (ERMITE CONSORTIUM, 2004). The water in some of these areas is highly acidic. Bell et al., 2001 reported high pollution levels in the Middelburg colliery in Witbank. The pH values were as low as 2.0 in March 1991 and the sulfates were as high as 2897 in February 1994 (Table 1.1). The pH value of the water from Blesbokspruit in the East Rand varied from pH 2,6, near were the water issuing from the mine, to 3,2 downstream of the wetland, sulfate concentrations were between 128 and 2250mg/l (Bell *et al*., 2000).

Table 1.1: Chemical composition of AMD from an abandoned mine in Witbank coalfield. South Africa (from Bell *et al*., 2001).

Determinant	May 1990	March 1991	Feb 1994	August 1996	SA guidelines (1996)
pH	2,3	2,0	2,8	2,95	6-9
Sulfate mg/l	2361	1692	2897	1730	0-200

The Oliphant's river is one of the main river systems in South Africa and has been described as one of the most polluted rivers in Southern Africa, with lake Loskop acting as a repository for pollutants from the upper catchment of the Olifants River system (Grobler *et al.*, 1994). Over the past 15 years isolated incident of fish mortality have been recorded at different times in lake Loskop, with increasing frequency during the past few years (Driescher, 2008), and have coincided with crocodile mortalities.

The crocodile population in the Loskop has declined from approximately 30 animals to 6 in 2008 (Paton 2008). The crocodile mortality in the Loskop during this period of time was ascribed to pasteatisis, which is associated with the intake of rancid fish fat after a fish die off, which appears to have resulted from sporadic incidents of AMD flowing into the lake (Paton, 2008). The destruction of plant life around water bodies that contains high levels of sulfate is also witnessed in places such as Robinson Lake in Randfontein on the west rand (Figure 1.2). The rocks, soil and plants on the river banks are often encrusted with sulfates (Figure 1.3), (Durand, 2012).

High levels of metals such as: manganese, aluminium, cobalt, zinc, radium have also been recorded in the Tweelopiespruit and Rietspruit. These levels were above those recommended by the World Health Organisation and were deemed fatal to organisms including humans if consumed (Durand, 2012). In this area almost all plant life has been killed (Figure 1.2). Dissolved aluminium ions in particular, are regarded as a major cause of plant toxicity in acid soils. According to Bell *et al.,* (2001), other than species of algae, no aquatic life appears to exist in the seepage area, in the pollution control ponds or in the streams around Middelburg colliery in Witbank.

The structural stability of the dolomite region in Gauteng and the North West is also threatened by the sulfuric acid contained in the AMD. Dolomite and limestone are highly soluble in acid, posing the threat of excessive karstification in this area with the formation of subterranean cavities in dolomite (Davies and Mundalamo, 2010). As the water containing AMD flows through the Karst aquifers in the dolomite, fissures may widen and sinkholes may result when the surface collapses into the void below (Hodson *et al.*, 2001).



Figure 1.2: Robinson Lake in Randfontein, the source of the Tweelospruit, note the absence of riparian plants. Image adapted from Durand, 2012.



Figure 1.3: Sulfate salts encrusting the rocks and soil adjacent to Tweelospruit. Image adapted from Durand, 2012.



Figure 1.4: Acid mine drainage water from abandoned mine. The orange coloration is due to the iron (Fe) that has been mobilised by the sulfur oxidation process.

1.6 Control of AMD generation.

The treatment of AMD usually cost more than the control of AMD, which could be practical at the early stage of the mining and may be required for many years after mining has ceased (Kuyucak, 2012). Since both oxygen and water are required for the formation of AMD, excluding either or both of them should possibly prevent or minimize AMD production (Johnson and Hallberg, 2005; Kuyucak, 2012). Water can be excluded by controlling its migration, diverting the water flowing towards the site of pollution, preventing ground water seepage into the affected area and proper management of acid generating wastes (Sangita *et al*., 2010). With the help of properly designed ditches, movement of contaminated water or clean water may be controlled. In theory, ground water migration could be controlled using

some interceptor structure such as grout curtains and slurry walls, as well as diversion ditches (Kuyucak, 2012).

The flooding and sealing of abandoned deep mines, also helps in preventing the formation of AMD, They serve to deprive the pyrite oxygen which is necessary for its formation. Atmospheric oxygen is prevented from entering by sealing the mine (Johnson and Hallsberg, 2005). Metals such as copper, lead and zinc are transported as dissolved oxide or sulfate complexes in acid water. In a flooded mine, dissolved oxygen that is consumed cannot be replaced by atmospheric oxygen. As this process continues the oxidation-reduction potential decreases and sulfide begins to precipitate. A reducing agent such as sulfur or organic carbon is needed to promote the precipitation of sulfides (Metesh *et al*., 1988). Another reliable method of preventing AMD is to submerge the waste rock or tailings under water. This will minimize the transport of oxygen and therefore limit or even prevent acid generation. The effectiveness of this method can be augmented if a thin layer of oily substance can be sprayed and maintained on the water surface (Saharan *et al*., 1995).

Another suggested method of control is to blend acid generating and acid neutralising material, producing benign composts. Johnson and Hallsberg (2005) suggested the use of solid phase phosphates (such as apatite) to pyritic mine waste in order to precipitate Fe^{3+} as ferric phosphate, thereby reducing its potential as an oxidant to pyrite oxidation.

Bactericides have also been used to reduce biological activity associated with oxidation of sulfide and the formation of AMD. Biocides are used to inhibit the activities of bacteria in mineral spoils and tailings (Motsi, 2010). Biocides are anionic surfactants such as sodium dodecyl sulfate (SDS), which is highly toxic to sulfide oxidizing bacteria. The anionic detergents are the most economical inhibitors of *T. ferrooxidants* (Saharan *et al*, 1995). The drawback for this method is that it cannot be applied in running water because most biocides are water soluble and in running streams they are washed away. If acid

formation cannot be prevented there are several treatment options which may be carried. These are briefly discussed in the following section.

1.7 TREATMENT OF AMD.

The overall goal of managing environmental impacts in South Africa is to implement mitigating measures that minimize residual impact of mining. Various strategies for AMD treatment and mitigation have been proposed including primary prevention (the prevention of acid producing processes), secondary control (the prevention of acid migrating after formation) and tertiary control (the collection and treatment of effluent). Primary prevention is not always feasible as the prediction of the potential of a process to create AMD is exceedingly challenging and costly. Furthermore this could vary from site to site and between mines as the AMD content differ. Secondary control is often not feasible as there is no standardised method of ranking, measuring, and reducing AMD. Tertiary control is typically conducted by a number of methods including (but not limited to) lime addition, Gypsum Cation-Anion Exchange (Sheridan *et al*., 2013).

To avoid significant environmental impacts, contaminated water must be collected and treated to remove metals and to increase pH before being discharged into the environment. There are a wide variety of conventional treatment methods for mine water effluents due to a variety of mine waters encountered in nature. Mine water treatment can be achieved through active, semi-passive or passive methods (Greben *et al*., 2009). Passive and semi-passive systems are often used, where active mining has stopped but where mine water is still generated (Pulles, 2000). Active treatment processes are mainly used in mines which are still in operation (Skousen, 1998; Brown *et al*., 2002). A broad range of passive and active treatment approaches are available for dealing with AMD. General treatment mechanisms which incorporate chemical and/or physical and/or biological processes are shown in Table 1.2 (Taylor *et al*., 2005).

Table 1.2. Chemical, physical and biological mechanisms for the treatment of AMD. Depending on site specific conditions, one or a combinations of these mechanisms may be suitable for AMD treatment. Adapted from Taylor *et al*., 2005.

AMD Treatment Mechanisms	
pH control	Oxidation
Adsorption	Electrochemical
Absorption	Sedimentation
Complexion	Flocculation
Chelation	Ion exchange
Biological mediation	Crystallisation
Reduction	

Neutralisation (pH control) is the AMD treatment mechanism for both passive and active systems. By increasing pH to create alkaline conditions, the solubility of most metals is significantly decreased by precipitation (Taylor *et al*., 2005)

1.7.1 Active AMD treatment.

The most widespread method used to mitigate acidic effluents is an active treatment process involving addition of a chemical-neutralising agent (Coulton *et al*., 2003b;). Addition of an alkaline material to AMD will raise pH, accelerate the rate of chemical oxidation of ferrous iron and causes many of the

metals present in solution to precipitate as hydroxides and carbonates (Sangita, 2010; Metesh *et al*., 1988). The result is the production of an iron rich sludge that may also contain various other metals, depending on the chemistry of the mine water treated (Johnson and Hallberg, 2005). Various neutralising reagents have been used, including lime, calcium carbonate, sodium carbonate, sodium hydroxide and magnesium oxide and hydroxide (Table 1.3). Neutralisation occur through dissolution of carbon dioxide from water (Sangita *et al*., 2010). Although active chemical treatment can provide effective remediation of AMD, it has the disadvantage of high operating costs and problems with disposal of the bulky sludge that is produced (Johnson and Hallberg, 2005, Cole *et al*., 1977, Neculita *et al*., 2007).

Active treatment can be divided into two main categories: (1) fixed plant and (2) *in situ*. The first category comprises conventional active treatment plants that are fixed in location and typically require pumping of AMD to the plant (Figure 1.5), reagent addition and mixing in one or more reactor tanks, collection/disposal of treatment sludge, and discharge of treated water*. In-situ* active treatment use portable land based or water based systems to conduct treatment within or adjacent to an affected water body (Taylor *et al*., 2005). The benefit of these systems is that they occupy a small area and they are effective and are a proven technology. Large volumes of water can be processed and the quality of water is unaffected by variations in temperature and metal contaminants (Burgers, 2002)

Of the broad range of treatment approaches available for dealing with AMD (Table 1.2), active systems (*in-situ* and fixed plants) utilise the following key chemical and physical processes:

a) pH control or precipitation
b) Electrochemical concentration
c) Biological mediation/ redox control (sulfate) reduction.
d) Ion exchange/ absorption or adsorption / flocculation and filtration

e) Crystallisation.



Figure 1.5: active treatment, oxidation, dosing and sedimentation.

Table 1.3. Neutralisation materials that can be used for treatment of AMD. Adapted from Taylor *et al.,* 2005.

Neutralisation Material		
Limestone ($CaCO_3$)	Lime kiln dust (Ca0, $CaCO_3$)	Ammonia (NH_3)
Quicklime (CaO)	Fly ash (Ca, Mg, Na and K oxides and hydroxides)	Potassium hydroxide (KOH)
Hydrated lime ($Ca(OH)_2$)	Fluidised bed ash (Ca, Mg, Na and K oxides and hydroxides	Calcium peroxide or Calcium dioxide (CaO_2)
Dolomite ($CaMg(CO_3)_2$)	Sodium carbonate (Na_2CO_3)	Cement kiln dust (CaO, $CaCO_3$)
Magnisite ($MgCO_3$)	Sodium hydroxide (NaOH)	Barium carbonate (BaCO3)
Caustic magnesia (MgO)	Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$	Barium Hydroxide($Ba(OH)_2$)

1.7.1.1 Other active treatment methods.

The other active treatment methods which may be employed for treating AMD are:

1.7.1.1.1 Iron exchange.

Ion exchange is the exchange of ions between a liquid phase and a porous solid, which may be synthetic or natural. Toxic metals can substitute for harmless ions. Ion exchange is able to remove base metals from AMD like zinc and copper and also raise the pH of AMD by adsorbing H^+ irons (Motsi, 2010; Taylor *et al*., 2005). The economic viability is limited due to the costs involved (Johnson and Hallberg., 2005).

1.7.1.1.2 Crystallisation.

Crystallisation is a treatment process that may be used to decrease sulfate concentration in AMD. The effluent AMD must be pretreated to adjust the pH and decrease metal concentrations prior to commencing the crystallisation process (Taylor *et al*., 2005). The principal of the process is as follows: when a solution containing dissolved contaminants is slowly frozen, ice crystals form and rise to the surface, while contaminants become concentrated in the remaining solution and eventually crystalize out at the eutectic temperature. The crystals can be separated from the major liquor, washed and melted to yield a nearly pure water (Lewis *et al*., 2010).

1.7.1.1.3 Adsorption.

Adsorption involves the movement or diffusion of solute molecules (adsorbate) from a bulk fluid to the surface of a solid (adsorbent) (Richardson *et al.*, 2002). There are two main types of adsorption processes; these are physical adsorption and chemisorption. Physical adsorption occurs when a solute is loosely bound to the solid surface via weak van de Waal forces or dipole interactions. Chemisorption involves the formation of stronger bonds between adsorbate and adsorbent. Some materials used as adsorbent include biomass, blast furnace slag, fly ash, clay, bark, tea leaves and natural zeolites (Motsi, 2010).

1.7.1.1.4 Reverse osmosis.

This process involves the use of semi permeable membrane to treat AMD. Pressure is applied on AMD (which is a more concentrated solution) and is forced through a membrane into a more dilute solution. The semi permeable membrane only allows the passage of solvent not solute. This leaves a more concentrated solution on the AMD side of the membrane (Motsi, 2010). The high costs involved in setting and operating this system are the major drawback.

1.7.1.1.5 Electrochemical technology

This involves the use of electrical energy to drive unfavourable reactions to extract metals from AMD. One of the main problem is its constant electrical supply (McGinness, 1999; Taylor *et al*., 2005)

Some of the treatment methods above, such as electrolysis and reverse osmosis are very expensive and are rarely used.

1.7.1.2.1 Advantages of active treatment (Sangita *et al*., 2010).

1) Effective and fast removal of metals

2) Frequent process monitoring

3) Precise process control

4) Can be accommodated at small sites.

1.7.1.2.2 Disadvantages of active treatment (Johnson and Hallberg., 2005).

Active treatment systems have a number of draw backs. These includes:

a) High capital costs

b) Chemicals used are expensive

c) Operational costs are high

d) Disposal of precipitated metal sludge.

1.7.2 Passive Treatment Systems.

Passive treatment systems offer low-cost, low-maintenance solution to AMD problems. While they may not always provide effluent that meets water quality regulations, they will improve the quality, which is a main goal of treating AMD from abandoned mines. Passive treatment systems for acid mine drainage are intended to renovate and improve the quality of water that passes through them. These systems are modelled after wetlands and natural processes (Zipper *et a*l., 2011). They provide a controlled environment in which natural, physical, chemical and biological reactions that help in the treatment of AMD can occur. The physical processes, such as filtration and sedimentation, are important in removing particulate metals. The biological and chemical processes mainly remove dissolved metals (Metesh *et al.*, 1988). In a constructed wetland, influent AMD drains by gravity through the wetland, progressively undergoing metal removal and neutralisation. The wetland plants are able remove metals from acid mine drainage by adsorption (especially iron exchange), consumption (plant uptake) and filtration (Sangita *et al*., 2010), while pH neutralisation is primarily by the activity of sulfate reducing bacteria (SRB), or the increase in alkalinity from the chemical and microbial reactions including limestone dissolution (MEND report 3.14.1., 1999). There are several systems used in passive AMD treatment, each type may be used on its own, or more than one may be used in sequence to optimize treatment of difficult effluent (Ford, 2003).

1.7.2.1 Aerobic wetlands

Aerobic wetlands (Figure 1.6) are the simplest type of passive treatment system but are limited to the types of waters they can treat. Aerobic wetlands are used to treat mildly acidic or net-alkaline water containing elevated iron concentration (Sangita *et al*, 2010). They have limited capacity to neutralise acidity. Their primary function is to allow aeration to mine waters flowing among the vegetation, allowing dissolved iron to oxidise, and to provide residence time where the water is slowed down for

iron oxide products to precipitate (Zipper *et al*., 2011; Taylor *et al*., 2005; Metesh *et al*., 1988). The reaction of oxygen with pyrite in the presence of water will reduce the pH of the wetland and can harm some plants that do not have a higher tolerance levels for pH changes. The best type of plants for aerobic wetlands are common cattail (*Typha latifolia*) and common reed (*Phragmites australis*) because of their ability to add oxygen to the soil through the root system which helps the oxidation process (Sadak, 2008).

1.7.2.2 Anaerobic wetlands.

Aerobic wetlands may be modified to allow these systems to add alkalinity and effectively treat net acid water. This include the addition of a bed of limestone beneath or mixed with an organic substrate, which encourage generation of alkali as bicarbonate, increasing the pH. The limestone is placed so that water must move through organic substrate prior to contacting the limestone, which allows bacteria in the organic material to remove oxygen from the percolating water. These systems are called anaerobic wetlands, they are capable of removing acid-soluble metals, especially iron and aluminium, and producing alkalinity (zipper *et al*., 2011, Metesh *et al*., 1988). Anaerobic wetlands neutralise AMD by encouraging the generation of bicarbonate alkalinity (HCO^{3-}) by both anaerobic microbial sulfate reduction (Equation 8), with CH_2O representing biodegradable organic compounds and limestone dissolution (Equation 9) (Motsi, 2010).

$$2CH_2O + SO_4^{2-} \longrightarrow H_2S + 2HCO^{3-} \qquad (8)$$

$$CaCO_3 + H+ \longrightarrow Ca_{2+} + HCO^{3-} \qquad (9)$$

Bicarbonate then increases the pH of the AMD (Equation 10), this allows the precipitation of acid soluble metals such as iron.

$HCO^{3-} + H^{+} \longrightarrow H_2O + CO_2$ (10)

In order for the wetlands to continue remediating environments affected by AMD there is need for the maintenance of the wetlands. The precipitates that form need to be disposed properly before the wetland reaches its maximum holding capacity and the plant life needs to be maintained to prevent overgrowth (Sadak, 2008).



Figure 1.6 Aerobic wetlands for the passive treatment of mine waters. Image from Taylor et al, 2005.

1.7.2.3 Anoxic Limestone Drains

Anoxic Limestone Drains (ALDS) can also be used to treat acidic water. ALDs are trenches filled with limestone through which acidic water is directed so the limestone can produce alkalinity via dissolution (Metesh, 1988). It allows the development of high CO_2 partial pressures which can produce high alkalinity concentrations beyond that possible under normal atmospheric conditions (MEND report 3.14.1., 1999). ALDS are capped with clay or compacted soil to prevent AMD contact with oxygen. The effluent is held in a settling pond to allow pH adjustment and metal precipitation prior to being discharged to natural water courses (Zipper *et al*., 2011; Taylor *et al*., 2005). ALD's are most commonly

used to treat seeps, but may be used for other effluent streams if special measures are used to avoid introduction of oxygen, such as routing the effluent through a pipe line (MEND report 3.14.1., 1999). ALDs are not appropriate for waters containing aluminium or ferric (Fe^{3+}) irons because these metals will form solids within the limestone aggregate, decrease its permeability, and eventually plug the system. For anoxic water containing iron and manganese, the ALD is the most effective and least costly method available for treating acidity (Hedin *et al*., 2013).

1.7.2.4 Limestone ponds

Limestone ponds are also used for the treatment of AMD. They provide a solution for places that have room to treat the AMD as the water comes from a seep or spring (Skousen *et al*, 1996a). Limestone is placed at the bottom of the pond and the water flows up through the limestone. The pond size and design are based on topography of the area and the water that emanates from the ground. The pond should retain the water for 1 to 2 days to enable the limestone to dissolve and to keep the seep and the limestone underwater (Metesh *et al*, 1988).

1.7.2.5 Bioreactors

Another potentially promising option is the use of bioreactors which utilize sulfate reducing bacteria to precipitate out dissolved metals (Wildeman *et al*., 1991). According to literature (Lindsay *et al*., 2011), sulfate reducing bacteria (SRB) form the catalyst in the dissimilatory sulfate reduction (DSR) process, and consuming organic carbon under strictly anaerobic conditions. Microbial bioreactors contain a biodegradable substrate (usually agricultural products such as mushroom compost or straw) which supports the microorganisms, which in turn treat AMD (MEND 3.14.1., 1999). The biodegradable substrates are used as nutrients, these are first metabolised and organic acids are produced which become available to SRB. The microbial activity within the bioreactor can be supplemented with

inorganic chemical reactions, such as pH neutralisation via limestone dissolution or other neutralising agents (Kuyucak, 2002). The bioreactors rely on several microbial reactions, which require different levels of oxygen to treat AMD.

Table 1.4 lists many of the passive treatment methods in use at present, including some new and emerging technologies.

Table 1.4. Methods for passive treatment of AMD. Table from Taylor et al., 2005)

Passive Treatment Methods	
Open/Oxic limestone drains (OLD)	Alkalinity producing system (APS)
Limestone diversion wells (LWD)	Permeable reactive barriers (PRB)
Anoxic limestone drains (ALD)	Microbial reactor systems
Pyrolusite limestone beds	Sulfide passive compounds
Aerobic and anaerobic wetlands	Alkalinity producing covers
Reverse alkalinity producing systems	Electrochemical covers
Vertical flow wetlands	Slag leach beds (SLD)

One drawback of the passive treatment systems is that many are carbonate based, this limit the number of metals which are precipitated due to maximum pH limitations, for example manganese is not completely removed (Taylor *et al*, 2005).

1.7.3.1 Advantages of the passive treatment systems as listed by Greben *et al*., 2000:

a) They can be used for more than 10 years with minimal requirement for operator intervention and costly maintenance.

b) Constructed wetlands provide wildlife habitat and have substantial social and ecological value.

c) Low capital and operational costs.

d) Use of non-hazardous material.

e) Can be integrated with surrounding systems.

1.7.3.2 Disadvantages of the passive treatment systems:

a) High surface area of land is needed.

b) Precise control of treatment effluent quality is not feasible since there is no day to day supervision.

1.7.3.3 Lignocellulose in AMD treatment

Lignocellulosic material have been investigated extensively in the remediation of AMD. Lignocellulose material have been used in biological treatment systems, such as constructed wetlands and bioreactors. The biological approaches involve the use of organic materials and anaerobic sulfate reducing bacteria (SRB), which reduce sulfate to sulfide by oxidising the organic source (Munawar and Riwandi, 2010) The sulfate reduction into sulfide is described in the following reaction:

$SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2HCO^{3-}$ (11)

CH_2O represent a simple organic carbon. Sulfate reduction reactions consume sulfate, produce hydrogen sulfide, and result in increasing alkalinity and pH. The released bicarbonate neutralises the acidity,

increases the alkalinity of AMD, and enhances precipitation of metal carbonate minerals. In the presents of metal ions as generally found in AMD, the released hydrogen sulfide will react to form insoluble metal sulfides, removing sulfates from water (Munawar and Riwandi, 2010).

Acid mine drainage generally contains relatively low concentrations of dissolved organic carbon (Kolmert and Johnson, 2001). Therefore availabity of carbon from an additional organic source is the most critical limiting factor for microbial activity (Gibert *et al*., 2004; Zagury *et al*., 2006). Different types of lignocellulose have been tested including hay and straw, spent mushroom compost, sawdust, oak chips, paper and peat (Munawar and Riwandi, 2010; Roman, 2004; Ramla, 2012).

1.7.3.4 Lignocellulosic filter systems in treating AMD.

The lignocellulosic fibres can be used as filters. Lignocellulosic materials are very porous and have a very high free surface to volume ratio that allows access of aqueous solutions to the cell wall component. Lignocellulosics are both hydrophilic (have an affinity for water) and hygroscopic (have the ability to adsorb water). Water is able to penetrate the non-crystalline portion of the cellulose and all of the hemicellulose and lignin. Thus through a combination of adsorption and sorption, aqueous solutions come into contact with a very large surface area of different cell wall components. The cell wall polymers of lignocellulosics contain phenolic hydroxyl, carboxyl and other hydroxyl groups that can act as ion-exchange sites. Thus, sorption of heavy metals can be accomplished by ion exchange, complexion, and precipitation. Heavy metal sorption capacity of lignocellulosics may be increased by several ways including solvent extraction, alkali treatment, sulfonation, acetylation, reactions with multifunctional carboxylic acids (McGraw hill year book of science and technology, 2004; Han *et al*., 2003). Han *et al* has reported the successful application of juniper fibre to AMD treatment. Juniper fibres were processed into a mat type filter medium and used to restore the watershed affected acid mine drainage in the Wayne National forest in Ohio. In general AMD contains iron, sulfates, aluminium, manganese, and

other dissolved and suspended solids because of its low pH (Drever, 1997). The juniper filter media were installed to remove dissolved metal ions from AMD. It was found that iron species was the primary metal deposited to the filter media, implying that the juniper can be a natural, novel, inorganic, and hybrid adsorbent. The potential of lignocellulosic fibres to act as filters is related to their sugar, extractives, and lignin contents and physical properties (Nawawi *et al.*, 2008).

Lignocellulosic material has also been used as a substrate in bioreactors. The substrate is degraded by cellulolytic bacteria, which generates free sugars and other metabolites. These are then used by SRB in the remediation of AMD (Kuyucak, 2002).

1.7.3.5 AMD treatment and sugar generation.

In biomass pretreatment and lignocellulose digestion, the objective is to use a novel method using existing technologies to provide amelioration of AMD by digesting cellulosic biomass (primarily) to fermentable sugars such as glucose and xylose (Sheridan *et al*., 2013). The hypothesis is that the lignin fraction which contains numerous reactive groups would bind to and remove metals from AMD. Meanwhile the sulfuric acid in the AMD will hydrolyse hemicellulose and will expose cellulose to a subsequent enzymatic hydrolysis step. The AMD feed will be combined with biomass, therefore removing sulfuric acid and metals from AMD and at the same time digesting lignocellulosic biomass. Following this treatment, an enzymatic step can be done using appropriate enzymes to further hydrolyse the solubilised material and the residue into simple sugars. These sugars are then in turn used by microbes in fermentation to produce bio-based products such as ethanol (*Baltz et al*., 2010; Sheridan *et al*., 2013).

2.0 Biomass and biofuels

Biomass refers to organic plant based material sourced from forest and forest residues, agricultural residues and dedicated energy crops (Hisham and Megeed, 2008). Biomass is an important part of a global clean power generation solution and is already the fourth largest energy source in the world after oil, coal and gas. Biofuels are fuels that are generated from biomass (Lee and Lavole, 2013).The two most common types of biofuels in use today are ethanol and biodiesel. Ethanol has been made from starches and sugars. Ethanol is generally produced from fermentation of C6 sugars (mostly glucose) using yeast strains such as *saccharomyces cerevisiae* (Lee and Lavole, 2013). Only a few different feedstocks such as sugarcane and corn are used for the generation of ethanol. Other feedstocks that are used or considered include, but are not limited to whey, barley, sugar beet, potatoes and rice (Cheng and Timilsina 2010; Lee and Lavole, 2013). Any biomass that contain starch or sugar is capable of being fermented to ethanol. Fuels produced from such plants (which can be used as food source) are termed first generation fuels. The first generation fuels are considered unsustainable because of the potential stress that their production places on food commodities. One of the reason of the increases in food prices is due to the increase in the production of these fuels (Naik *et al*., 2010; Taylor, 2005; Davidson, 2008). Biofuels production and consumption has been increasing rapidly in the last few years. Led by Brazil and the United States, global production of ethanol more than doubled (Carriquiry *et al*, 2010). According to Licht (2009), the production of biofuels increased from 31.3 billion litres in 2005 to 72.8 billion litres in 2009. Currently biofuels account for over 1.5% of the energy used for transport (Carriquiry *et al*, 2010). In terms of market potential, the International Energy Agency (IEA) projects that sugarcane ethanol and advanced biofuels could provide up to 9.3% of total transport fuels by 2030 and up to 27% by 2050 (IEA-ETSAP and IRENA 2013). Research has been ongoing in developing new technologies to allow biofuels to be obtained from lignocellulosic material, the fibrous material that make the bulk of most plants. Biofuels produced from these non-food plant material are termed second

generation biofuels (Taylor, 2005; Naik *et al.*, 2010; Havlik *et al.*, 2011). Production of cellulosic biofuels is attractive and sustainable because it is a renewable source of energy and it does not compete with food crops, is less land and water intensive and it also helps reducing gas emissions (N.S.F., 2008; Carriquiry *et al*., 2010). Economically lignocellulosic biomass has an advantage over other agricultural important biofuels feedstocks such as corn starch, soybeans and sugarcane, because it can be produced quickly and at significantly lower cost. Lignocellulosic biomass is also an important component of the major food crops. It is the non-edible portion of the plant which is currently underutilised, but could be used for biofuels (N.S.F., 2008).

Although research has been ongoing on lignocellulosic biofuels, at the moment there is no sufficient process or technology available to economically convert the lignocellulosic structure to bioethanol. According to N.S.F., 2008.The limiting factor of lignocellulosic biofuel is that a low cost processing technologies to efficiently convert large fraction of lignocellulosic biomass energy into liquid fuels does not yet exist. It is important therefore to continue developing technologies from cost effective conversion of non-edible lignocellulosic biomass to biofuels.

The whole process of the production of lignocellulosic ethanol primarily comprises, the hydrolysis of lignocellulosic structure to fermentable sugars, followed by fermentation then distillation of the fermented broth. The hydrolysis of the lignocellulosic material is the critical stage, which determines the overall efficiency of the process (Binod *et al.*, 2011).

2.1 Lignocellulose

Lignocellulosic biomass is abundantly present in sub Saharan Africa and has the highest growth potential when modern agriculture methods are implemented. In South Africa alone, 12.4 million tons of agricultural residues are left in the field after harvest every year. This has an energy equivalent of 214

PJ; almost half of South Africa's energy demand for transport (Lynd *et al*., 2003).The raw material is usually considered a waste with no value or a negative value considering the environmental cost of burning it (Villasden, 2003).

It can be based on any substance, including but not limited to roadside grass bales, alfalfa, woodchips, sugarcane bagasse, etc. (Lynd *et al*., 2003). Plant cell wall biomass contains cellulose, hemicellulose and lignin, but different species of plants have significant differences in the proportions of the main compositions and important differences in the types of hemicellulose and/or the ratios of monomers in lignin. Every plant consists of different ratios of cell wall compositions in different parts of the plant (Zhao *et al*., 2012). The components of some typical plant biomass are shown in Table 2.0.

Table 2.0: Main chemical compositions of several typical grass biomass feedstocks. (Data obtained from Zhao *et al.*, 2012)

Feedstock	Cellulose (%)	Xylan (%	Galactan (%)	Araban (%)	Lignin (%)	Mannan (%)	Extractives (%)	Ash (%)
Grass biomass	25–50	20–50	0.5–1.0	1.8–3.0	10-30	1.2-0.6	4-25	2-12
Corn cob	36.4	18.0	1.0	3.0	16.6	0.6	7.3	9.7
corn stover	40.9	21.5	1.0	1.8	16.7	NA	NA	6.3
sugarcane bagasse	40.2	21.1	0.5	1.9	25.2	0.3	4.4	4.0
wheat straw	38.2	21.2	0.7	2.5	23.4	0.3	13.0	10.3
rice straw	34.2	24.5	NA	NA	11.9	NA	17.9	16.1
switch grass	31.0	20.4	0.9	2.8	17.6	0.3	17.0	5.8

2.2 Chemical composition and physical structure of lignocellulose.

The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin (Taherzaden and Karimi, 2007). Cellulose is the major structural component of cell walls, and it provides mechanical strength and chemical stability. Plants absorb solar energy through photosynthesis and stores it in the form of cellulose (Raven *et al.*, 1992). Hemicellulose is a copolymer of different C5 and C6 sugars that also exist in the plant cell wall. Lignin is a polymer of aromatic compounds which forms a protective

layer for the plant walls. Apart from these basic components, water is also present in the complex. Furthermore minor amounts of proteins, minerals and other components can be found in lignocellulose composition as well (Harmsen *et al*., 2010). An analysis of the structure of each of the three major component is given below.

2.2.1 Cellulose

Cellulose is an unbranched, crystalline micro fibril constructed from 7000 to 15000 alpha-D-glucose molecules embedded in hemicelluloses. The molecules are held together by *β*-glycosidic bonds (Fig 2.1). Cellulose fibres are arranged in bundles of parallel chains held together by hydrogen bonding between hydroxyl groups and hydrogen atoms, forming a crystalline structure with great mechanical and high chemical stability (Zhul *et al*., 2008). Cellulose acts as reinforcements similar to iron rebar in concrete to give strength to the cell wall. Many properties of cellulose depend on its degree of polymerisation (DP), i.e. the number of glucose molecules that make up one polymer molecule (Harmsen *et al*., 2010).

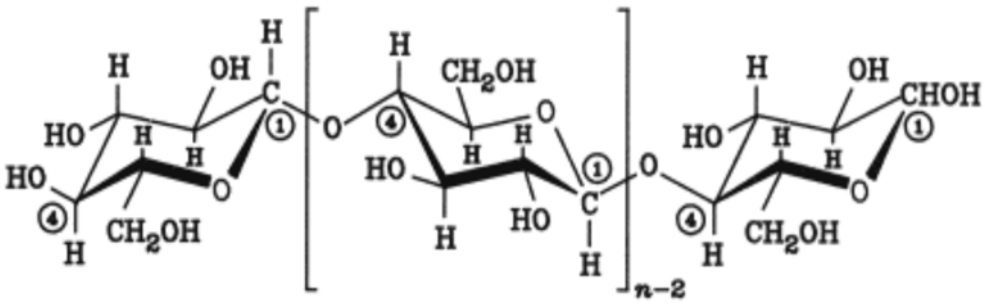


Figure 2.1: Structure of single cellulose molecule. Image from Harmsen *et al.,* 2010.

Cellulose is a relatively hygroscopic material absorbing 8-14% water under normal atmospheric conditions. Nevertheless it is insoluble in water, where it swells (Harmsen *et al*., 2010).

2..2.2 Hemicellulose

Hemicelluloses are composite branched polysaccharides polymerized from 500-3000 D-C5 sugar units to form an amorphous structure of xylan, arabinose, glucomannan and others (Figure 2.2). The backbones of the chains of hemicellulose can be a homopolymer (generally made up of single sugar repeat unit or heteropolymer (made up of a mixture of different sugars). According to the main sugar residue in the backbone hemicellulose has different classifications, for example, xylans, glucans, glucoronoxylans, arabinoxylans, galactomannas, galactoglucomannans and xylomannans (Musatto and Texeira, 2010). Xylan is the predominant hemicellulose in most plant cell walls, generally comprising one third of the biomass (Prade, 1996). Xylans form cross links between cellulose, lignin and pectin by hydrogen bonding to the other polysaccharides and by covalent linkages through the arabinofuranoyl side chains to the ferulic and coumaric acid found in lignin. The composition of the side chains determine the specific variety of xylan. In contrast to cellulose, hemicelluloses are relatively easy to hydrolyse by acid treatment or by enzymes to form monomers with the C5 sugar D-xylose being the most abundant pentose derived from the material (Decker *et al*, 2005).

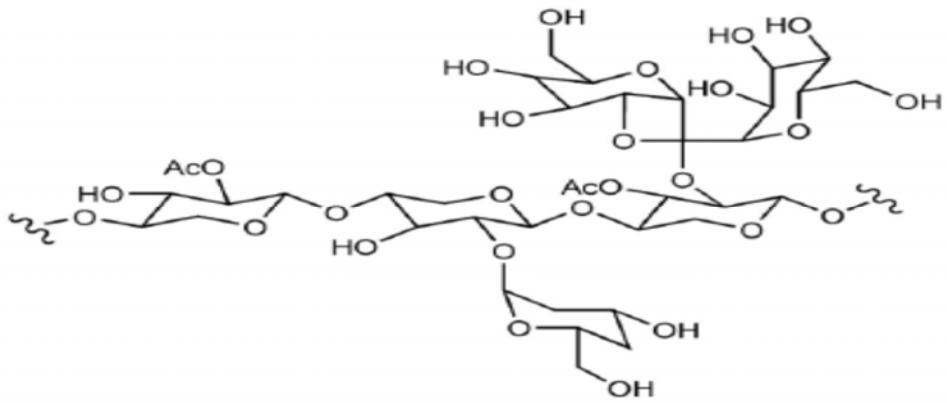


Figure 2.2: A schematic representation of hemicellulose. Image from Harmsen et al, 2010

2.2.3 Lignin

Lignin (Figure 2.4) is an amorphous hydrophobic polymer into which cellulose micro fibrils are embedded. It adds resistance to chemical and microbial attack to the network of cellulose and hemicelluloses. The building blocks of the very complex lignin polymer change from one lignocellulosic biomass to another, but generally they are aromatic in character, dominated by derivatives of phenyl propane (Villasden, 2003; Palmqvist *et al*., 2000; Howard *et al*., 2003). More specifically, p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol are the most commonly encountered derivatives (Figure 2.3).

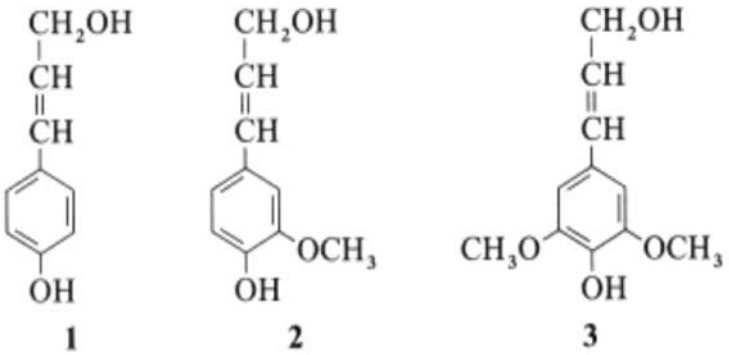


Figure 2.3: P-comaryl, coniferyl and sinapyl alcohol: dominant building block of the 3-dimensional polymer lignin (Image from Harmsen *et al*., 2010).

Higher plants are divided into two categories depending on the proportional compositions of the constituent alcohols, hardwood (angiosperm) and softwood (gymnosperm). Lignin from softwood is made up of more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol. Lignin in hardwood is made up of varying ratios of coniferyl and sinapyl alcohol type units (Kirk-Othmer, 2001).

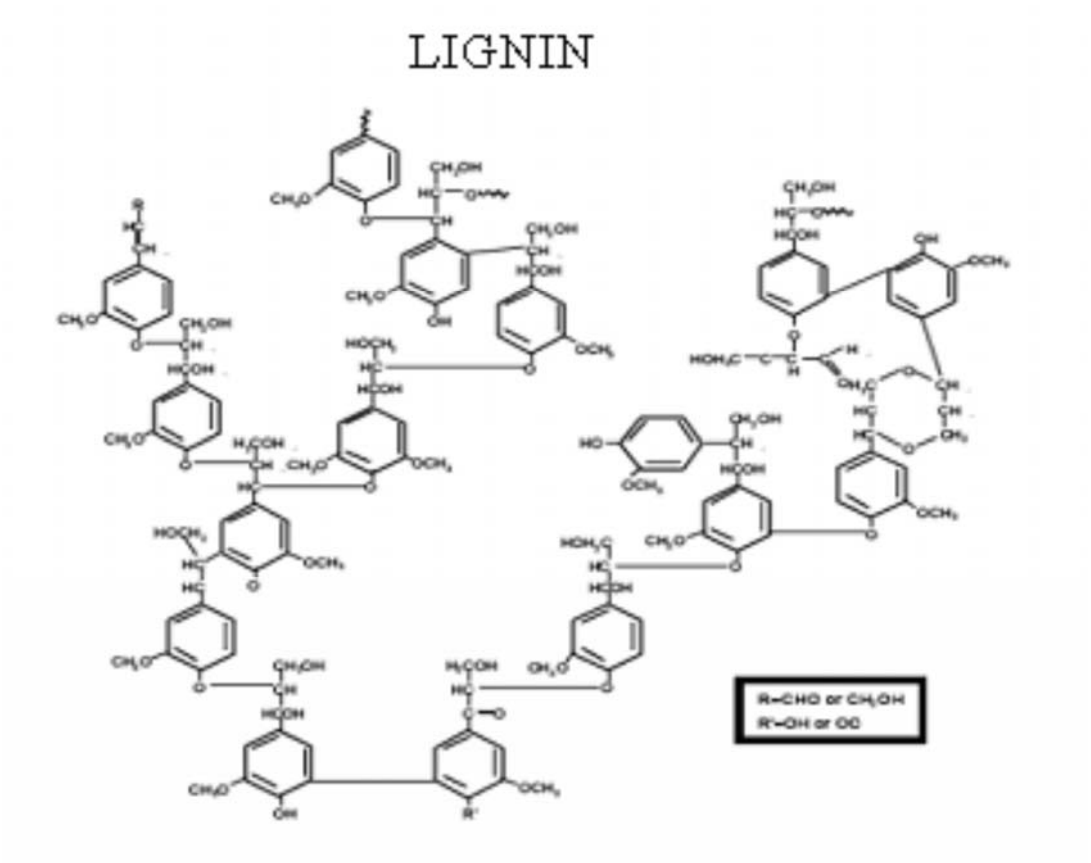


Figure 2.4: lignin from gymnosperm showing the different linkages between phenyl-propane units: +Image from Perez *et al*., 2002.

Lignin plays an important role in the cells' endurance and development, it affects the transport of water, nutrients and metabolites in the plant cell. It acts as a binder between cells creating a composite material that has a remarkable resistance to impact, compression and bending (Figure 2.5), (Harmsen *et al*, 2010)

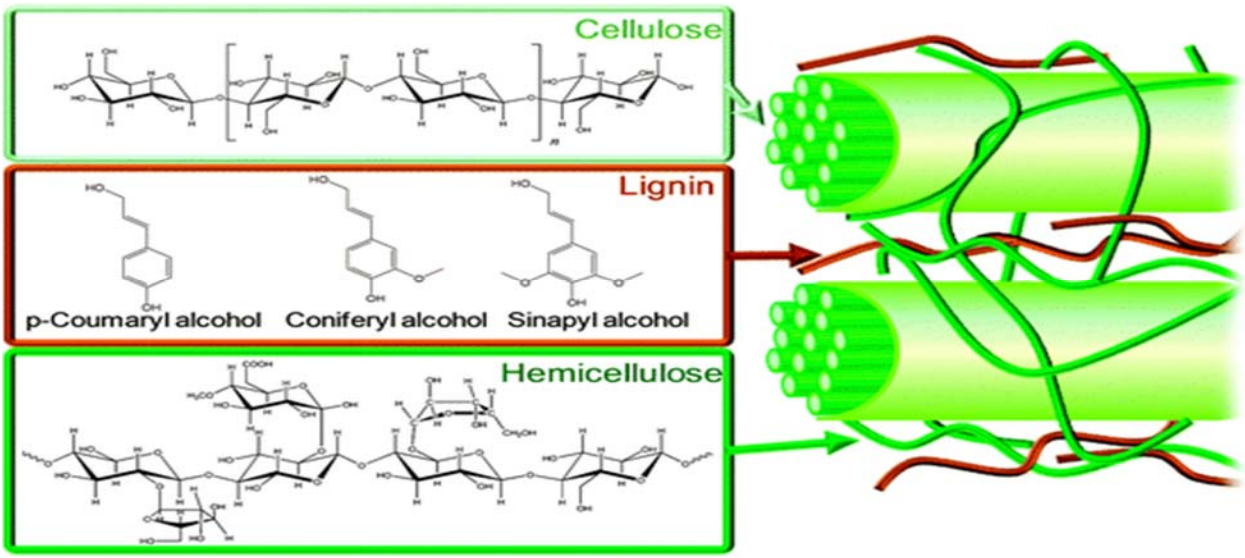


Figure 2.5: The arrangement of lignin, cellulose and hemicellulose to form a micro fibril.

Lignocellulosic biomass is a renewable resource and has great potentials for the production of fuel ethanol because it is less expensive than starch (e.g. corn) and sucrose (e.g. sugarcane) producing crops and is available in large quantities (Petrova and Ivanova, 2010). For lignocellulose to be amenable to fermentation, it has to undergo treatment to release its monomeric sugars, which can then be converted by microorganisms. The two main steps are: (1) a pretreatment (by physical or chemical procedures) that release hexoses and pentoses; and (2) an enzymic treatment (or alternatively hydrolysis by chemical procedures) that generates glucose from cellulose. The cellulose hydrolysis is the main bottleneck in the process, as there is no microorganism currently available that can utilize lignin monomers for ethanol production (Petrova and Ivanova, 2010). Thus chemicals are needed to pre-treat the lignocellulose prior to biological hydrolysis and fermentation.

Following pretreatment, the individual polymers are ready for enzymatic digestion, which results in monomeric sugars of mainly glucose and xylose which can be utilized by microbes in industrial processes, most importantly cellulosic ethanol production (Rumbold *et al*., 2010). Therefore the

pretreatment process is a key step in the production of fermentable sugars. However the implementation of such a process in industry requires large amounts of sulfuric acid, plus the current price of sulfuric acid has increased so that the economically feasibility of dilute acid pretreatment might need to be considered (Zheng, 2009). This makes the use of AMD in biomass pretreatment an attractive option. This research connects the issue of AMD treatment and biomass pretreatment by combining the AMD feed with biomass, therefore (a) removing sulfuric acid from AMD and (b) digesting cellulose biomass Sheridan *et al*., 2013).

2.3 Cellulosic Bio-processes.

Following pretreatment of lignocellulosic feedstock, an enzymatic step can be employed further to hydrolyse the solubilised material and residue into simple sugars. These sugars are then in turn used by microbes in fermentation to produce bio-based products such as ethanol (Baltz, 2010). The use and production of bio fuels has risen dramatically in recent years. Bio-ethanol comprises 85% of global bio-fuels' production, with benefits including reduction in emissions of greenhouse gases and promotion of energy independence and rural economic development. Ethanol is primarily made from corn grain, and sugarcane juice, however ethanol production from current technologies will ultimately be limited by land availability, government policy and alternative use of these agricultural products. Biomass feed stocks are an enormous and renewable source of fermentable sugars that could potentially provide a significant proportion of transport fuels globally (Sainz, 2008). According to the National Academy of science (USA) there is no commercially viable bio-refinery in existence to convert lignocellulosic biomass to fuel. Bio-conversion of lignocellulosic biomass is significantly hindered by structural and chemical complexity of biomass (Figure 2.5; Table 2.1), which makes these materials a challenge to be used for cellulosic ethanol production. Cellulose and hemicelluloses , when hydrolysed into their component sugars ,can be converted into ethanol through well-established fermentation technologies, however

sugars necessary for fermentation are trapped inside the cross linking structure of lignocelluloses. Hence, pretreatment of biomass is always necessary to remove and/or modify the surrounding matrix of lignin and hemicelluloses prior to enzymatic hydrolysis of the polysaccharides (cellulose and hemicelluloses) in the biomass. Pretreatment refers to a process that converts lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme systems, into a form which cellulase hydrolysis is much more effective (Figure 2.6). The effects of pretreatment are usually focused on lignin and hemicellulose removal, cellulose crystallinity reduction and accessible surface area increase. There are some widely accepted requirements that pretreatment should meet (Sun *et al*., 2002):

a) Improve the formation of sugars or create a reactive cellulose fibre for enzymatic attack;
b) Minimize the degradation or loss of carbohydrates;
c) Avoid formation of by products that are inhibitory to enzymatic/microbial activity.
d) Minimize the use of energy, chemicals and capital equipment to produce a cost effective process that is scalable to industrial size.

In general pretreatment methods can be classified into three categories, including physical, chemical and biological pretreatment (Zheng *et al*., 2009).

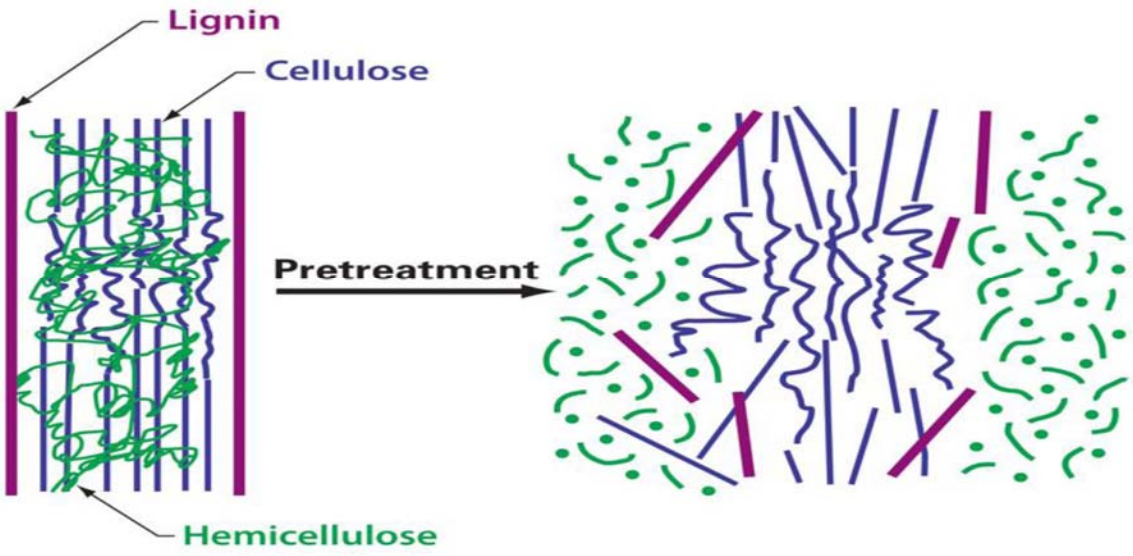


Figure 2.6: Schematic representation of effects of pretreatment on lignocellulosic biomass (Adapted from Hsu *et al*., 1980)

Table 2.1: Effects of chemical compositions and physical structures on enzymatic digestibility (ED) of lignocellulosic biomass. Adapted from Zhao *et al*., 2012.

Factors	Description
Lignin	Lignin acts as a physical barrier to restrict the access of cellulases to cellulose. The structure of lignin plays a significant role in determining the magnitude of inhibition. Lignin also can irreversibly adsorb cellulase enzymes. Hydrophobic interactions, electrostatic interactions and hydrogen bonding contribute to the adsorption of cellulases onto lignin matrix.
Hemicellulose	Hemicelluloses can play as physical barriers to limit the accessibility of cellulase, but compared to the effect of lignin, the limitation by hemicelluloses seems to be less important. Removing hemicelluloses can increase the porosity of the feedstock.
Accessible and Specific surface area (ASA and SSA)	Accessible surface area is the most important factor to limit the accessibility of cellulose to enzymes. It is the direct factor relevant to enzymatic digestibility of lignocellulosic biomass. Increasing the accessible surface area of cellulose is the objective of pre-treatments, which can be achieved by altering the chemical compositions and physical structures of the feedstock. SSA is important to the accessibility of cellulose. The enzymatic digestibility of cellulose is increased with surface area, but the total SSA of substrates is not an independent factor. It is strongly related with particle size, the porosity and pore volume of the feedstock. SSA is not equal to accessible surface area, because not all the surface is accessible to enzymes. The interior surface is believed to be more important than exterior surface to the accessibility. Only the pores with size larger than 5.1 nm can accommodate cellulase enzymes to act the hydrolysing behaviour.
Crystallinity	Amorphous celluloses are typically 3–30 times faster to hydrolyse than high crystalline cellulose. For biomass, decreasing crystallinity can tremendously increase the initial hydrolysis rate and reduce the hydrolysis time or the amount of enzyme required to attain high digestibility. However, the biomass crystallinity index (CRI) is closely relevant to contents of lignin and hemicelluloses. CRI is also not an independent factor. Decrease of CRI is always accompanied by decrease of particle size with associated increase of accessible surface area.
Degree of polymerisation	The hydrolysis of cellulose actually is the depolymerisation process of cellulose chain. Lower DP can provide more binding sites to cellulase enzymes, thus increasing the hydrolysis rate; however, the initial DP does not significantly affect the final extent of hydrolysis. DP actually is not an independent factor, because altering DP is always accompanied by change of crystallinity or porosity of the substrates.

2.3.1 Physical pretreatment.

Physical pretreatment affects the physical structure of biomass. It reduces crystallinity, increases pore size and reduces polymerisation. It can involve mechanical methods (milling) which decreases the size of particles and therefore the crystallinity and pyrolysis which involves subjecting the biomass to higher temperatures above 300 degrees Celsius. This causes cellulose to rapidly decompose (Carvalho, 2009).

2.3.1.1 Milling

Milling or comminution, or mechanical particle size reduction is an essential mechanical pretreatment step. The final target size for milling is 0.2 to 2mm. the size reduction is accompanied by a decrease in cellulose crystallinity, making it more accessible to cellulases. It also disrupts the lignin-carbohydrate complexes, which further aids the enzyme hydrolysis (Mais *et al*., 2002; Canam *et al*., 2013). There are several types of milling, such as ball-milling, knife milling and hammer milling. Regardless of the method employed, particle size reduction requires energy input, therefore, strategies that facilitate the production of biomass in the proper size range minimizing energy input will provide positive benefits to the overall economics of biofuels processes (Canam *et al*., 2013. One limitation of milling is that it is unable to remove lignin. This restricts access of enzymes and inhibits their activity, preventing the hydrolysis of cellulose from reaching close to the maximum theoretical values (Calvalho, 2009).

2.3.1.2 Steam explosion

In steam explosion biomass can be briefly exposed to high temperatures (~200°C), under high pressure, then subjected to a rapid pressure drop that renders the biomass more penetrable by enzymes for subsequent hydrolysis (Chandra *et al*., 2007; Hisham and Mageed, 2008). In some cases steam explosion is enhanced by addition of an acid catalyst such as sulfuric acid (Ballesteros *et al*., 2006). For lignocellulosic agricultural waste steam explosion under optimised conditions has been shown to be an

effective pretreatment for enzymatic saccharification (Rosgaard *et al*., 2007). It can also be successfully used in combination with physiochemical chemical pretreatment such as acid/water impregnation of cereal straws (Rosgaard *et al*., 2007).

2.3.1.3 Irradiation

In the presence of lignin, radiation affects directly the cellulose component, breaking glycosidic bonds, thus creating fragile fibres and low molecular weight oligosaccharides. Excess of radiation can lead to decomposition of glucose ring structure. The drawback of using irradiation is that they are expensive and difficult to apply on an industrial scale (Calvalho, 2009).

2.3.2 Chemical pretreatment.

Chemical pretreatments are purely initiated by chemical reactions for the disruption of biomass structure. The treatments include the use of water at high temperature and pressure, terms for this process are, hydrothermosis or hydrothermal pretreatment, dilute and strong acid and alkaline hydrolysis among others (Harmsen *et al*., 2010). During chemical pretreatment, hemicellulose and lignin may be hydrolysed to their monomeric constituents and lignin-cellulose-hemicellulose interactions are partially destroyed, thus increasing the enzymatic digestibility of cellulose (Mcmillan, 1994; Mussatto and Robert, 2006). Acid hydrolysis uses concentrated or dilute acid, at high and low temperatures (Chartuvedi and Verma, 2013). Nitric acid and phosphoric acid may also be used. When dilute acid hydrolysis is applied the xylose yield is significantly higher (Petrova and Ivanova, 2010). Dilute acid pretreatment usually use acid concentrations (sulfuric acid) of 0,5 to 1.0% at moderate temperatures (140-180°C) and can effectively remove and recover most of the hemicellulose as dissolved sugars (Zhang B and Shahbazi A, 2011). The limitations of acid treatment is that neutralisation must be done

before sugars are fermented, the neutralisation salts produced are usually insoluble providing another problem with waste disposal (Mosier *et al.*, 2005).

2.3.2.1 Dilute Acid pretreatment.

Acid treatment works primarily on hemicellulose, hydrolysing to monomeric sugars to a great extent. Lignin is not significantly removed. Although some part of it is solubilized, it recondenses forming an altered lignin polymer (Torget *et al*., 1991). Still this destruction and redistribution of lignin weakens the carbohydrate-lignin matrix, increasing cellulose digestibility (Yang *et al*., 2004). Effects on crystallinity vary. Chemical treatments remove amorphous lignin and hemicellulose components, decreasing biomass crystallinity. Conversely, they loosen the highly packed crystalline structure through swelling, and so decrease crystallinity. As such the change in biomass crystallinity depend on which of the two is predominant (Calvalho, 2009).

The acid treatment offers good performance in terms of recovering hemicellulose sugars but there are also some drawbacks. The soluble sugar products are primarily xylose, and mannose, arabinose, and galactose. The cellulose bulk will be converted in a separate step. The product is filtered and pressed, solids (cellulose and lignin) go to cellulose hydrolysis, and the liquid go to a fermenting process (Hisham and Mageed, 2008). The hemicellulose sugars might be further degraded to furfural and hydoxymethyl furfural, strong inhibitors of microbial fermentation (Harmsen *et al*., 2010; Chaturvedi and Verma, 2013, Canam *et al*., 2013, Zhang and Shahbazi, 2011). This reduces the ethanol yield and productivity. The major inhibitory substances for fermentation include:

a) Sugar degradation products: Subsequent to hemicellulose hydrolysis pentose monomers may dehydrate to the inhibitor furfural, hexose sugars may degrade to hydroxymethyl-furfural

(HMF). These affect cell growth and respiration. Extensive degradation of cellulose is responsible for the inhibitor compounds (Harmsern *et al.*, 2010; Chartuvedi and Verma, 2013)

b) Lignin degradation compounds: A variety of compounds including aromatic, polyaromatic, phenolic and aldehydic may be released from the lignin fraction. Phenolic compounds have a considerable inhibitory effect and are more toxic even in lower concentrations. Phenolic compounds cause partition and loss of integrity of cell membranes of the fermenting organisms reducing cell growth and sugar assimilation (Hamsern *et al.*, 2010)

c) Acetic acid is derived from the acetyl group of hemicellulose. At a pH around 7.4, the acid dissociates causing a lowering of pH that inhibits cell activity.

2.3.2.2 Concentrated acid hydrolysis.

Concentrated acid pretreatment is conducted with mineral acids such as H_2SO_4 or HCl, in upwards of the 10% concentration range, at temperatures above 60°C and pressures above 10 atmospheres (Sun and Cheng, 2002; Kumar *et al*., 2009). The harsh conditions are needed to liberate glucose from the tightly associated chains. In this process acid concentration, temperature and time are crucial factors, and must be controlled to avoid the sugars and lignin degradation by-products (McMillan, 1994)

2.3.2.3 Pretreatment of biomass under alkaline conditions.

In this treatment biomass is treated with alkali such as sodium hydroxide, potassium hydroxide, calcium hydroxide and ammonium hydroxide at normal temperature and pressure (Chartuvedi and Verma, 2013). Alkaline hydrolysis is efficient in removing lignin from biomass. Lime or sodium hydroxide is the most commonly used. The reaction conditions are generally mild, which prevents condensation of lignin leading to its high solubility and greater removal (Chartuvedi and Verma, 2013). In these conditions, degradation of sugars is minimal (Sharma *et al*., 2012). Aqueous ammonia treatment can also be used at

elevated temperatures. The process sufficiently removes lignin content and removes some hemicellulose, while cellulose is decrystallised, lignin is separated in the form of a rich phenolic liquor that represents the process effluent (Chartuvedi and Verma, 2013; Fensel and Wegener, 1989; Mussatto *et al.*, 2007).

A number of other options exist, including ammonium fibre explosion in which biomass is treated with liquid anhydrous ammonia at 60-100°C and high pressure (250-300 psi) for five minutes. The pressure is then released rapidly (Chartuvedi and Verma, 2013). The combined effect of ammonia and high pressure leads to swelling of lignocellulose biomass, destruction of lignocellulosic architecture leading to hemicellulose hydrolysis and decrystallisation of cellulose (Fan *et al*., 1987). Lignin however remains unaffected during the process. Pretreatment with ammonia is thus suitable for biomass with low lignin content (Bradshaw *et al*., 2007; Fensel and Wegener, 1989). A major drawback of using ammonia is the high cost involved in pretreatment process. Ammonia is also toxic to the environment, proper handling and maintenance is required to avoid leakages into the environment. This adds more cost to the process.

2.3.2.4 Substrate cleaning

During the pretreatment and hydrolysis process, some degradation products of C5 and C6 sugars are formed. These includes furfural, hydroxyl-methyl furfural (HMF), weak acids and acids from pretreatment and hydrolysis. The degradation products are toxic and inhibit enzymatic hydrolysis and fermentation. In order to remove these inhibitors and increase the hydroxylate fermentability several chemical and biological methods have been used. These include liming, charcoal adsorption, ion exchange, detoxification with laccasse and biological detoxification. The detoxification of acid hydroxylate has been shown to increase fermentability, however the cost is often higher than the

benefits. Recycling of the acid can add some economic value to the process cost (Hisham and Mageed, 2008)

2.3.3 Biological pretreatment.

Biological pretreatment uses microorganisms such as white, brown and soft rot fungi to degrade hemicellulose and lignin. This modifies the biomass structure so that it is more amenable to enzyme digestion. Advantages of this process are low energy requirements and mild operating conditions. It is also ecologically benign and cheap. Nevertheless the rate is usually low, so pretreatment requires a long resident time in the order of weeks or even months (Harmsen *et al*., 2010; Petrova and Ivanova, 2010). The biological pretreatment of lignocellulosic biomass is usually performed by employing cellulolytic microorganisms which synthesise potent cellulolytic enzymes during hydrolysis. White rot fungi have been shown to be the most effective for the pretreatment of lignocellulosic biomass such as wood chips, wheat straw, Bermuda grass and soft wood (Akin *et al*., 1995). These fungi are capable of degrading cellulose, hemicellulose and lignin and are considered major degraders of woods in forest ecosystems. They are able to completely mineralise lignin to carbon dioxide and water (Shretha, 2008). White rot fungi produce laccases and peroxidases like lignin peroxidase and manganese peroxidase for delignification processes (Tuor *et al*., 1995). The ligninolytic enzymes are further complimented by consortia of cellulose and hemicellulose degrading enzymes (Shretha, 2008). Another class of fungi, commonly known as brown rot fungi can selectively degrade cellulose and hemicellulose, without affecting lignin. Soft-rot causes degradation of cellulose and hemicellulose but only partially digests lignin. Soft rot fungi are particularly prevalent at the early stages of wood decay and in conditions of high moisture and increased nitrogen content (Shretha, 2008).

None of the pretreatment processes are able to achieve 100% yield of reducing sugars from all types of biomasses (Chartuvedi and Verma, 2013).The efficiency depends mainly on the biomass used as raw

material, its structure and lignin content (Mckendry, 2002) using two or more pretreatment processes has been proven to be efficient as compared to a single pretreatment process.

Another approch for applying the power of microbial metabolism to the challenges of biofuels involves ensiling, which is a commonly used means of enhancing the digestibilty of forage and biomass for ruminants(Canam *et al*,., 2013). It is based on anareobic fermentation by lactic acid bacteria (LAB) that produce organic acids, reduce ph, and prevent growth of yeasts, fungi and competing bacteria.Enlising is nomally carried out in anaerobic conditions in which organic acids, especially lactic acid and acetic acids produced by endogenous microflora, decrease pH, preserve the substrate against growth of fungi, bacteria or yeasts and prevent carbohydrate loses. Enlising treatments have been used as a mild pretreatment method to improve the disassembly and hydrolysis of lignocellulosic material. The organic acids produced during enlising have been found to enhance the production of biogas or bioethanol by decreasing crystallinity of cellulosic material, by increasing accessible surface area of plant substrate and by altering the lignin structure (Pakarinen *et al*., 2011). This natural modification of lignin can reduce the severity of subsequent pretreatment steps (Ambye-Jensen *et al*., 2013).

2.4 Enzymatic hydrolysis.

In this process, cellulose is converted to glucose using cellulase. This process is known as enzymatic saccharification or enzymatic hydrolysis. Hemicellulose, a branched polymer composed of pentose (5-carbon) and hexose (6-Carbon) sugars can be hydrolysed by hemicellulases or acids to release its component sugars, including xylose, arabinose, galactose glucose and/or mannose. Hexoses such as glucose, galactose and mannose are readily fermented to ethanol by naturally occurring microorganisms, but the pentoses including xylose and arabinose are fermented to ethanol by native strains, and usually produces low yields of ethanol (Petrova and Ivanova, 2010). A cellulase enzyme preparation is a mixture of enzymes (catalytic proteins) that work to breakdown cellulose fibres into

cellobiose and soluble gluco-oligomers and ultimately into glucose monomers (Humbird *et al*, 2011). Enzymatic hydrolysis cannot be successful alone, since the lignocellulose is resistant to enzymatic attack. Therefore it is used between a first pretreatment and the anaerobic digestion, in order to convert cellulose and hemicellulose to simple sugars, obtaining an easily –metabolisable sugar rich liquid fraction (Calvalho, 2009). The lignocellulose degrading enzymes work under mild conditions therefore have lower utility costs, the enzymes themselves however are costly. This limits their application at a commercial scale (Duff and Murray, 1996)

2.4.1 Lignocellulose degrading enzymes.

Cellulose is degraded by a multi-enzyme complex involving at least 3 enzymes: exo-(1, 4)-β-cellobiohydrase, an endo-(1, 4)-β-glucanase and glucosidase. Cellobiohydrolase has the greatest affinity for microcrystalline cellulose. This group of enzymes is called cellulases (Sweeney and Xu, 2012).

The endo-β-(1, 4) glucanases randomly hydrolyses internal bonds of the β-(1, 4) - glucan chains producing glucose, cellobiose and other sugar oligomers of higher molecular weight (Figure 2.7). It is thought that the endo-1, 4-β-glucanase cannot attack the highly ordered microcrystalline cellulose but can act on amorphous regions in the fibre, rendering the chains more amenable to attack by cellobiohydrolase (Ramesh, 2005; Wood, 1991; Cullen and Kersten 1992; Wyman *et al*., 2005). The exo-(1, 4) glucanases act only on the exposed ends of β-(1, 4) glucan chains, releasing a disaccharide cellobiose or single glucose molecules. β-glucosidase enhances the overall hydrolysis process by converting cellobiose into glucose. The cellulose enzyme components act synergistically in the hydrolysis of crystalline cellulose. This synergism has been explained by the attack of endo glucanases on amorphous cellulose, forming sites for exo glucanases to hydrolyse cellobiose units from the crystalline cellulose. Then β-glucosidase prevent the accumulation of cellobiose by hydrolysing it, as exoglucanase

is inhibited by an accumulation of cellobiose (Leschene, 1995; Howard *et al*., 2013; Sweeny and Xu, 2012).

Hemicellulose degradation involves different enzymes depending on the monomer composition of the hemicellulose backbone. Xylan is the most abundant hemicellulose and xylanases are one of the major hemicellulases, which hydrolyse β-1-4 bond in the xylan backbone yielding short xyloligomers which are further hydrolysed into single xylose units by β-xylolidase (Prates, 2001; Wyman *et al*., 2005; Ni and Tokuda, 2013; Binod *et al*., 2011). β-mannanase hydrolyse mannan based hemicellulose and liberate short β1-4-manno-ligomers which further hydrolyse to mannose by β-mannosidases. Hemicellulolytic esterases, include acetyl esterases, which hydrolyse acetyl substitutions on xylose moieties and feruloyl esterases which hydrolyse the ester bond between arabinose substitutions and ferulic acid. Feruloyl esterase aid the release of hemicellulose from lignin and renders the free polysaccharides more amenable to degradation by other hemicellulases (Prates, 2001; Howard *et al*., 2003; Cullen and Kersten, 1992). Hemicelluloses are of particular industrial interest since these are readily available bulk sources of xylose from which xylitol and furfural can be derived (Howard *et al.*, 2003).

Unlike cellulose and hemicellulose, the lignin polymer is not principally linear nor does it have a repeating hydrolysable interunit bond, instead it is a complex three dimensional non-stereoregular aromatic polymer composed of phenylpropanal units linked through several major types of C-C and ether bonds (Figure 2.4) (Cullen and Kersten, 1992). Lignin is degraded through oxidative processes that involve peroxidases, polyphenols and laccases. Through different mechanisms they catalyse the oxidation of lignin producing phenolic intermediates that further react leading to the formation of polymeric products of increasing complexity (Fioretta and Fuggi 2005). Lignin peroxidase catalyses a variety of oxidations, all of which depends on H_2O_2. These include C_x-C_β cleavage of propyl side chains of lignin and lignin models, Hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the

corresponding aldehydes and ketones, phenol oxidation, and even non-phenolic cleavage of non-phenolic lignin model compounds (Cullen and Kersten, 1992; Ni and Tokuda, 2013; Sweeny and Xu, 2012).

Laccases catalyse the oxidation of phenolic units in lignin and a number of phenolic compounds and aromatic amines to radicals, with molecular oxygen as the electron acceptor that is reduced to water (Binod *et al*., 2011)

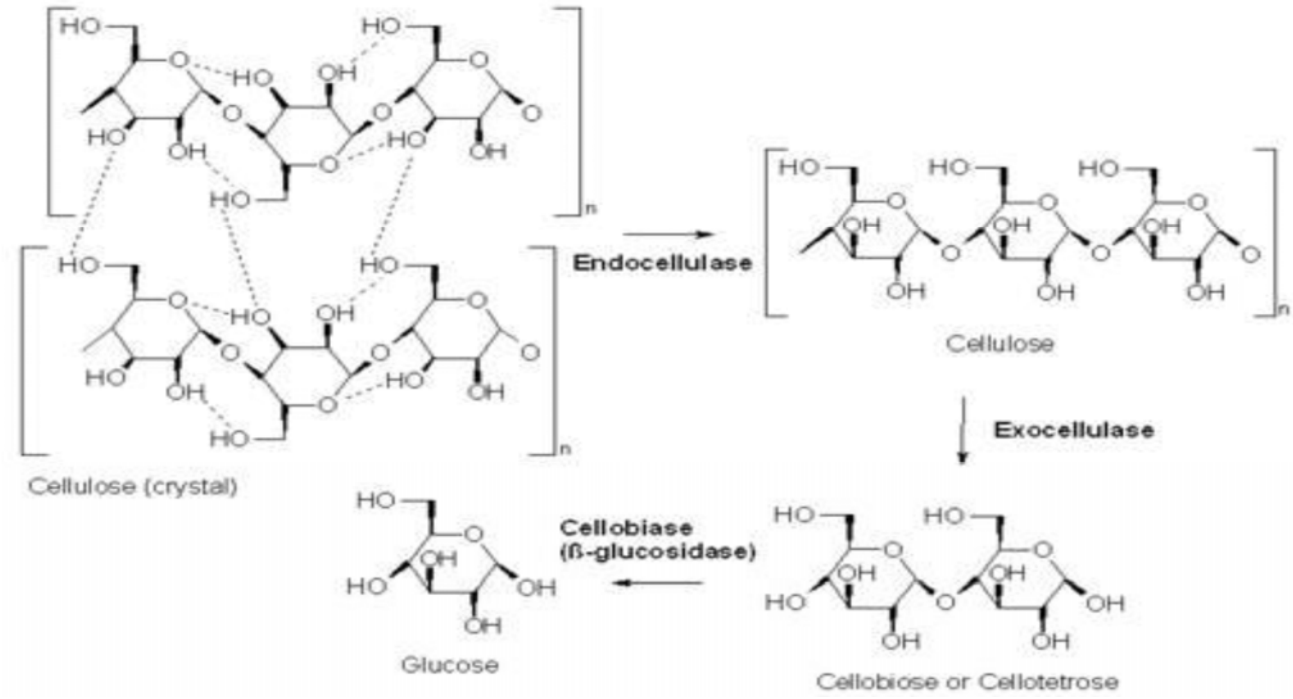


Figure 2.7: Reaction pathway from cellulose to glucose (enzymeindia2008)

2.4.2 Sources of lignocellulose degrading enzymes.

Most research in the production of cellulases is focused on fungi. Even though cellulotic bacteria , particularly the cellulotic anaerobes, produce cellulases of high specificity; they have low growth rates and require anaerobic growth conditions. Conversely, the anaerobic mesophilic filamentous fungi

Trichoderma reesei has shown good results with some strains releasing up to 30g/l of extracellular enzymes, mostly cellulases. It also produces enzymes from the three cellulase groups. Commercial cellulases are available mainly from fungi including *Trichoderma*, *Aspergillus*, *Penicillium* and *Basidiomycetes* species (Ramesh., 2005), bacteria and actinomycetes. While several fungi can metabolize cellulose as an energy source, only a few strains are capable of secreting a complex of cellulase enzymes, which could have practical applications in the hydrolysis of cellulose (Cowlin., 1975).

2.5 Overview of ethanol fermentation

Ethanol is produced by the fermentation of carbohydrate material. Fermentation is a series of chemical reactions that convert sugars to ethanol. The fermentation reaction is caused by yeast or bacteria which feed on the sugars. As the sugars are consumed ethanol and carbon dioxide are produced (Figure 2.8). Ethanol is both renewable and environmental friendly and is one of the best choices as alternative fuel (Sanchez and Cardona, 2008). *Saccharomyces cerevisiae* is the most commonly used microorganism for the production of ethanol due to its robustness and high productivity (da Cunha-Pereira *et al*., 2011; Laulce *et al.,* 2012; Limayen and Ricke, 2012). It is better than other yeasts with respect to tolerance to both ethanol and inhibitors present in the hydrolysates and shows higher efficiency of sugar conversion to ethanol (Laulce *et al.*, 2012, Sveinsdottir and Baldursson, 2009). Under aerobic conditions and in the presents of high glucose concentration *Saccharomyces cerevisiae* grows well, but produces no alcohol. Under anaerobic conditions, growth is slow and pyruvate from the catabolic pathway is split with pyruvate decarboxylase into acetaldehyde and carbon dioxide. Ethanol is then produced from the acetaldehyde by reduction with alcohol dehydrogenase (Figure 2.8). After fermentation, ethanol can be recovered from the fermentation broth by distillation or distillation combined with adsorption or filtration, including drying using lime or salt, addition of entrainer molecular sieves, membranes and pressure reduction. The distillation residue, including lignin, ash, enzyme, organism debris, residue

cellulose and hemicelluloses, and other components may be recovered as solid fuel or converted to various value added co-products (Zheng, 2009).

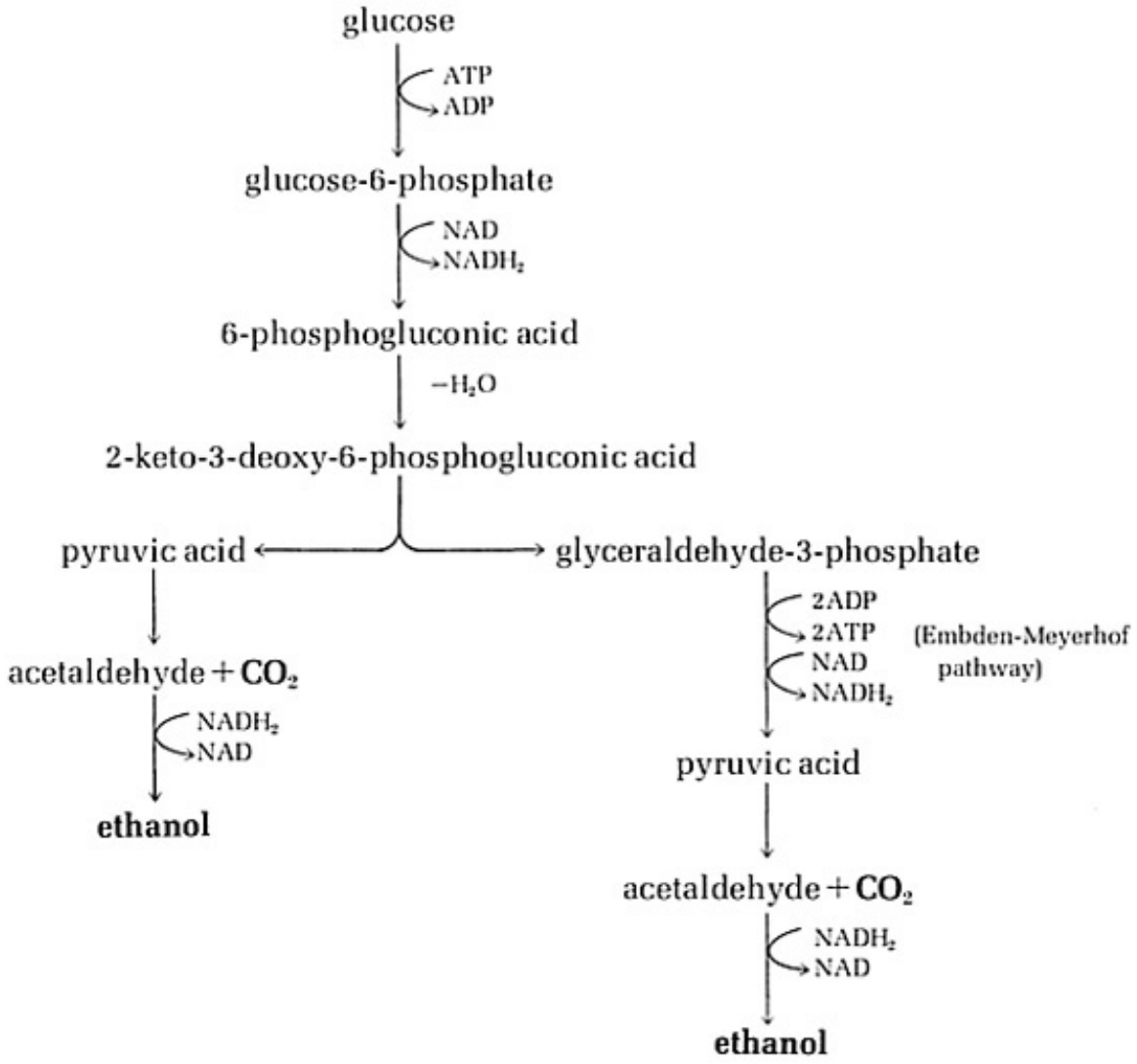


Figure 2.8: Schematic representation of the fermentation process. (Image adapted from Purves et al., 2004).

2.5.1 Ethanol fermentation from lignocellulosic biomass.

In the processing of lignocellulosics to ethanol, several approaches have been examined for hydrolysis of cellulose and fermentation of glucose into ethanol. These are sequential hydrolysis and fermentation (SHF), direct microbial conversion (DMC), Simultaneous saccharification and fermentation (SSF) and Simultaneous saccharification and co-fermentation (SSCF) (Wyman, 1994; Mosier *et al*., 2005). These processes are briefly explained below.

2.5.1.1 Sequential hydrolysis and fermentation (SHF)

In this approach cellulase is added to the bulk of pretreated material to hydrolyse cellulose into C6 sugars. Upon completion yeast will ferment the glucose to ethanol (Wyman, 1994 ; Chiaramonti, 2007). The positive aspect of this sequential approach is the ability to guarantee optimal conditions for both enzyme and microorganism (pH, Temperature and oxygen). The disadvantage is that two distinct reactors are needed (Ong, 2004; Binod *et al.*, 2011). Also the accumulation of glucose and cellobiose during hydrolysis inhibits cellulase activity (Ohgren *et a.l*, 2007; Taherzadeh and Karimi, 2007). Another possible problem for SHF is that of contamination. The hydrolysis process is long, it can take one to four days ,and a dilute sugar solution always has a risk of microbial contamination, even at higher temperature such as 45-50°C (Taherzadeh and Karimi, 2007).

2.5.1.2 Direct microbial conversion (DMC).

In this approach, microorganisms with the ability to produce cellulase enzymes, hydrolyse the cellulose and ferment glucose to ethanol are used (Wyman, 1994). Cellulase synthesizing bacteria such as *C. thermocellum*, *C. therosaccharolyticum* and fungi such as *Fusarium oxysporium* have been used to convert cellulose directly to ethanol (Wyman, 1994; Philipines, 1996, Ong, 2004). The technology

combines all three processes (cellulase production, cellulose hydrolysis and fermentation) into one step. The disadvantage is the low yield and high by product formation (Philipines, 1996; Abril and Abril, 2009).

2.5.1.3 Simultaneous saccharification and fermentation (SSF).

In this step, the cellulose hydrolysis and glucose fermentation are carried out in a single reactor (Wyman, 1994; Piccolo and Bezo, 2007)). Since cellulase is inhibited by glucose, rapid conversion of the glucose into ethanol by yeast results in faster rates and yields (Taherzadeh and Karimi, 2007). Obviously the optimisation of process conditions for both enzymes and microorganisms at the same time is critical issue of this solution (Chiaramonti, 2007; Ohgren *et al*, 2007). The optimum temperature which has been used is 37-38°C (Wyman, 1994). This is a compromise temperature between the optimum temperature for hydrolysis (between 45 and 50C), and fermentation (between 35 and 45°C). *Saccharomyces cerevisiae* is inactive at more than 40°C (Taherzadeh and Karimi, 2007). SSF has the following advantages as compared to a two stage process.

1) Lower enzyme required (Abril and Abril, 2009).
2) Increased rate of hydrolysis by conversion of sugars that inhibit cellulase activity (Sun and Cheng, 2002).
3) Higher product yield (Abril and Abril, 2009; Krishna and Chowdary, 2000).
4) Lower requirement for sterile conditions since glucose is removed immediately and ethanol is produced (Ohgren *et al*., 2007; Sun and Cheng 2002).
5) Less reactor volume because a single reactor is used (Sun and Cheng, 2002; Krishna and Chowdary, 2000).

2.5.1.4 Simultaneous saccharification and co-fermentation (SSCF).

SSCF can ferment both hexoses and pentose in a single bioreactor with a single microorganism. The production of the cellulase, the rate of saccharification, and the co-fermentation of hexose and pentose sugars are crucial parameters for SSCF (Chen *et al*, 2011; Mosier *et al*., 2005; Piccolo and Bezzo, 2007). Genetically modified microorganisms (*Saccharomyces cerevisiae*) are generally used in SSCF as they can ferment pentose sugars as well as mono- and disaccharides (Jin *et al*., 2012). SSCF is generally considered to be superior to SHF, although it suffers from similar problems to SHF such as differences in optimum fermentation and saccharification temperatures in addition to inhibition of enzymes by ethanol (Carr, 2012)

2.5.1.5 Ethanol from pentose (C5) sugars

Unlike in the hydrolysis of cellulose where glucose is produced, the pretreatment of hemicellulose produces pentose sugars. Many microorganism cannot easily convert the five carbon sugars typically comprising hemicellulose to ethanol. If this portion is not used it adds substantial costs for waste disposal to the process and results in significant loss of potential revenue. The hemicellulosic hydrolysate generated in the pretreatment (usually dilute acid or steam explosion) will be converted to ethanol. A number of yeasts such as *Candida shehatae, Pichia stipilis and Pachysolen tannophilus* convert xylose to ethanol (Silva *et al.*, 2010). Various bacteria and fungi can also ferment xylose and other pentose sugars into ethanol (Wyman, 1994). Another approach that has been used to convert xylose to ethanol is to produce an enzyme known as xylose isomerase that will convert xylose into an isomer called xylulose (Chaing *et al*., 1981). The enzyme can be produced by genetically engineering the common intestinal bacterium *Escherichia coli* so that it can produce large quantities of xylose isomerase under controlled conditions (Taherzadah *et al*., 2007). The xylose, the enzyme and the yeast are added to the vessel simultaneously because isomerisation reaction equilibrium is limited to 5-10% conversion

levels (Lastic *et al*., 1980), by having yeast present the equilibrium is shifted towards ethanol production, ethanol yields of up to 70% can be achieved. Some bacteria including *Escherichia coli* have been directly engineered to ferment xylose and other 5-C sugars to ethanol (Wyman, 1994).

Butanol is another biofuel which can also be obtained from hemicellulose bioconversion. Butanol is an excellent feedstock and a superior fuel as compared to ethanol. Butanol can be produced by fermenting dilute sulfuric acid hemicellulose hydrolysates produced from wastes such as barley straw, corn stover and switch grass. A variety of microorganisms are able to convert hemicellulose sugars to butanol, the most commonly used strains are *Clostridium acetobutylicum* and *Clostridium beijerinkii* (Qureshi *et al.*, 2010; Qureshi *et al*., 2010).

Xylitol, a five carbon sugar alcohol that can be used as a natural food sweetener, and as a sugar substitute for diabetics, may be produced by the fermentation of xylose present in hemicellulose hydrolysate (Musato and Robert, 2004). Many microorganisms are able to generate xylitol from xylose, among them are*, Candida guilliermondii* yeast, which has been the most employed due to its high conversion efficiency. Besides xylitol, arabitol, a polyalcohol, may also be produced by fermentation of hemicellulose hydrolysates. Yeasts such as *Candida entomaea* and *Pichia guilliermondii* have demonstrated the ability to convert both pentoses, xylose and arabinose to xylitol and arabitol respectively (Saha *et al*., 1996).

Recent technologies are focusing on development of recombination yeast, which can mobilize all forms of sugars, to improve ethanol production and reduce operational costs. Two approaches have been used: the first approach has been to genetically modify yeast and other natural ethanologens to have additional pentose metabolic pathways. The second is to improve yields by genetic engineering in microorganisms that have the ability to ferment both hexoses and pentoses (Hisham and Mageed, 2008).

2.5.1.6 Ethanol purification

Bioethanol obtained from the above mentioned fermentation processes requires further separation and purification of ethanol from water through a distillation process. Fractional distillation is the process implemented to separate ethanol from water based on their different boiling points. This process consists mainly of boiling the water-ethanol mixture, because water has a higher boiling point (100°C) than ethanol (78.3^{o}C), ethanol will be converted to vapour before water, thus water can be separated via a condensation procedure and ethanol distillate recaptured at a maximum concentration of 95% (Limayem and Ricke, 2012). Anhydrous ethanol (99.0%), which can be mixed with gasoline, can be obtained by the use of pervaporation or membranes (Hisham and Mageed, 2008). Lignin represents the main residual solid from the process. Other minor residues include holocellulose compounds and cell mass. A number of co-products from lignin, such as vanillin, phenol and high octane hydro-carbon fuel may be important to the competitiveness of the process. Production costs and market values of these products are complex. However all the residual solids can be deployed for production of heat and electricity (Hisham and Mageed, 2008).

2.5.1.7 Bioethanol application

Ethanol is used for production of alcoholic beverages, for industrial purposes (as a solvent, detergent, or chemical feedstock) and in recent years as a blending agent with gasoline to increase octane and reduce carbon monoxide and other smog causing emissions (Petrova and Ivanova, 2010).

Fuel ethanol can be used in a variety of ways. Ethanol is commonly used as an oxygenated fuel additive to reduce emissions of carbon monoxide, nitrous oxide and hydrocarbons. Numerous ethanolic motor fuel formulations are being used with increasing frequency. Ethanol has a higher octane rating than petroleum fuels enabling combustion engines to run at higher compression ratios and thus give superior

net performance. In addition ethanol exhibits higher vapour pressure and heat of vaporisation than gasoline and therefore increased power outputs observed while using ethanol (Petrova and Ivanova, 2010; Zaldivar *et al*., 2001).

2.6 Other products from lignocellulose metabolism

Analogous with the raw materials such as petroleum and natural gas in the oil refinery, the biorefinery operates with cellulose, hemicelluloses, lignin and starch as raw materials. The sugar polymers are converted to mono or dimeric sugars. Six carbon sugar platforms can be accessed from sucrose or through the hydrolysis of starch or cellulose to give glucose. Glucose can be used as a feedstock for (biological) fermentation processes providing access to a variety of important chemical building blocks. Mixed six and five carbon platforms are produced by the hydrolysis of hemicelluloses. The fermentation of these carbohydrates streams can in theory produce the same products as six carbon sugar streams.

These sugars may be used for the production of organic acids (Villasden, 2003). Microbial production of organic acids may be used to produce building blocks for industrial processes. Most of the acids are natural products of microorganisms or at least natural intermediates in major metabolic pathways. Of special interest in bio refining are these platform intermediates chemicals from fermentation that can be converted into numerous consumer and industrial products, including, succinic acid, butanol, itaconic acid, 1,3 propanediol, polyhydroxyalkanoates, 3-hydroxypropionic acid, lactic acid, citric acid, acetic acid, gluconic acid, malic acid, some of which are briefly discussed below. . Because of their functional groups organic acids are extremely useful as starting materials for the chemical industry.

2.6.1 Lactic Acid

Lactic acid is the most widely occurring multifunctional organic acid. It is used in the food and pharmaceutical industry. One of its most promising applications is in its use for biodegradable and biocompatible polylactate polymers an environmentally friendly alternative to non-biodegradable plastics derived from petrochemicals. The application of lactic acid polymers range from packaging material to textile fibres and biomedical applications, these products have the advantage that they are biodegradable (Neurieiter *et al*., 2004). The economics of lactic acid production by fermentation is dependent on many factors, of which the cost of raw materials is very significant. It is expensive when sugars such as glucose, sucrose and starch are used as feedstock for lactic acid production. Therefore lignocellulosic biomass is a promising feedstock in the production of lactic acid considering its great availability, sustainability, and low cost compared to refined sugars (Abdel-Raman *et al*., 2011). Lactic acid bacteria are usually used in the production of lactic acid. Bioconversion of hemicellulosic sugars to lactic acid requires a strain which is capable of fermenting sugar mixtures of hexoses and pentoses. *Lactobacillus pentosans* has been successifully used (Dien *et al*., 2002; Picatagio *et al*., 1998). *L. pentosans* ferments hexoses using the Embden Meyerhof-Parnas (EMP) pathway (Figure 2.9b) and pentoses using the phosphokinase (PK) pathway (Figure 2.9a) (Buyondo and Liu, 2011).

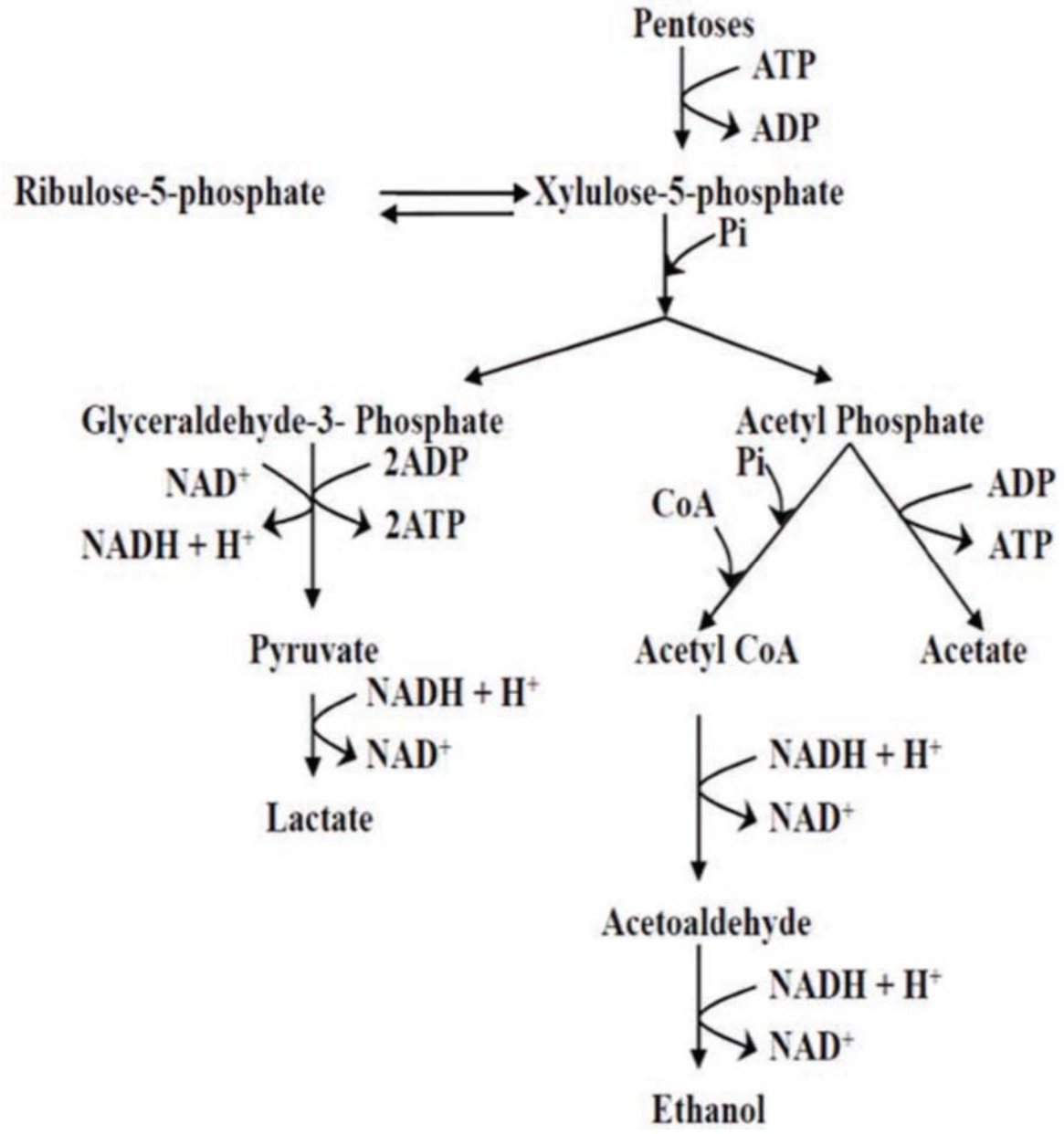


Figure 2.9a Heterolactic fermentation of pentoses. (Adapted from Buyondo and Liu, 2011)

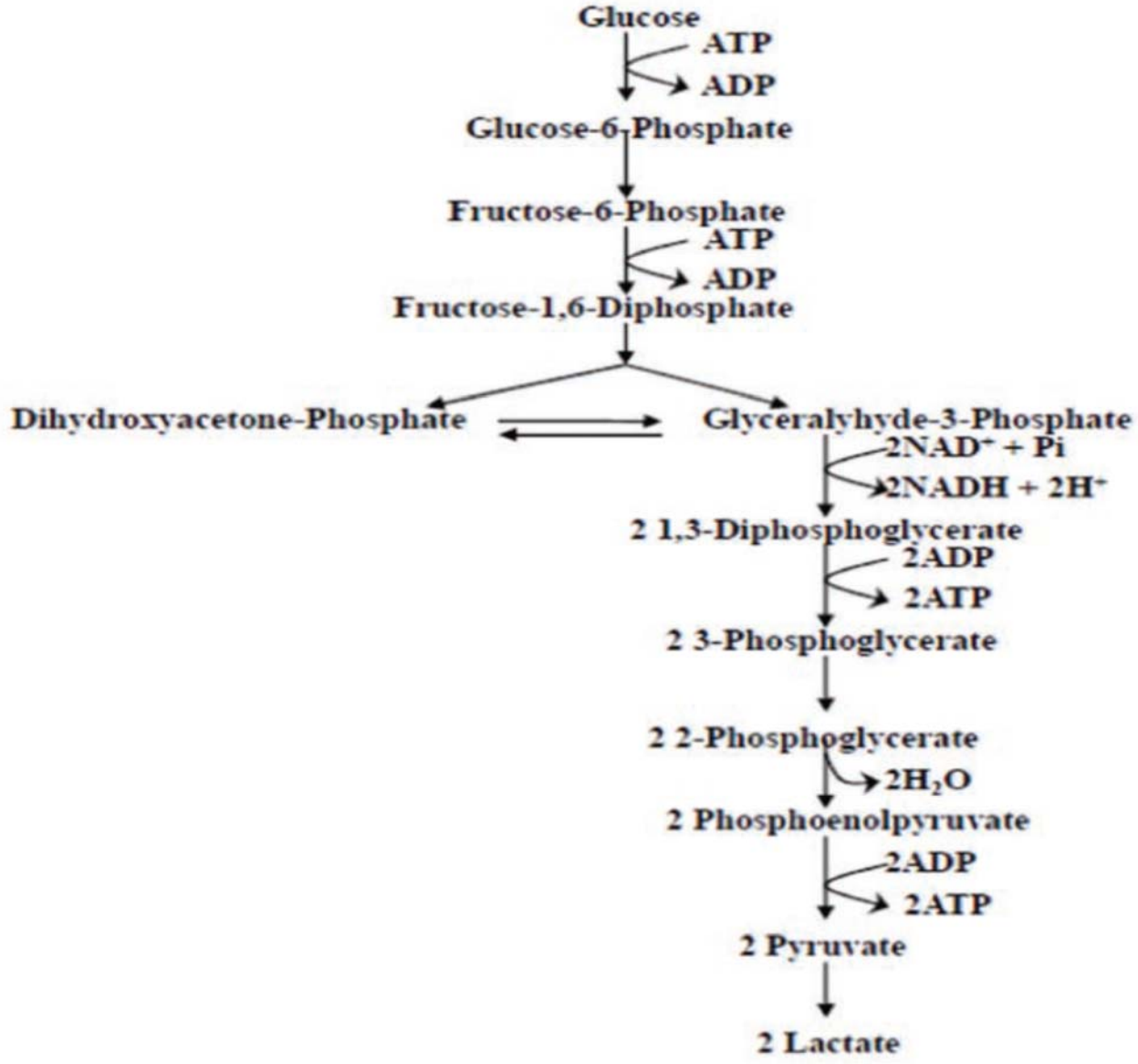


Figure 2.9b: Homolactic fermentation of glucose. (Adapted from Buyondo and Liu, 2011)

2.6.2 Citric acid

Citric acid has been produced for thousands of years from *Aspergillus Niger. A. Niger* can access glucose, starch and cellulose based carbohydrates as carbon sources. The production of citric acid from *A. Niger* has now exceeded that from lemon extraction. Citric acid is used in the food and beverage industry, the

carbonated beverage industry accounted for 50% of the total citric acid consumption in 1990 (Magnuson and Lasure, 2004; Alvarez-vaquez *et al*., 2000). *A. niger* generates citric acid through the tricaboxylic acid (TCA) metabolic pathway. In addition to well established filamentous fungal species, yeast *Yarrowia lipolytica* has been recently developed as a microbial cell factory for citric acid production (Papanikolaou *et al*., 2006). Several other moulds such as *Penicillium luteum, P. citrium* and *Aspergillus wentii* are able to produce this acid from glucose (Mussato and Teixeira, 2010).

2.6.3 Itaconic acid

Itaconic acid (Figure 3.0) is one of the most promising substances within the organic acids. Itaconic acid can be regarded as a substituted acrylic or methacylic acid and is isomeric with cistaconic and mesaconic acids (Blatt, 1943). Itaconic acid is industrially produced by fermentation of starch hydrolysates or molasses by *Aspergillus niger* and *A. itaconicus* in yields of more than 200g/l (Kubicek and Christian, 2013). Its polymerised esters (methyl, ethyl and vinyl) are used in adhesives and coatings. Itaconic acid is also used in emulsion paints to aid in polymer adhesion and hardening agent for organosiloxanes which are used in contact lenses (Okabe *et al*., 2009),. Itaconic acid has two carboxyl groups (Figure 3.0), this makes it suitable to be incorporated into polymers. The problem with production from lignocelluloses is the necessity of high sugar concentration (Kubicek and Christian, 2013).

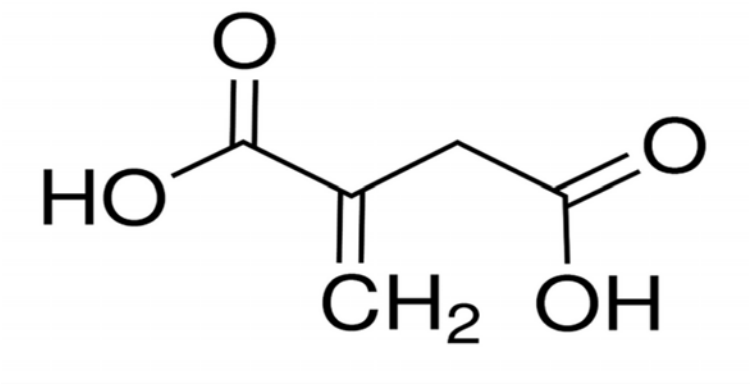


Figure 3.0: formula of itaconic acid. Adapted from MuralidharaRao et al, 2007

2.7 The advantages of using lignocellulosic biomass as a passive AMD treatment system.

a) Low operating costs and usually low capital costs (Greben *et al*., 2000).
b) Low maintenance and little supervision (Greben *et al*., 2000).
c) Uses ecological material to promote naturally occurring chemical and biological processes (Greben *et al*., 2000).
d) The AMD sludge containing cellulose can serve as a source of sugars for further bioprocessing (Sheridan et al., 2013).

2.8 Benefits of biofuels.

a) Lignocellulosic biofuels are made from non-edible plant material. Using lignocellulose to produce ethanol avoids the competition with the food industry as opposed to using first generation sugar and starch crops. Making food available and reducing food prices (Binod et al., 2011).
b) Cellulosic ethanol sources produce more than ten times as much energy as that is required to make them, this compares favourably to corn ethanol which produces 1, 36 times as much energy as the process energy put into corn ethanol production. Coal and gasoline provide

slightly less than the energy put into them, while electricity only produces half the energy put into it (Nigam and Sigh, 2011).

c) Cellulosic ethanol is better than corn ethanol because of the comparative accessibility and abundance to forest, agricultural and other cellulosic resources (Demers *et al*., 2009).

d) Cellulosic biofuels are considered the most strategically important sustainable fuel sources and are considered an important way of progress for limiting greenhouse gas emissions, improving air quality and finding new energy sources. They also encourage better waste utilisation, reduce local waste pollution and reduction in landfill sites (Nigam and Sigh, 2011).

e) Important in meeting rural development goal, employment creation and price stability (Nigam and Sigh, 2011) .

3. Linking bioremediation to bioprocessing.

The literature review has highlighted the need for a cheaper and effective way of treating AMD. The use of lignocellulosic material in the remediation of AMD has been investigated. Lignocellulosic material which has been used include: sawdust, hay, alfalfa and wood chips. The efficiency of the cellulosic substrates for the biological treatment of AMD have been confirmed by several studies (Tuttle *et al*., 1969a, 1969b; Chang *et al.,* 2000; Johnson and Hallberg 2005b; Neculita *et al*., 2007). The residual lignocellulosic biomass left after AMD treatment still contains a lot of cellulose, which is a potential source of reducing sugars for bioprocesses. At present this residue is treated as waste and needs to be disposed. In this study, the proposal is to take advantage of the structural changes caused by the AMD to the switch grass to further process this waste residue and recover the sugars in the cellulose. The morphological structure of the residual lignocellulose is altered by the sulfuric acid during incubation with AMD. This morphological alteration of the lignocellulosic material is similar to the changes effected during the pretreatment of lignocellulosic material using sulfuric acid. Acid pretreatment involves dilute and concentrated acid to breakdown the rigid structure of cellulose. The chemical pretreatment usually consists of diluted acids between 0.2-2.5%w/w to the biomass, followed by mixing at between 130-210°C. Depending on the conditions of the pretreatment, the hydrolysis can take a few minutes to hours (Brodeur *et al*., 2011). Sun and Cheng (2005), studied Bermuda grass and rye straw, after 48hrs of enzymatic hydrolysis of pretreated Bermuda grass and rye straw with 1,5% sulfuric acid, the total reducing sugars were found to be 197.1mg/g and 229.3mg/g of biomass respectively.

The changes which occur during acid pretreatment include the solubilisation of hemicellulose to sugars and possibly the rearrangement of lignin in the lignocellulose. These changes in the physical and chemical structure reduces the resistance of lignocellulose to both enzymatic and microbial hydrolysis.

Acid mine drainage contains sulfuric acid. The goal of this study is to use the sulfuric acid in the AMD to effect morphological changes in the lignocellulosic material, and to use a further enzymatic step to recover the sugars from the AMD treated lignocellulose residue (Sheridan *et al*., 2013). This study will therefore integrate the AMD treatment process to the lignocellulosic biofuel production process.

If successful this this investigation will be able to:

1) Treat AMD using lignocellulosic material
2) Use the lignocellulose residue from AMD for further enzyme hydrolysis and bioprocessing (Sheridan et al., 2013)

This project will reduce the cost of both AMD treatment and biofuels production by using readily available material in the two processes.

3.1 AIM.

1) To successfully use lignocellulosic biomass for the remediation of AMD.
2) To successfully use AMD for the pretreatment of cellulosic biomass
3) To utilize cellulases to further hydrolyse the pretreated biomass.
4) To successfully ferment the AMD residue containing hydrolysed cellulose to produce bioethanol.

3.2 Hypotheses and questions

1) What is the maximum capacity of cellulosic feedstock needed to treat a given volume of AMD?
2) Is it possible to ferment the hydrolysed cellulose contained in the AMD residue to produce bioethanol?

4.0 Research Design and Methods

4.1 Materials.

4.1.1 Biomass:

Sugarcane bagasse was originally obtained from Illovo Sugar Company based in Kwazulu Natal. Its harvest date was unknown. It was previously stored at the MCB laboratory at room temperature for some months.

The switch grass, *Panicum virgitum* (an indigenous perennial grass) was obtained milled from the chemical engineering laboratory. The second batch of switch grass was collected from the African Leadership Academy garden. It was obtained already dry.



Figure: 4.1 Dried and milled switch grass



Figure: 4.2 dried switch grass cut into 2-5cm particles

4.1.2 Enzymes:

The enzyme (cellulase) was supplied by Yakult Pharmaceutical Ind. Co., Ltd from Tokyo, Japan. The enzyme was obtained in powdered form. The product name of the enzyme is Cellulase “Onozuka” FA. The enzyme was composed of cellulase 12% and lactose 88%.

4.1.3 Equipment.

The equipment used for this study included an easy sense software pH meter, a bench top centrifuge-model HKSC-220 globe, Prestige medical autoclave.2100 classical clinical autoclaves and water bath located at African Leadership Academy biology laboratory in Honeydew, a desk top electronic pH meter, a 37 degrees Celsius incubator and Shaker located in the MCB laboratory. The High Performance Liquid Chromatography (HPLC) and the UV-VIS Spectrophotometer (spectroquant 300 from Merck) are located in the chemical engineering building at the University of Witwatersrand in Johannesburg. An FEM Quanta 400 scanning electron microscope located in the Microscopy and microanalysis unit in the biology building at the University of Witwatersrand in Johannesburg.

4.2 Biomass pre-treatment and lignocellulosic digestion.

4.2.1 Pre-treatment experiments and enzyme hydrolysis.

a) **Remediation of AMD**: Dried and untreated sugarcane bagasse and switch grass were used as the lignocellulose source in the remediation of AMD. One batch of switch grass was manually cut into 2-5cm sizes. The other batch was obtained already milled into a course powder and was passed through a 2mm sieve. Prepared bagasse was obtained in particles of less than 5cm. The composition of the Simulated AMD used in this study is as shown in table 4. The pH, sulphate and iron content were modelled on the findings of Bell et al., 2001. The components making up the simulated AMD are shown in Appendix 1. The characterization of the SAMD included pH measurement using an electronic pH meter. Iron concentration was done using spectrophotometry and glucose was measured by HPLC. The organic waste material was mixed with simulated AMD in 5 liter containers. A parallel series was run using distilled water. The

liquid to solid ratio was 13:1. A control experiment with only simulated AMD was also set up. The containers were sealed and allowed to incubate anaerobically at room temperature for increasing periods of time (Figure 4.3 and 4.4). During the experiment pH and dissolved iron were measured at increasing time intervals.

Table 4: Characteristics of simulated AMD

Parameter	pH	Iron	Sulphate
Unit	2,11	500mg/l	3000mg/l





Figure 4.3: Switch grass at day 5 of treatment; Figure 4.4: Switch grass at day 20 of treatment

b) **Measuring dissolved iron**: The dissolved iron concentration was analyzed using the Merck test kit number 114761 and the Merck Spectroquant Pharo 300 according to the manufacturer's manual.

c) **Release of glucose during pretreatment**: Samples were taken to determine the release of glucose during pretreatment. The samples were then incubated at 95°C for three hours in a bench top water bath to complete the hydrolysis. Sodium hydroxide was then used to neutralize the samples to pH between 5,5-6,5.The sample were then centrifuged for 20 minutes at 6000RPM in a desk top centrifuge. Centrifuging was done to remove the precipitated sulfate and

other solids (lignin) from the liquid containing glucose. The liquid was then sterile ultra-filtered using a 0.45 micrometer filter. The amount of glucose was determined by HPLC.

d) **HPLC**: was carried out using a 1200 series Agilent HPLC. The HPLC was operated using a 0,0001M solution of sulphuric acid as the mobile phase at a flow rate of 0,8ml/min, a biorad fermentation monitoring column of 60 degrees and a RID detector at 35 degrees. The chromatographs were analysed using chemsoft software. The analysis was calibrated using glucose and xylose. Four calibrations were made for each compound using dilutions of the standard. Chromatograms for the standards are shown in Appendix 3 and 4. Four known concentrations of glucose were analysed and used to form a linear calibration curve for glucose using the software (Appendix 5).

e) **Enzymatic hydrolysis**: The milled switch grass series was chosen for enzymatic hydrolysis since it effected the greatest remediation on the simulated AMD (pre-treated). The parallel series with distilled water was used as a control (untreated). The simulated AMD treated sample was divided into four:

   1) 8g pre-treated switch grass + 0% enzyme+ buffer.
   2) 8g pre-treated switch grass + 2, 5% enzyme + buffer.
   3) 8g pre-treated switch grass + 5% enzyme +buffer
   4) 8g pre-treated switch grass +10% enzyme + buffer.

   The series with distilled water was also divided into 4 subsets, one without enzymes and the others containing 2,5%, 5% and 10% enzyme/buffer solutions. The buffer solution, pH 6, was made from KH_2PO_4 and K_2HPO_4.

   Prior to the addition of the enzyme and buffer to the reaction vessels, the vessels and the weighed biomass were autoclaved at 121°C for 20 minutes. The buffer components were autoclaved separately. This was done to kill and inhibit the proliferation of microbes which

would deplete the glucose released from the hydrolysis. The reaction flask were tightly sealed and incubated at 50 degrees Celsius with shaking at 120 RPM for 24 hours. Clear filtrates containing the digest were obtained by ultrafiltration (0.45 micrometres). Filtrates were analysed for glucose by HPLC.

F) **Scanning Electron Microscope (SEM):** The scanning was done using an FEI Quanta 400 E-SEM. The samples from both the water treated and the AMD treated before and after enzyme hydrolysis were sun dried for one week. The samples were carbon coated in a vacuum chamber in order to make them conductive and to enable better scanning. The Samples were placed on a brass stage and put into a vacuum chamber of the microscope and analysed using different magnifications.

5. Results

5.1 Dissolved iron and pH changes

Figures 5.1 to 5.3 below shows the decrease in the dissolved iron concentration and increase in pH during treatment of AMD using different lignocellulosic biomass (milled switch grass (2mm), switch grass (particle size <5cm) and sugarcane bagasse) over the 14 week experimental period. In the control, Figure 5.4, the pH and the dissolved iron concentration remained relatively constant over the experimental period.

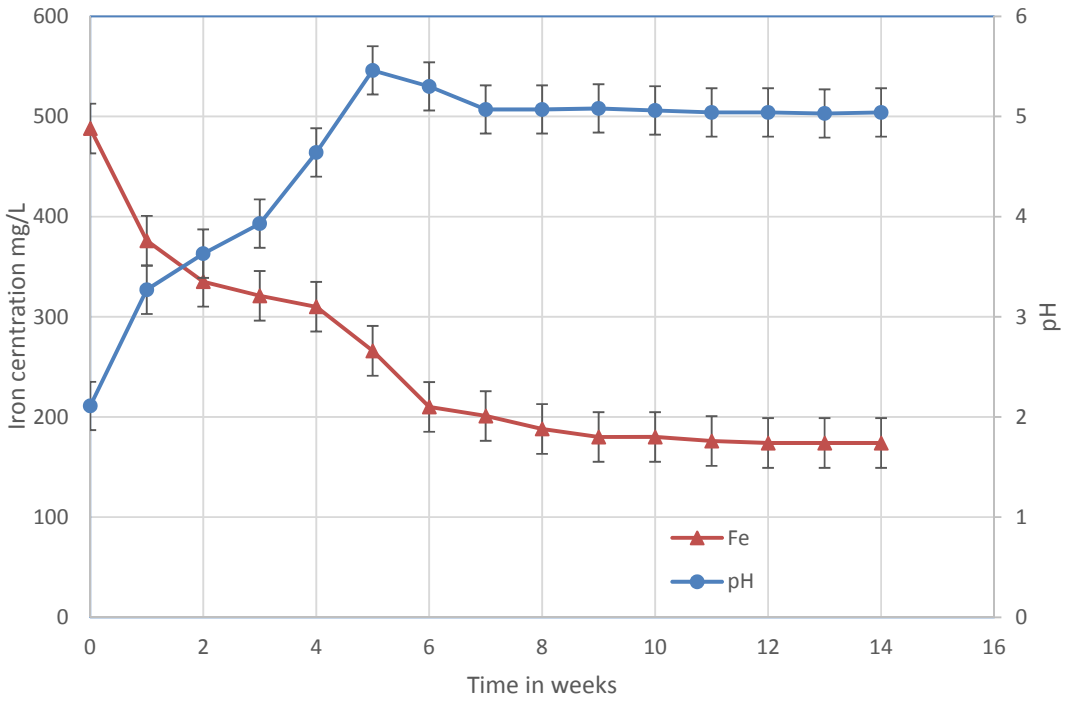


Figure 5.1: Changes in pH and dissolved iron concentration as effected by milled switch grass (2mm)

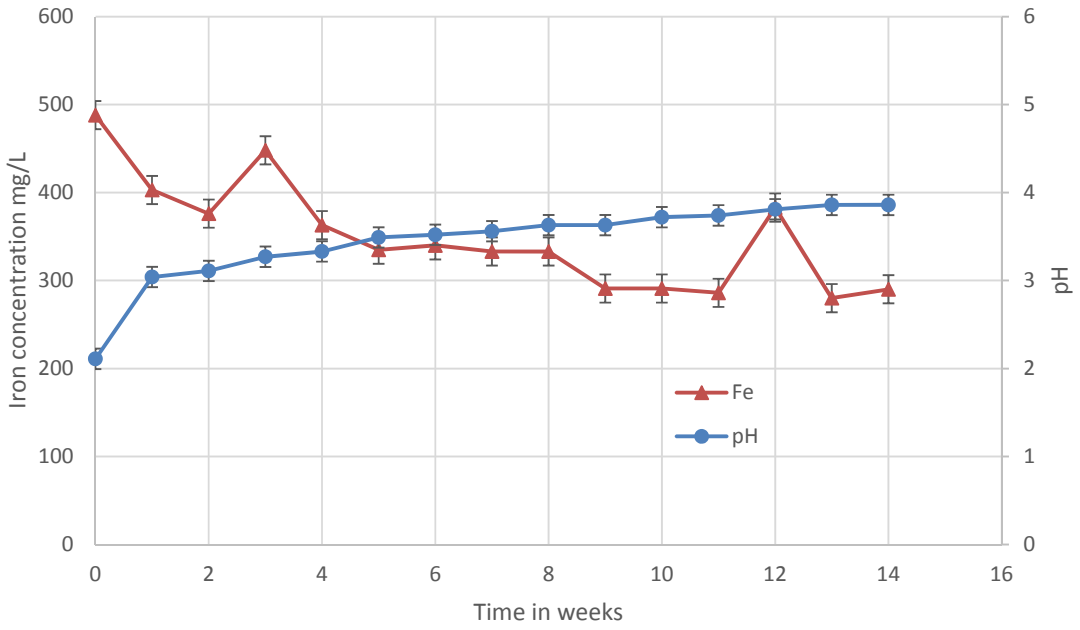


Figure 5.2: Changes in pH and iron concentration as effected by switch grass (5cm particle size)

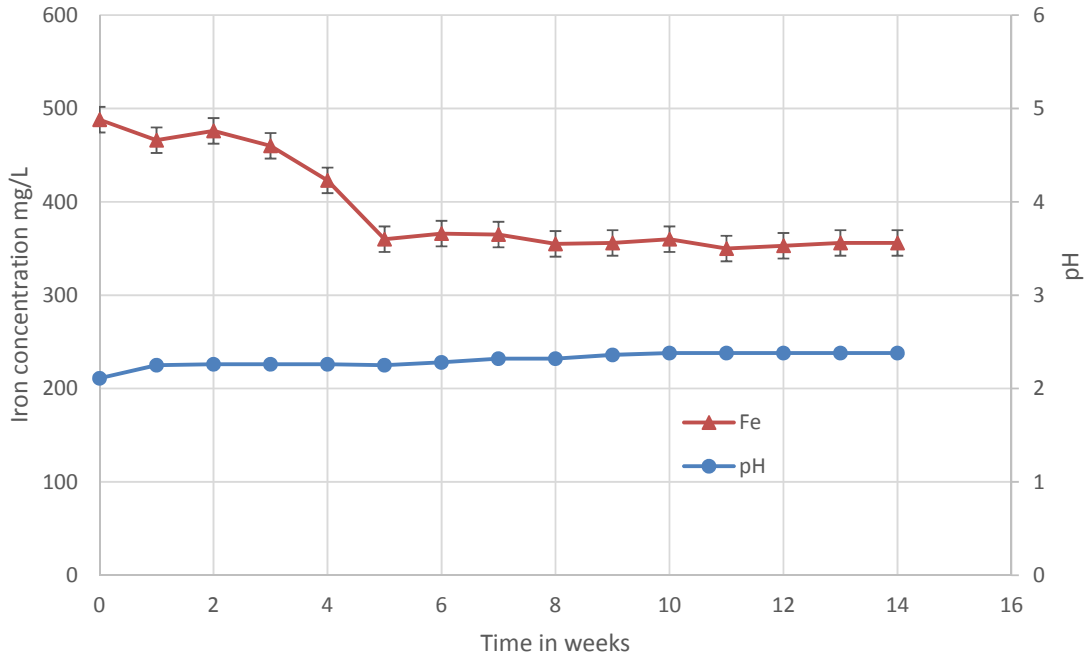


Figure 5.3: Changes in pH and iron concentration as effected by sugarcane bagasse.

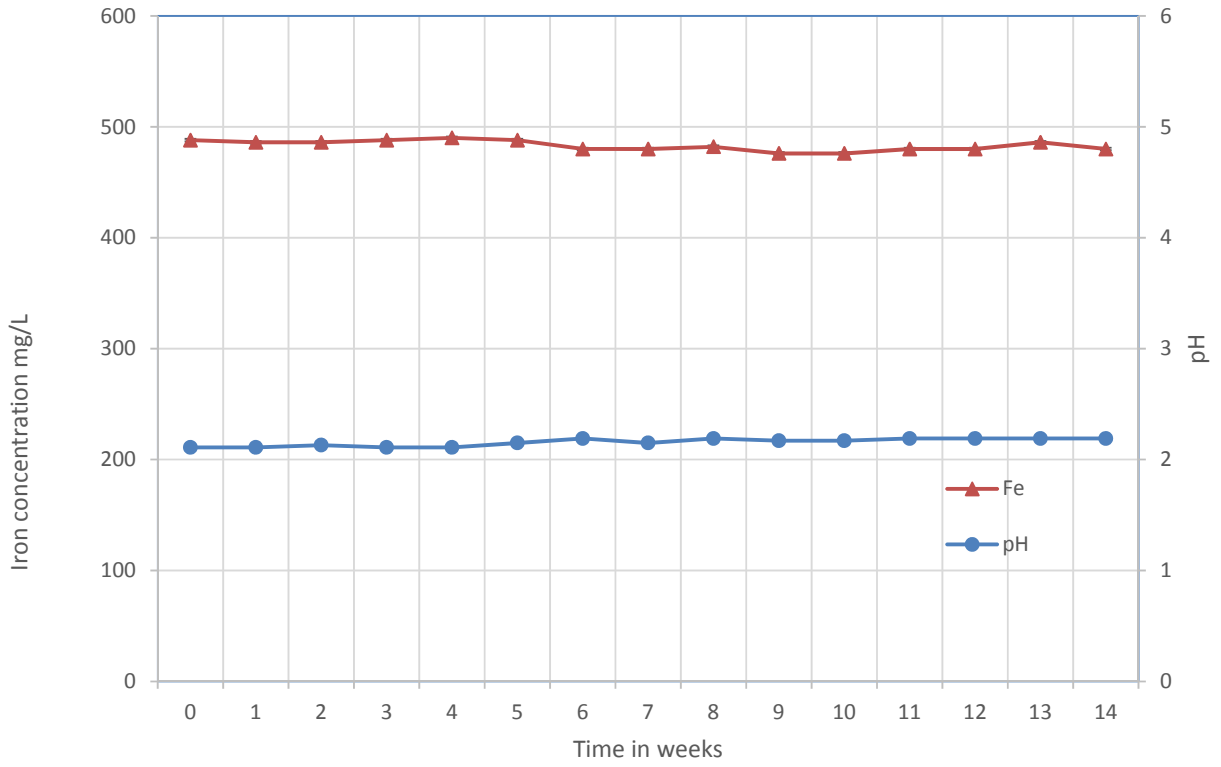


Figure 5.4 Changes in pH and iron concentration in the control reactor. The control reactor contained only AMD at an initial pH of 2.11 and iron concentration of 488.

The data in Figures 5.1 to 5.3 indicate that the three sources of cellulosic biomass were able to effect some remediation of AMD. In all three cases (Figure 5.1 to 5.3) dissolved iron concentration was reduced and pH was increased. The milled switch grass (2mm) was the most effective in the remediation of AMD. The milled switch grass was able to remove 64% of dissolved iron and increase the pH from 2.11 to 5.30 (Figure 5.1). The maximum pH was obtained in a relatively shorter period of time (5 weeks). The switch grass (<5cm) was able to remove 42% of dissolved iron and increased the pH from 2.11 to

3.86 (Figure 5.2). Sugarcane bagasse had the least remedial effect on AMD. Sugarcane bagasse removed 28% of iron from AMD and increased the pH from 2.11 to 2.36, this was achieved in 14 weeks (Figure 5.3). The pH and the iron concentration of the control which contained only AMD did not change much during the experimental period, indicating that the Biomass (switch grass and bagasse) caused the increase in alkalinity and the decrease in dissolved iron concentration. Similar results were obtained by Ramla (2012). Ramlas' investigation used two indigenous grass species, *Hyparrheia hirta* and *Setaria spacenta*. Maximum pH values as high as 8.48 and percentage removal of iron from the AMD solution was reported at a maximum value of 90%.

The pH of the AMD seem to be critical for the efficient removal of metal ions from AMD as increase in pH resulted in a marked decrease in iron concentration (Figure 5.1). Santos *et* al., (2003) worked with different biomass concentrations and showed that increase of biomass concentration of up to 30g/l leads to an increase in ferrous ion concentration and a reduction in the total concentration of iron (ferric and ferrous) in the AMD solution. Chockalingam *et al*., (2005) reported sulfate reduction from 550mg/l to 100mg/l and iron reduction of 86% in 20 days using rice husk filtrate to treat AMD.

These values are comparable with the ones reported in literature for the biosorption and precipitation of metals on switch grass and other biomasses. According to literature the main mechanisms of metal ion removal in bioreactors are precipitation in the form of sulfides, hydroxides and carbonates and sorption mechanisms such as adsorption (Neculita *et al.*, 2007; Hamla, 2012). The adsorption mechanism is supported in this investigation by the fact that decrease in particle size of switch grass was accompanied by a large reduction in the dissolved iron concentration and the high values of pH obtained, also the milled switch grass took a relatively shorter time to reach the maximum pH values.

The observed increase in the amount of dissolved iron removed from the AMD solution can be explained by the presence of a larger number of active sites on the milled switch grass due to the increased

surface area effected by the milling action. Functional groups capable of metal sorption in biomass include phenolic, hydroxyl and carboxylic groups. Milling exposes these functional groups to the dissolved ions.

These functional groups in the biomass are protonated at higher pH and presumably available for binding dissolved metals (Neculita *et al*., 2007). This explains the fact that the increase in pH is accompanied by a marked decrease in dissolved iron concentration (Figure 5.1).Increases in pH of acidic water effectively removes some metals due to the precipitation of metals in the form of hydroxides (Goatham, 2013).

Microbial activity seem to play a role in the remediation of the AMD. Microbial activity was observed in the two switch grass series with the milled switch grass seeming to support more growth. This microbial population could be sulfate reducing bacteria (SRB). As explained by Ramla (2012), the increase in pH can be directly related to SRB population growth. The substrate may have been providing nutrients to sulfate reducing bacteria which contributed to the growth of the microbial population. The SRB activity reduces sulfate to sulfide and produces hydrogen carbonate (Equation 12). The hydrogen carbonate partially neutralises the AMD, while the hydrogen sulfide reacts with dissolved metals in the AMD, resulting in the precipitation of metals as metal sulfides (Equation 13) (Taylor *et al*, 2005).

$SO_4^{2-} + 2CH_2O + 2H^+ \rightarrow H_2S + 2H_2CO_3$ (Equation 12)

$H_2S + M^{2+} \rightarrow MS + 2H^+$ (Equation 13)

Where CH_2O are the sugars and M^{2+} represents metal ion.

The partially treated AMD may be further neutralised in a limestone reactor to near neutral pH and discharged into water ways.

5.2 The release of glucose during the treatment of AMD using milled switch grass (2mm)

The release of glucose was observed only in the milled switch grass, suggesting that milling was a significant factor in the generation of glucose. Milling reduces cellulose crystallinity and disrupt the lignin-carbohydrate complexes, which aids the acid treatment process and the subsequent enzyme hydrolysis (Mais *et al*., 2002: Carmen *et al*., 2013). The glucose increased initially after two weeks of incubation (Figure 5.5). A decrease in glucose concentration occurred after 8 weeks of incubating the switch grass with AMD. The generation of glucose and other reducing sugars during AMD treatment is attributed to acid catalysis, where the sulfuric acid in the AMD catalyses the breakdown of cellulose and hemicellulose to release glucose, xylose and other degradation products. The AMD pretreatment of lignocellulose allows the recovery of the hemicellulose sugars. The hemicellulose, mainly mannan and xylan, accounts for up to a third of the total carbohydrate in many lignocellulosic material (Pingali *et al*., 2010).

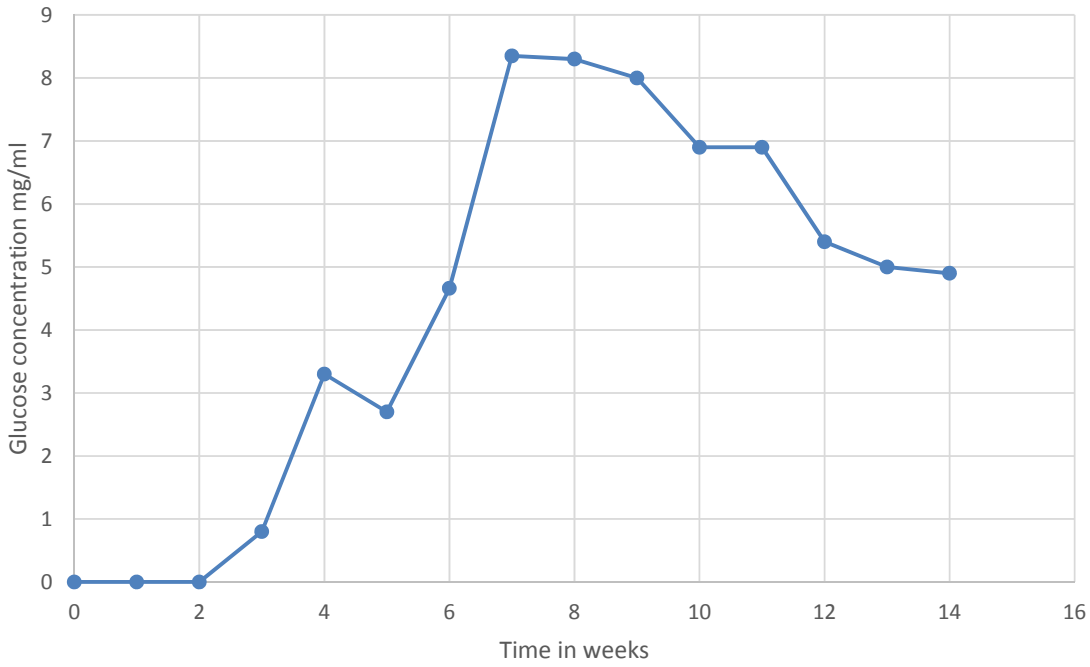


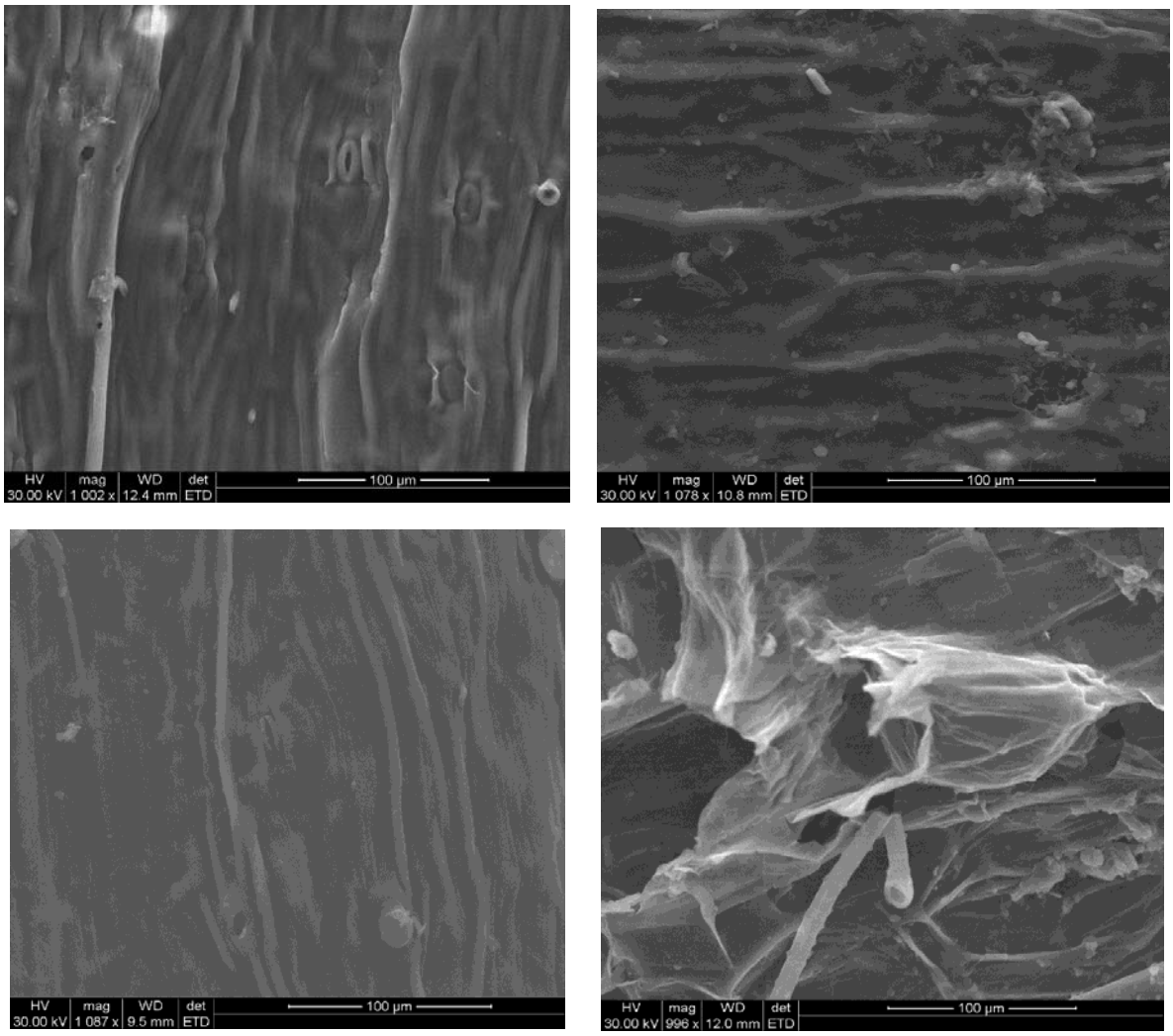
Figure 5.5: The release of glucose during treatment of AMD using milled switch grass.

In Figure 5.5, glucose started to appear in the third week of treatment. A rapid increase in glucose concentration was observed. The maximum amount of glucose obtained was 8.35mg/ml in the seventh week. A small decrease in glucose concentration occurred which was at 4.9mg/ml after 14 weeks. The decrease in glucose concentration may be explained by the consumption of glucose by SRB. A study by Roman (2004) reported high levels of reducing sugars (306g/l) production which was followed by consumption. This was accompanied by the increase in pH from 5.8 to 7. The increase in pH corresponded to the consumption of reducing sugars and may be related to sulfate reduction and the production of alkalinity within the bioreactor.

5.3 Scanning electron microscope (SEM) analysis of switch grass treated by water and AMD before and after enzymatic hydrolysis.

SEM was used to study the morphological features and surface characteristics of the switch grass treated by water and AMD before and after enzyme hydrolysis (Figure 5.6).

a *b*



c *d*

Figure: 5.6 Scanning electron microscope images of switch grass (a) water treated, (b) AMD treated, (c) water treated followed by enzyme treatment and (d) AMD treated followed by enzyme treatment.

The SEM images revealed that pretreatment induced morphological changes in the switch grass. Water treated switch grass exhibited rigid and highly ordered fibrils (Figure 5.5a). The fibres of the AMD treated switch grass appear to be distorted with some holes evident on the surface (Figure 5.5b). This renders the switch grass more accessible to cellulase, resulting in higher enzymatic hydrolysis rates and cellulose digestibility (Moxley *et al.,* 2008: Remli *et al*., 2013). The molecular structure of the water pretreated and enzyme treated switch grass remained intact, showing no digestion occurred during the hydrolysis of the water treated switch grass. The molecular structure of the switch grass which was pretreated by AMD followed by enzyme digestion was completely destroyed (Figure 5.5d). The improved/altered morphological properties generated by AMD pretreatment of switch grass appeared to be the primary source for the enhancement of enzymatic hydrolysis (Goshadrou *et al*., 2011). These micrographs are in agreement with the HPLC results shown in Table 5.1 and 5.2 which show high digestibility of AMD treated switch grass and no or very little digestibility in the water treated switch grass.

5.4 Enzymatic hydrolysis of AMD treated and untreated switch grass

Tables 5.1 shows the HPLC analysis results, which show the concentrations of xylose and glucose after the enzymatic hydrolysis of the water treated switch grass.

Table 5.1: The release of glucose and xylose obtained from the enzymatic digestion of water treated switch grass using different enzyme concentrations.

Enzyme (%)	Xylose mg/ml	Glucose mg/ml
0	0	0
2,5	0	0
5	0	0
10	1.36	0

The water treated sample was taken as a control. It can be noted from Table 5.1 that the glucose yield for the water treated switch grass was zero for all the enzyme concentrations. The xylose yield was zero except for the 10% enzyme concentration which had xylose concentration of 1.36 mg/ml. The SEM images (Figure 5.6) showed that the water treated switch grass displayed no obvious signs of mechanical disruption of the general surface morphology. This rigid and compact structure of untreated lignocellulosic biomass hinders the accessibility of cellulase and prevent it from digesting the grass.

In table 5.2 HPLC analysis results of the enzymatic digestion of the AMD treated switch grass are shown. The results show the concentrations of glucose and xylose in mg/ml. The maximum sugar concentration achieved with the milled switch grass were 4.825 mg/ml glucose at 5% enzyme concentration and 3,35 mg/ml xylose at 10% enzyme concentration.

Table 5.2: The release of glucose and xylose from different enzyme concentrations on AMD treated biomass**.**

Enzyme%	Xylose mg/ml	Glucose mg/ml
0	0	0
0.5	1.97	0
2.5	3.29	3.795
5	0	4.825
10	3.35	3.693

The chromatograms of the results shown in Tables 5.1 and 5.2 are shown in Figure A2 to Figure A14 in the appendix.

As expected from literature, the enzymatic hydrolysis of AMD pretreated switch grass yielded greater yields of both glucose and xylose than water pretreated switch grass. The maximum yields of glucose was 4.85mg/ml and 3.35mg/ml xylose for the AMD pretreated switch grass, while no glucose and 1.36mg/ml xylose production was observed in the water treated switch grass. The enzyme concentration has a great effect on the amounts of glucose and xylose obtained, there is a general increase of the glucose and xylose concentrations with the increase in enzyme concentration. Small quantities of enzymes were ineffective in effecting digestion to the treated switch grass, for example the switch grass treated with the 0.5% enzyme concentration produced 1.97 mg/ml xylose but did not produce any glucose. The AMD which contains sulfuric acid was able to effect some changes in the supramolecular structure of switch grass. According to literature (Zhang *et al*., 2007) hemicellulose is

removed when sulphuric acid is added to biomass and this enhances digestibility of cellulose in the residual solids. The AMD treatment of the switch grass was therefore able to significantly improve the accessibility of the cell walls to enzymatic digestion, leading to greater sugar yields compared with the water treated switch grass.

Table 5.3 provides a summary of results showing maximum values of pH, percentage iron removal and glucose concentration obtained during the treatment of AMD with switch grass and bagasse. The table also shows the maximum concentration of glucose obtained after the hydrolysis of the milled switch grass. Refer to Appendix 1+- for the comprehensive data recorded during the course of this investigation.

5.5 Summary of results

Table 5.3: Summary of pH, %Fe and sugar production during pretreatment and after enzyme hydrolysis.

Feedstock	pH	%Fe	Glucose (mg/ml) during pretreatment	Glucose(mg/ml) after enzyme hydrolysis
Switch grass(2mm)	5.30	62%	8.35	4.83
Switch grass(5cm)	3.86	42%	0	Not done
Bagasse	2.38	28%	0	Not done

6.0 Discussion

Switch grass has been shown in thi**s** study to have the capacity to effectively remove iron and acidity from AMD. The results from the three series used in this investigation indicate that milled switch grass (2mm) was more effective in remediating AMD than sugar cane bagasse or the large particle size switch grass (<5cm) (Table 5.3). The ability of the switch grass, the sugar cane bagasse and other lignocellulosic material to remove metals by adsorption is expected since such materials contain functional groups such as phenolic, hydroxyl, carboxyl and carboxylic (Harman *et al*., 2007). This theory is supported by Minawar and Riwandi (2010) who attributed the decrease in dissolved iron in the first two weeks of AMD treatment to iron retention by the organic matter rather than iron precipitation. In our study the milled switch grass which had a higher surface area was able to retain more iron resulting in lower dissolved iron concentration. Zagury *et al*., 2006 (cited in Munawar and Riwandi, 2010) explained that metal removal tends to occur earlier compared to sulfate reduction, through adsorption of metals to organic matter. Once reducing conditions are established metal precipitation becomes predominant. Munawar and Riwandi (2010) reported the decrease of sulfate concentration in the third week of their study, using chicken manure mixed with AMD. This was attributed to sulfate reduction to metal sulfides such as iron sulfide. In our study microbial activity seemed to dominate in the third week of AMD treatment supporting Munawar and Riwandis' findings that sulfate reduction occurs at a later stage of AMD treatment. As explained in section 1.7.3.3, SRB activity produces hydrogen carbonate which increases the alkalinity of the AMD.

The milled switch grass was able to increase the pH of the AMD from 2.11 to 5.30 and to remove dissolved iron from the AMD by 64% by the fifth week. Both the pH and the dissolved iron content started to stabilise thereafter. To further increase the alkalinity and to reduce the dissolved iron

concentration, periodic substrate loading may be considered in future for the long term remediation of AMD (Ramla 2012).

Santos *et al*., (2004) worked with different biomass concentrations and showed that an increase of the biomass (grape stalks) concentration up to 30g/L leads to a 20% reduction of the total content of dissolved iron in the AMD. In a similar study Greben *et al*., (2008) used cut grass serving as a carbon and energy source for continuous sulphate removal and obtained between 80 and 90% removal efficiency by adding cut grass loadings every two weeks. The increase in grass loadings provide new surface area for adsorption of metal ions, further reducing their concentration.

In this study the residue switch grass obtained from the AMD treatment process was prepared for a further enzymatic hydrolysis step. The preparation included washing to remove metal ions adsorbed to the surface. SEM was used to study the effects of the AMD on the switch grass. The results showed that AMD induces morphological changes in the switch grass (Figure 5.6). These changes are due to the hydrolysis of hemicellulose and to a lesser extent cellulose by the sulfuric acid in the AMD. The hemicellulose fraction is converted to its monomeric sugars which include pentose and glucose. This treatment is analogous to the pretreatment of lignocellulosic biomass using acid. In literature dilute acid pretreatments of lignocellulosic material are done at higher temperatures and pressure and are able to recover relatively higher amounts of fermentable sugars from the hemicellulose fraction of lignocellulose. Barrier et al., (1985) reported the conversion and recovery of sugars from the hemicellulose fraction treated with dilute acid to be more than 90% efficient. Dilute sulphuric acid in the range 0.5-1.5% and temperatures above 160 has been found the most favoured for industrial applications. Under these conditions high sugar yields from hemicellulose are obtained (Hisham and Mageed, 2008). In our study the conditions are mild (room temperature and pressure). Acid mine drainage is used instead of dilute sulphuric acid.

Glucose was detected during the treatment of AMD in the course of this study (Figure 5.5). A maximum glucose concentration of 8.35 g/ml was obtained in the seventh week of the study. After AMD treatment it is necessary to recover some of the sugars from the AMD/sugar liquor. The sugars in solution in the AMD are highly contaminated with metal ions and will need purification if they are to be recovered and to be used for further bioprocessing. It is difficult to recover the hemicellulose fraction of sugars from the AMD/sugar solution. The AMD/sugar liquor can be fermented to produce ethanol which can be recovered by distillation, or alternatively the AMD/sugar liquor could be used as a carbon and energy source for SRB in subsequent treatments. The remaining cellulose residue instead of being disposed, will be channelled to enzymatic hydrolysis. The cellulose residue contains the bulk of the glucose. Cellulases enzymes were used in our study to recover some of the sugars in the lignocellulose, with maximum values of glucose and xylose at 4.825 mg/ml and 3.35 mg/ml respectively. The glucose produced can be used to produce bioethanol. The xylose produced in the processing may also be fermented or used to produce other value added products. One possible use of the unconverted xylose is to produce fodder yeast (single cell protein) for animal feed (Barrier *et al*., 1985).

7.0 Conclusion

Lignocellulosic material has the capacity to effectively treat acid mine drainage by removing dissolved metal content and increasing its pH, if appropriate pretreatments such as milling of the material are provided. Comparison of the two grass species used in this study showed that switch grass had more capacity to remediate AMD than sugarcane bagasse. Milled switch grass was even more effective than unmilled switch grass, indicating that particle size is an important consideration in the remediation of AMD. As explained in section 5.1, decrease in particle size increases the surface area for dissolved metal ions to adsorb to. The milling process will add costs to the treatment of AMD, however it is necessary if maximum remediation effects on the AMD is to be realised. In this study the maximum pH obtained was 5.30 and iron removal was at 64% after 84 days. To increase the pH to neutral and to further reduce the iron concentration to levels acceptable to human consumption, further periodic loadings of switch grass can be done or alternatively a further neutralisation step can be done before the water is discharged into the waterways. Alternative lignocellulosic feedstocks can be tried to determine the most effective material for maximum remediation.

The AMD causes solubilisation of the hemicellulose. The solubilisation of hemicellulose changes the structural integrity of the cell walls making them amenable to further enzymatic hydrolysis. The solubilisation of hemicellulose was done under room temperature and ambient pressure making it impossible to recover all the hemicellulose sugars. Complete recovery of hemicellulose would require an additional cost of heating. The energy for heating could be provided by burning the digested residual switch grass (Demers *et al*., 2009). This could also improve the enzymatic digestion step. Recovery of the hemicellulose from the AMD-sugar liquor will require all the metal ions in solution to be precipitated, further increasing the cost of the process.

This study has evaluated the potential of AMD treated switch grass in the production of fermentable sugars. The results indicated that the AMD treated switch grass has the potential to produce usable amounts of glucose for ethanol production. AMD is not only being remediated but assists in a bioprocess to prepare second generation feedstock for industrial biotechnology.

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Appendix. 1

Table A1: Set up target for synthetic AMD composition.

Targets	
volume	20L
Fe	500mg/L
SO_4^{2-}	3000mg/L
pH	2,0
H^+	0,01mol/L
Iron/acid soln	2.0L
Base soln vol	1.0L

Table A2: Amounts required in the makeup of synthetic AMD

	g/L	mols
Fe	0,5	0,0089
$H_2SO_4^{2-}$	2,245	0,023
$FeSO_4.7H_2O$ added	2,246	0,0081 moles
SO_4^{2-} to be added w/H_2SO_4	2.2	0,0081
SO_4^{2-} added w/$FeSO_4$	0.8	0,9796
H_2SO_4 added	2,245	0,023

TableA3: Changes in pH as effected by the control experiment

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2,11	2,11	2,13	2,09	2,09	2,18	2,22	2,17	2,23	2,23	2,18	2,22	2,22	2,23	2,23
2	2,11	2,11	2,15	2,14	2,13	2,14	2,2	2,14	2,2	2,16	2,16	2,19	2,19	2,19	2,18
3	2,11	2,11	2,1	2,11	2,11	2,15	2,15	2,14	2,13	2,11	2,16	2,15	2,16	2,16	2,19
Mean	2,11	2,11	2,13	2,11	2,11	2,16	2,19	2,15	2,19	2,17	2,17	2,19	2,19	2,19	2,20
stdev	0,0000	0,0000	0,0205	0,0205	0,0163	0,0170	0,0294	0,0141	0,0419	0,0492	0,0094	0,0287	0,0245	0,0287	0,0216
Std Error	0,0000	0,0000	0,0119	0,0119	0,0094	0,0098	0,0170	0,0082	0,0242	0,0284	0,0054	0,0166	0,0141	0,0166	0,0125

Table A4: Changes in pH as effected by the milled switch grass

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2,11	3,29	3,7	3,99	4,7	5,47	5,44	5,08	5,08	5,08	5,04	5,04	5,04	5,04	5,05
2	2,11	3,29	3,61	3,92	4,63	5,42	5,2	4,98	5	5,1	5,09	5,04	5,04	5,04	5,04
3	2,11	3,22	3,57	3,89	4,59	5,48	5,26	5,15	5,13	5,06	5,04	5,04	5,04	5,06	5,03
Mean	2,11	3,27	3,63	3,93	4,64	5,46	5,30	5,07	5,07	5,08	5,06	5,04	5,04	5,05	5,04
Stdev	0,0000	0,0404	0,0666	0,0513	0,0557	0,0321	0,1249	0,0854	0,0656	0,0200	0,0289	0,0000	0,0000	0,0115	0,0100
Standard Error	0,0000	0,0233	0,0384	0,0296	0,0321	0,0186	0,0721	0,0493	0,0379	0,0115	0,0167	0,0000	0,0000	0,0067	0,0058

Table A5: Changes in pH as effected by bagasse

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2,11	2,22	2,24	2,21	2,21	2,25	2,3	2,29	2,3	2,33	2,35	2,32	2,33	2,33	2,29
2	2,11	2,31	2,23	2,33	2,22	2,22	2,25	2,29	2,3	2,4	2,41	2,4	2,41	2,4	2,42
3	2,11	2,22	2,3	2,25	2,32	2,28	2,28	2,37	2,37	2,36	2,42	2,42	2,41	2,42	2,42
Mean	2,11	2,25	2,26	2,26	2,25	2,25	2,28	2,32	2,32	2,36	2,39	2,38	2,38	2,38	2,38
Stdev	0,0000	0,0520	0,0379	0,0611	0,0608	0,0300	0,0252	0,0462	0,0404	0,0351	0,0379	0,0529	0,0462	0,0473	0,0751
Standard Error	0,0000	0,0300	0,0219	0,0353	0,0351	0,0173	0,0145	0,0267	0,0233	0,0203	0,0219	0,0306	0,0267	0,0273	0,0433

Table A6: Changes in pH as effected by the larger particle (5cm) switch grass

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	2,11	3,09	3,26	3,36	3,43	3,56	3,6	3,64	3,68	3,71	3,72	3,75	3,81	3,89	3,89
2	2,11	2,97	3,01	3,15	3,28	3,41	3,44	3,5	3,56	3,56	3,68	3,7	3,78	3,86	3,86
3	2,11	3,05	3,06	3,29	3,29	3,5	3,52	3,55	3,64	3,62	3,76	3,76	3,85	3,83	3,83
Mean	2,11	3,04	3,11	3,27	3,33	3,49	3,52	3,56	3,63	3,63	3,72	3,74	3,81	3,86	3,86
Stdev	0,0000	0,0611	0,1323	0,1069	0,0839	0,0755	0,0800	0,0709	0,0611	0,0755	0,0400	0,0321	0,0351	0,0300	0,0300
Standard Error	0,0000	0,0353	0,0764	0,0617	0,0484	0,0436	0,0462	0,0410	0,0353	0,0436	0,0231	0,0186	0,0203	0,0173	0,0173

Table A7: Changes in Fe concentration as effected by the control

Table A8: Changes in Fe concentration as effected by sugarcane bagasse

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	488	470	480	463	426	364	368	363	352	359	360	351	356	351	353
2	488	478	458	456	421	361	363	364	355	355	359	348	349	358	360
3	488	471	472	462	423	355	366	368	358	352	362	351	355	359	355
Mean	488,00	473,00	470,00	460,33	423,33	360,00	365,67	365,00	355,00	355,33	360,33	350,00	353,33	356,00	356,00
stdev	0,0000	4,3589	11,1355	3,7859	2,5166	4,5826	2,5166	2,6458	3,0000	3,5119	1,5275	1,7321	3,7859	4,3589	3,6056
Std.error	0,0000	2,5166	6,4291	2,1858	1,4530	2,6458	1,4530	1,5275	1,7321	2,0276	0,8819	1,0000	2,1858	2,5166	2,0817
stdev	0,0000	3,4641	2,3094	2,3094	3,6056	2,6458	6,6583	4,0415	3,0551	8,6217	4,5092	3,2146	5,5076	3,5119	6,0000
Std.error	0,0000	2,0000	1,3333	1,3333	2,0817	1,5275	3,8442	2,3333	1,7638	4,9777	2,6034	1,8559	3,1798	2,0276	3,4641

Table A9: Changes in Fe concentration as effected by the larger particle (5cm) switch grass

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	488	398	378	449	357	336	339	335	330	293	294	284	281	282	293
2	488	401	373	446	366	334	334	331	332	287	289	286	283	280	288
3	488	410	376	449	366	336	338	332	336	292	290	280	286	278	290
Mean	488,00	403,00	375,67	448,00	363,00	335,33	337,00	332,67	332,67	290,67	291,00	283,33	283,33	280,00	290,33
stdev	0,0000	6,2450	2,5166	1,7321	5,1962	1,1547	2,6458	2,0817	3,0551	3,2146	2,6458	3,0551	2,5166	2,0000	2,5166
Std.error	0,0000	3,6056	1,4530	1,0000	3,0000	0,6667	1,5275	1,2019	1,7638	1,8559	1,5275	1,7638	1,4530	1,1547	1,4530

Table A10: Changes in pH as effected by milled switch grass

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	488	377	339	321	303	268	215	203	194	184	170	180	171	181	181
2	488	375	336	318	315	269	206	200	187	180	172	175	176	171	171
3	488	377	331	325	311	261	210	200	182	176	168	173	175	171	170
Mean	488,00	376,33	335,33	321,33	309,67	266,00	210,33	201,00	187,67	180,00	170,00	176,00	174,00	174,33	174,00
stdev	0,0000	1,1547	4,0415	3,5119	6,1101	4,3589	4,5092	1,7321	6,0277	4,0000	2,0000	3,6056	2,6458	5,7735	6,0828
Std.error	0,0000	0,6667	2,3333	2,0276	3,5277	2,5166	2,6034	1,0000	3,4801	2,3094	1,1547	2,0817	1,5275	3,3333	3,5119

Table A5: Appearance of glucose during the pretreatment of the three biomass series using AMD.

t	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
bag	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
SG(2)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
SG(m)	0,0	0,0	0,0	0,8	3,3	2,7	4,66	8,35	8,3	8,0	6,9	6,9	5,4	5,0	4,9

Appendix 2

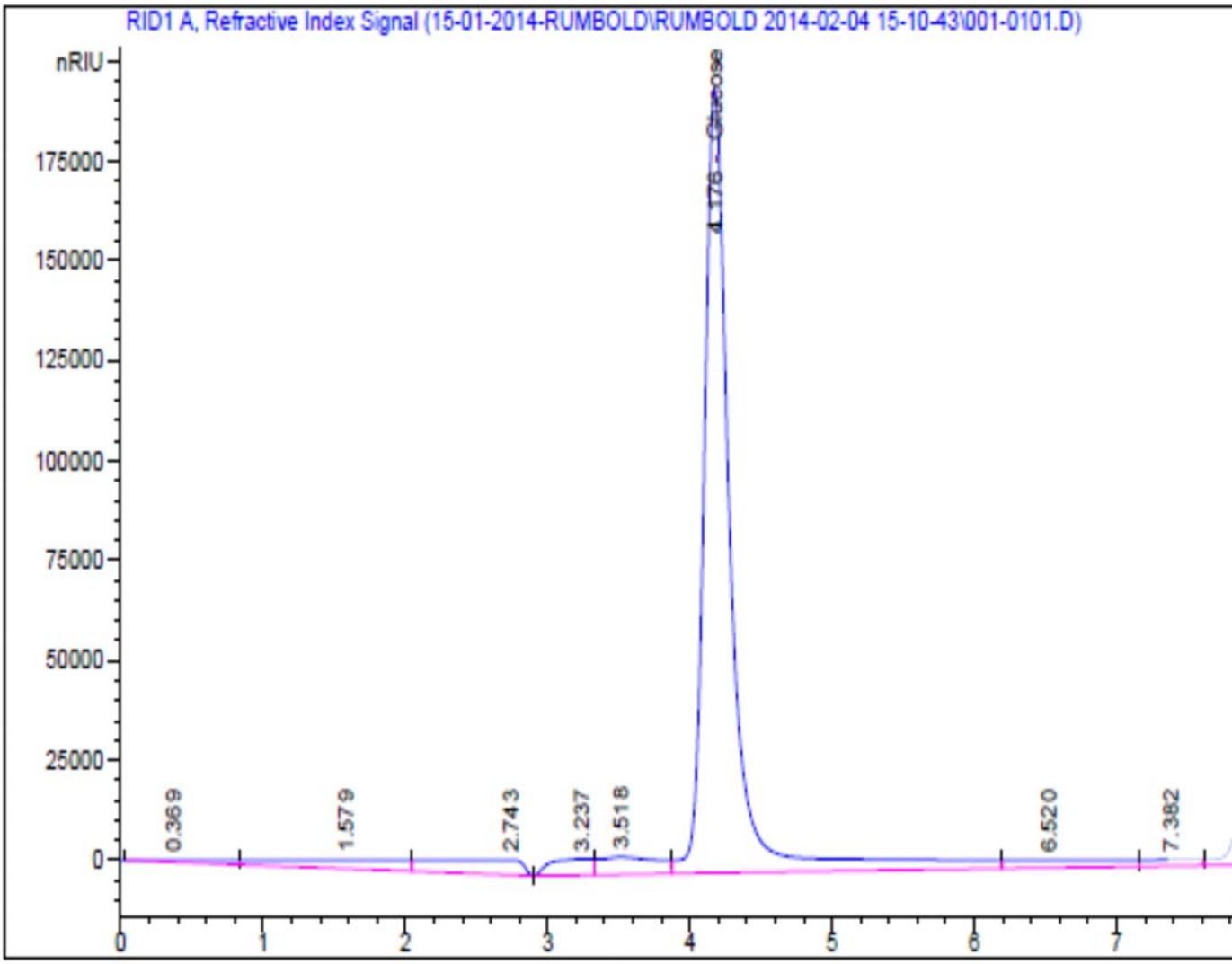


Figure A2: Chromatogram of glucose standard

Appendix 3

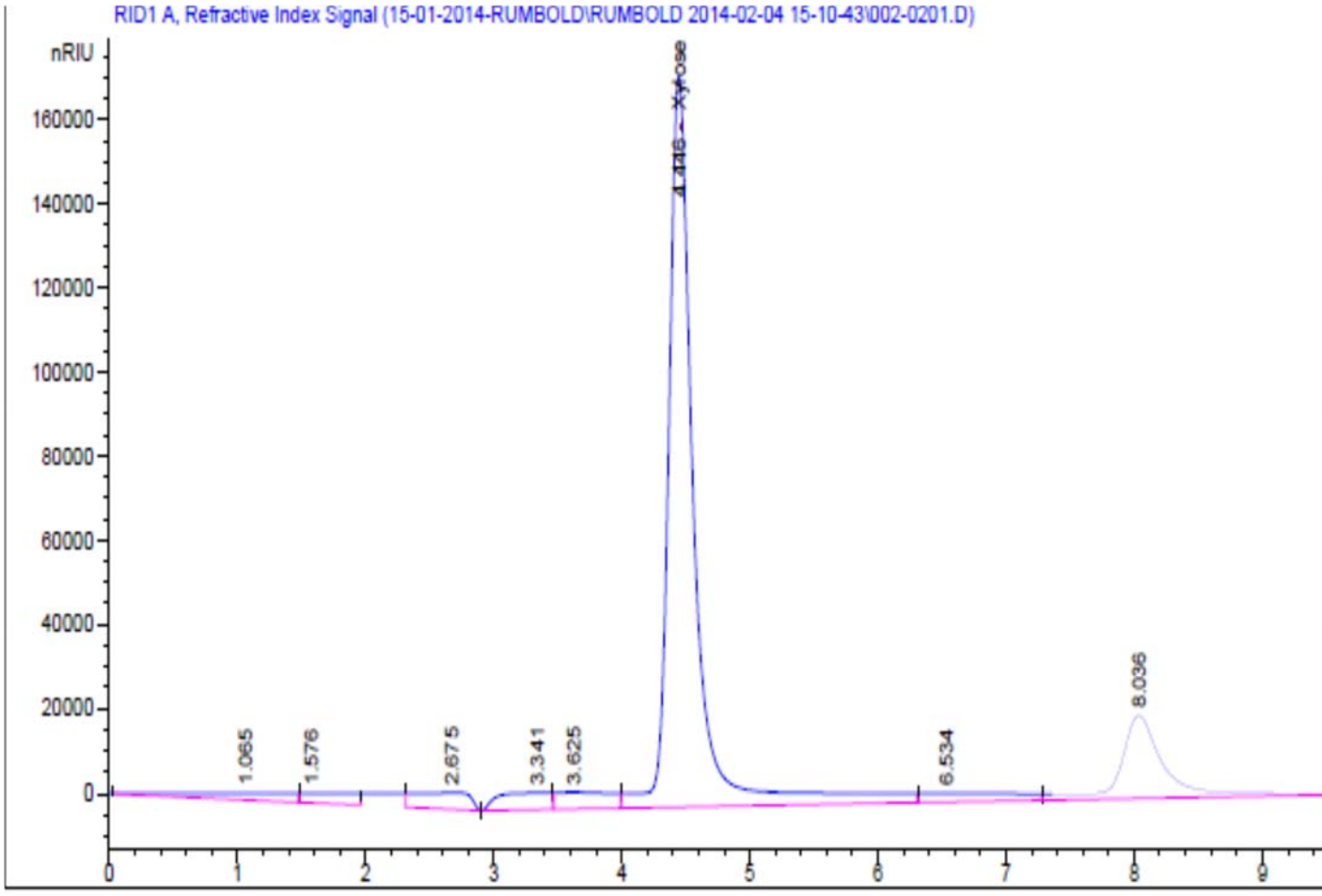
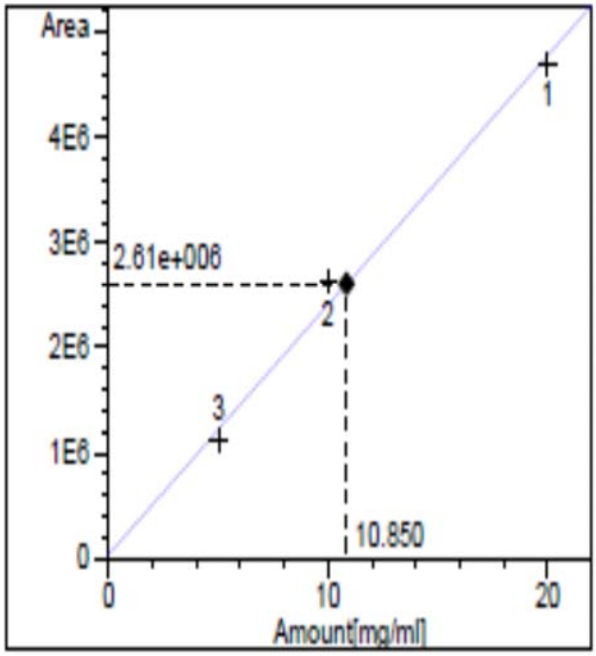
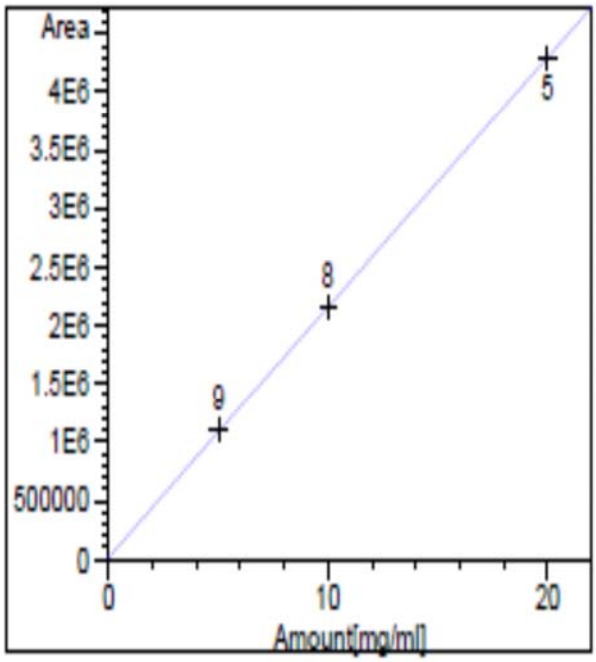


Figure A3: Chromatogram of Xylose standard

Appendix 4



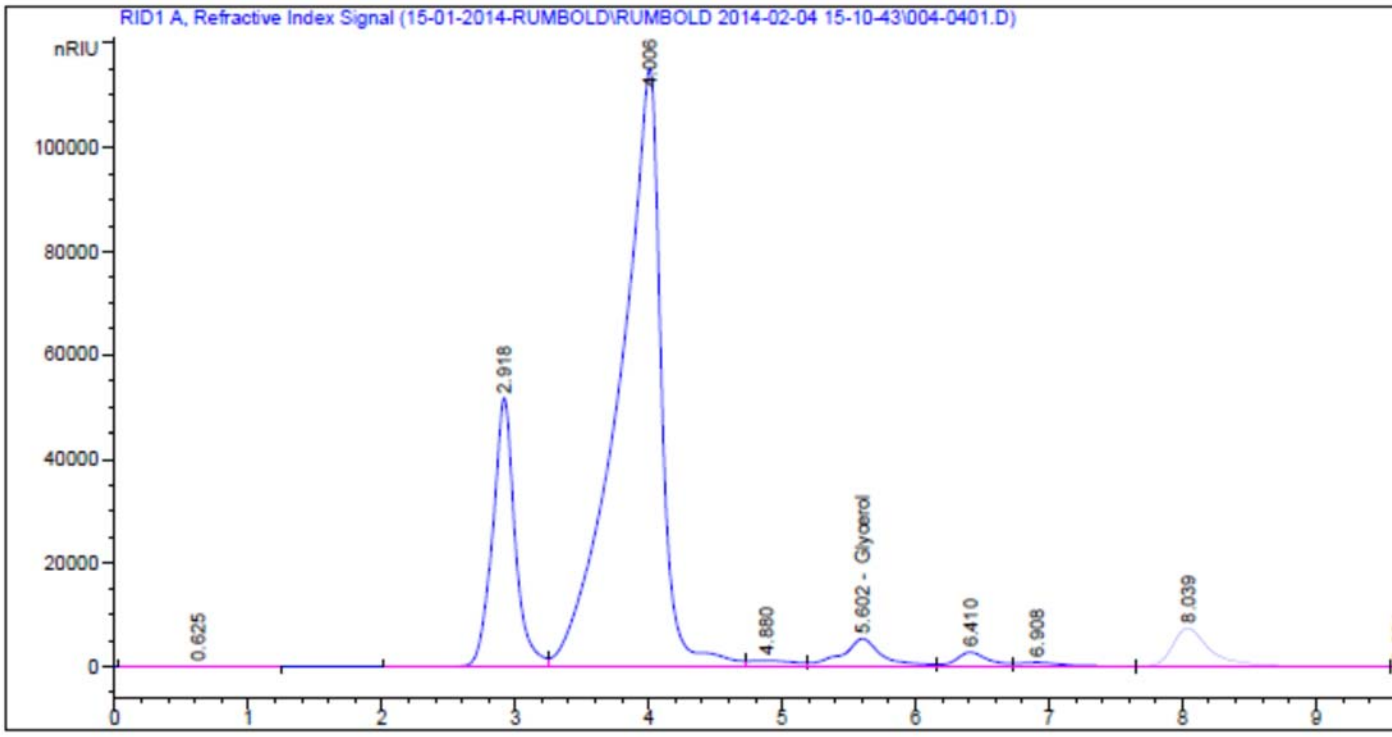
Glucose at exp. RT: 4.168
RID1 A, Refractive Index Signal
Correlation: 0.99720
Residual Std. Dev.: 186591.51399
Formula: y = mx + b
m: 237972.44643
b: 30735.50000
x: Amount
y: Area



Xylose at exp. RT: 4.432
RID1 A, Refractive Index Signal
Correlation: 0.99995
Residual Std. Dev.: 22151.52269
Formula: y = mx + b
m: 214318.13429
b: 16298.20000
x: Amount
y: Area

Figure A4: Glucose (top) and Xylose (bottom) standard curves

Appendix 5

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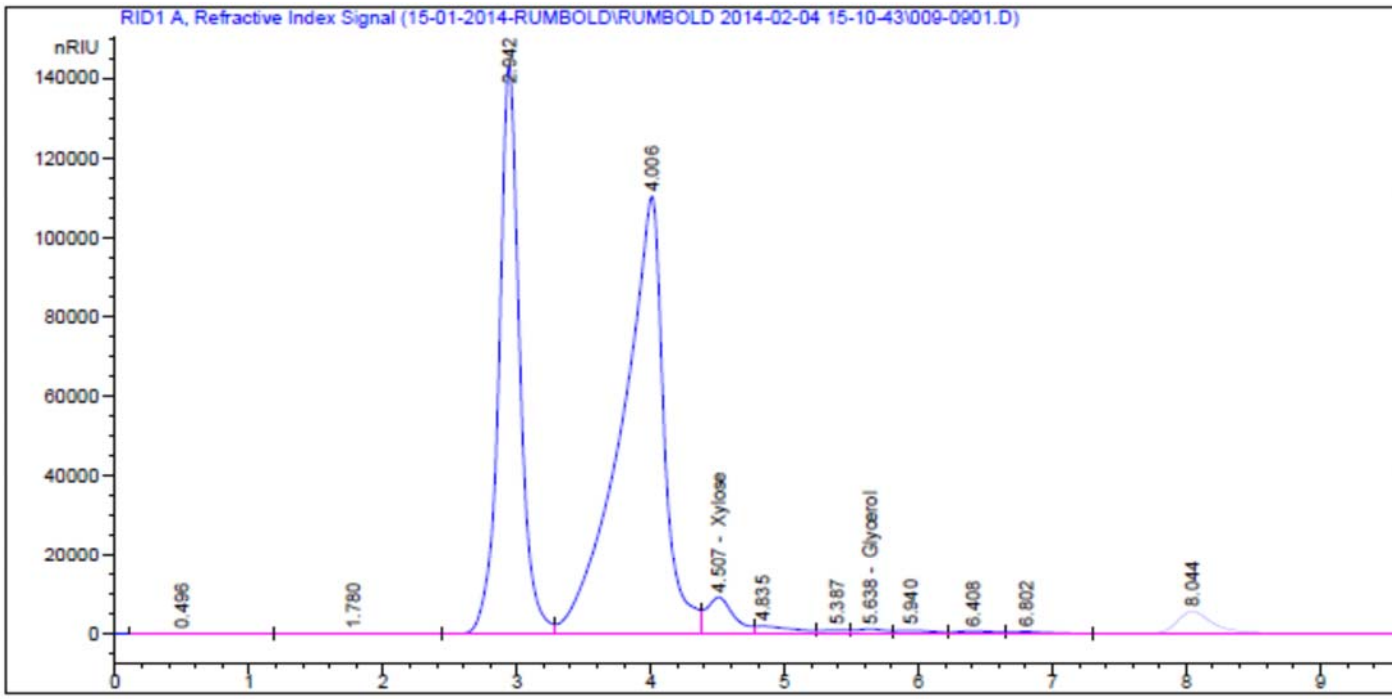
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area    Amount    Grp    Name
 [min]          [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|------------------
  0.625 BV      810.42432    0.00000    0.00000    ?
  2.918 VV     6.03759e5     0.00000    0.00000    ?
  4.006 VV     2.52215e6     0.00000    0.00000    ?
  4.168             -            -          -      Glucose
  4.432             -            -          -      Xylose
  4.880 VV     2.82305e4     0.00000    0.00000    ?
```

Figure A5: Chromatogram and report of water treated biomass.

Appendix 6

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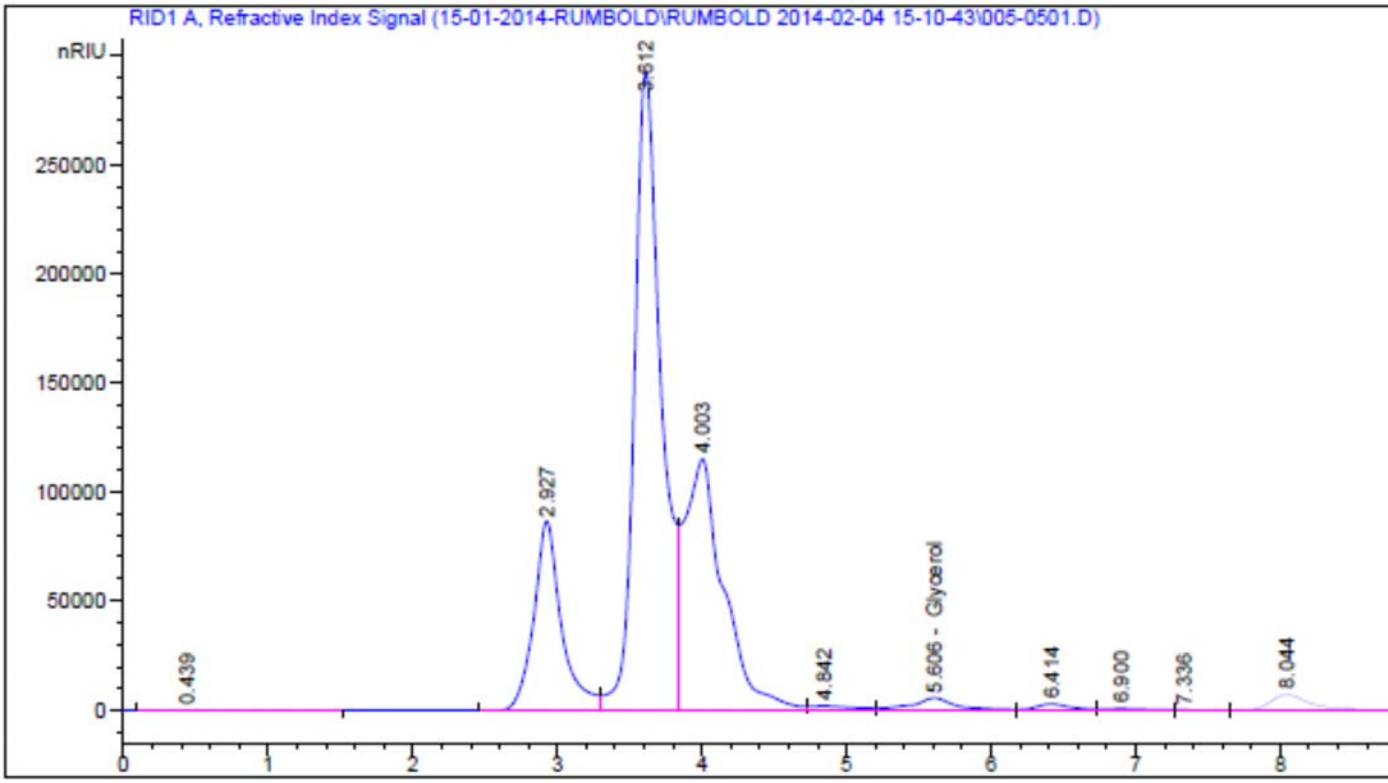
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area    Amount    Grp    Name
 [min]           [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|------------------
  0.496 BV       745.90668    0.00000    0.00000    ?
  1.780 VV       785.77783    0.00000    0.00000    ?
  2.942 VV      1.54366e6     0.00000    0.00000    ?
  4.006 VV      2.42864e6     0.00000    0.00000    ?
  4.168             -            -          -       Glucose
  4.507 VV      1.30985e5  4.08538e-6 5.35124e-1    Xylose
  4.835 VV      3.71357e4     0.00000    0.00000    ?
```

Figure A6: Chromatogram and report of AMD treated biomass.

Appendix 7

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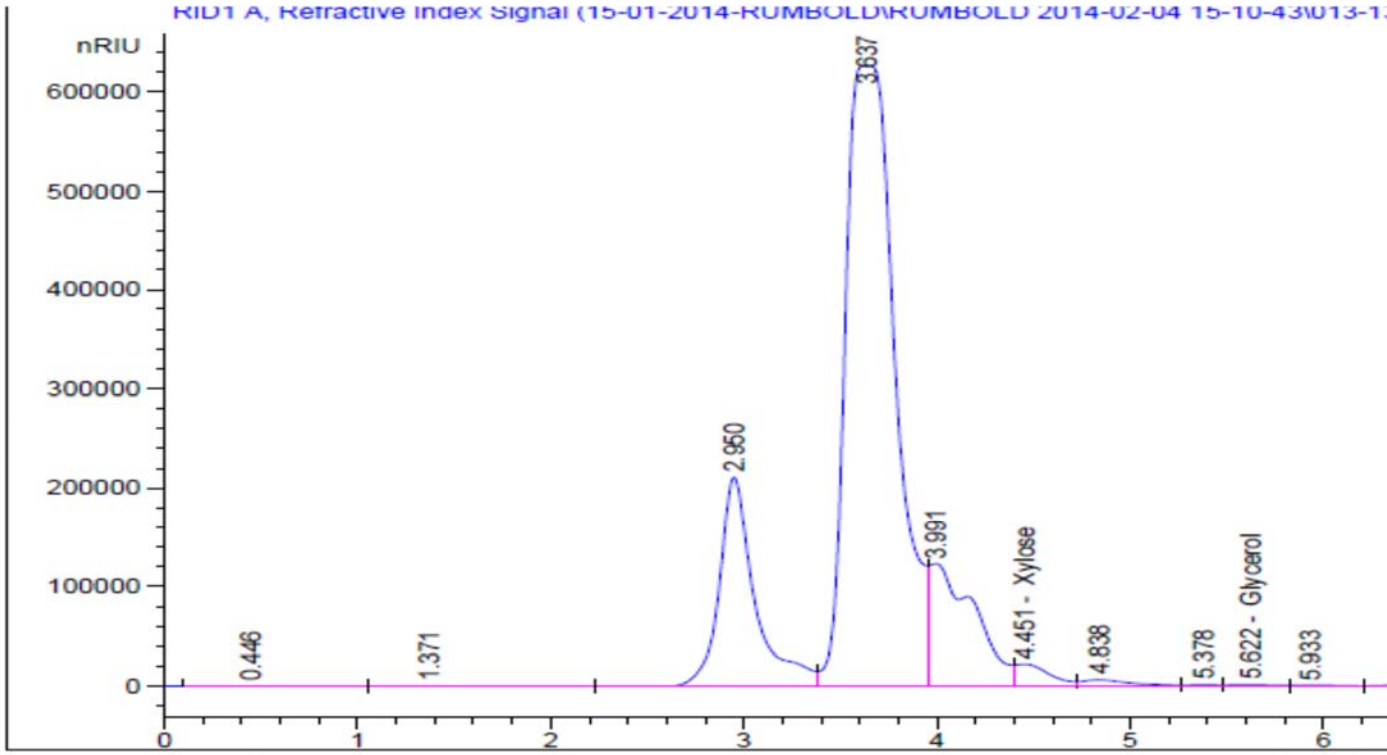
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|-----------------
  0.439 BV       840.20807    0.00000    0.00000    ?
  2.927 BV      1.15421e6     0.00000    0.00000    ?
  3.612 VV      3.80977e6     0.00000    0.00000    ?
  4.003 VV      2.15642e6     0.00000    0.00000    ?
  4.168             -          -          -        Glucose
  4.432             -          -          -        Xylose
  4.842 VV      4.57367e4     0.00000    0.00000    ?
```

Figure A7: Chromatogram of water treated switch grass with 0.5% enzyme

Appendix 8



External Standard Report

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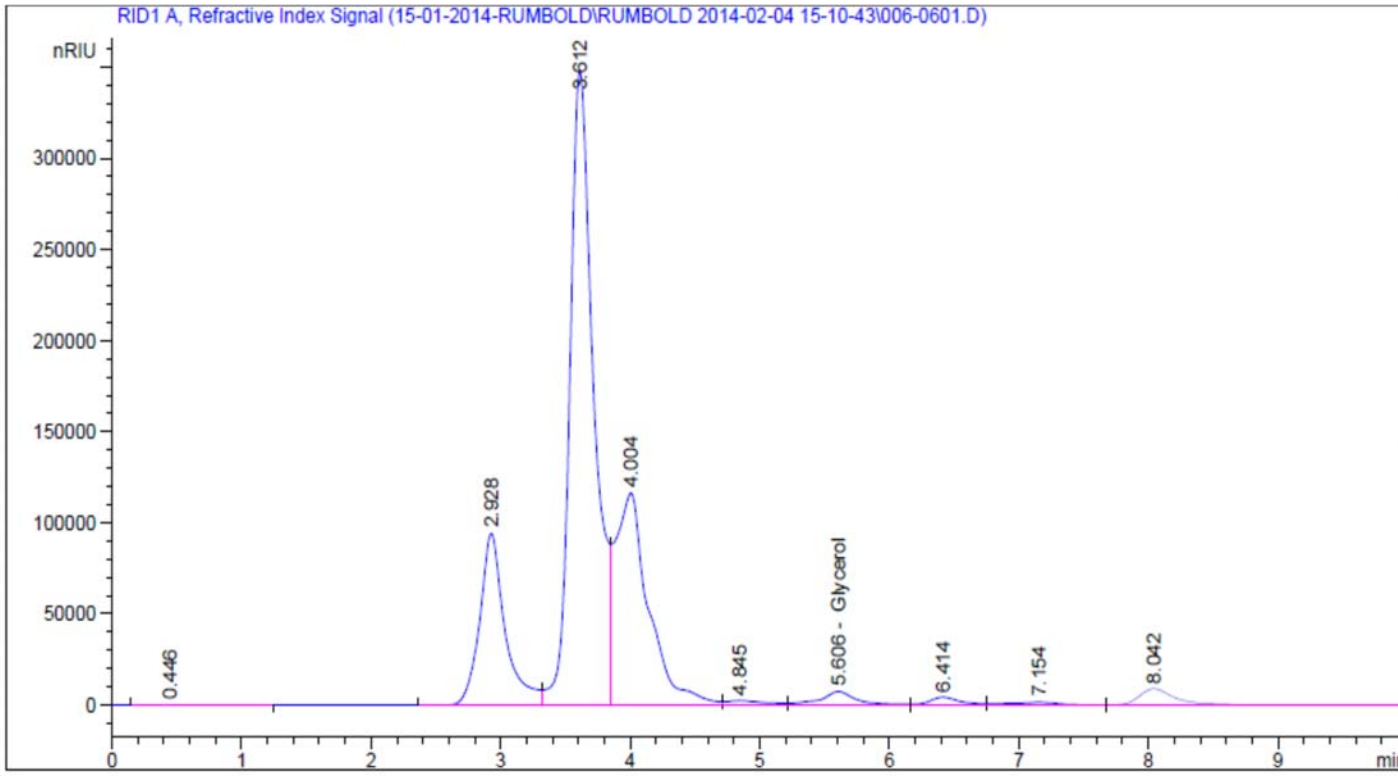
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks : compound name not specified

RetTime [min]	Type	Area [nRIU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
0.446	BV	1077.79663	0.00000	0.00000	?	
1.371	VV	753.58630	0.00000	0.00000	?	
2.950	VV	2.70266e6	0.00000	0.00000	?	
3.637	VV	1.11992e7	0.00000	0.00000	?	
3.991	VV	1.99555e6	0.00000	0.00000	?	
4.168		-	-	-	Glucose	
4.451	VV	2.78556e5	4.39296e-6	1.22369	Xylose	
4.838	VV	1.21354e5	0.00000	0.00000	?	

Figure A8: Chromatogram of AMD treated switch grass digested with 0.5% enzyme

Appendix 9

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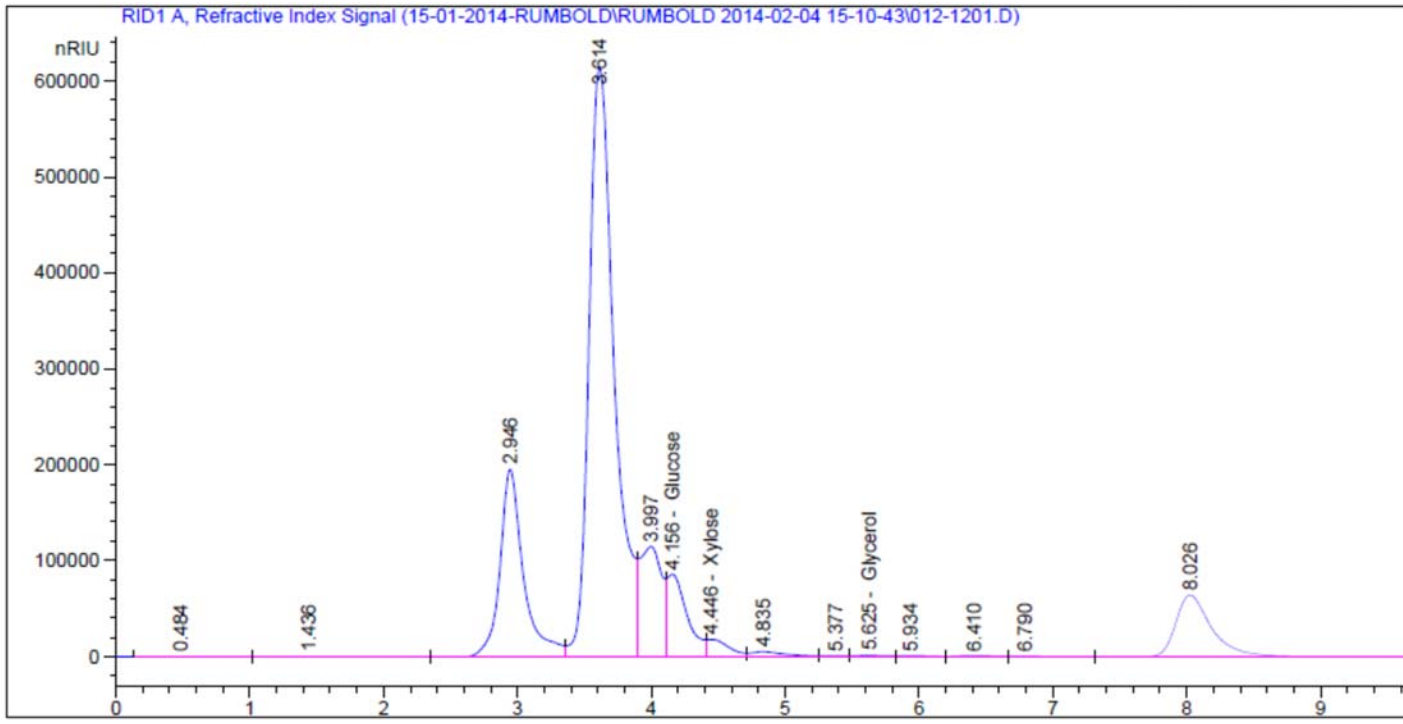
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area    Amount    Grp   Name
 [min]           [nRIU*s]               [mg/ml]
-------|------|----------|----------|----------|--|-----------------
  0.446 BV       580.39606    0.00000    0.00000    ?
  2.928 VV      1.28538e6     0.00000    0.00000    ?
  3.612 VV      4.49313e6     0.00000    0.00000    ?
  4.004 VV      2.06838e6     0.00000    0.00000    ?
  4.168             -          -          -        Glucose
  4.432             -          -          -        Xylose
```

Figure A9: Chromatogram of water treated switch grass digested with 2,5% enzyme

Appendix 10

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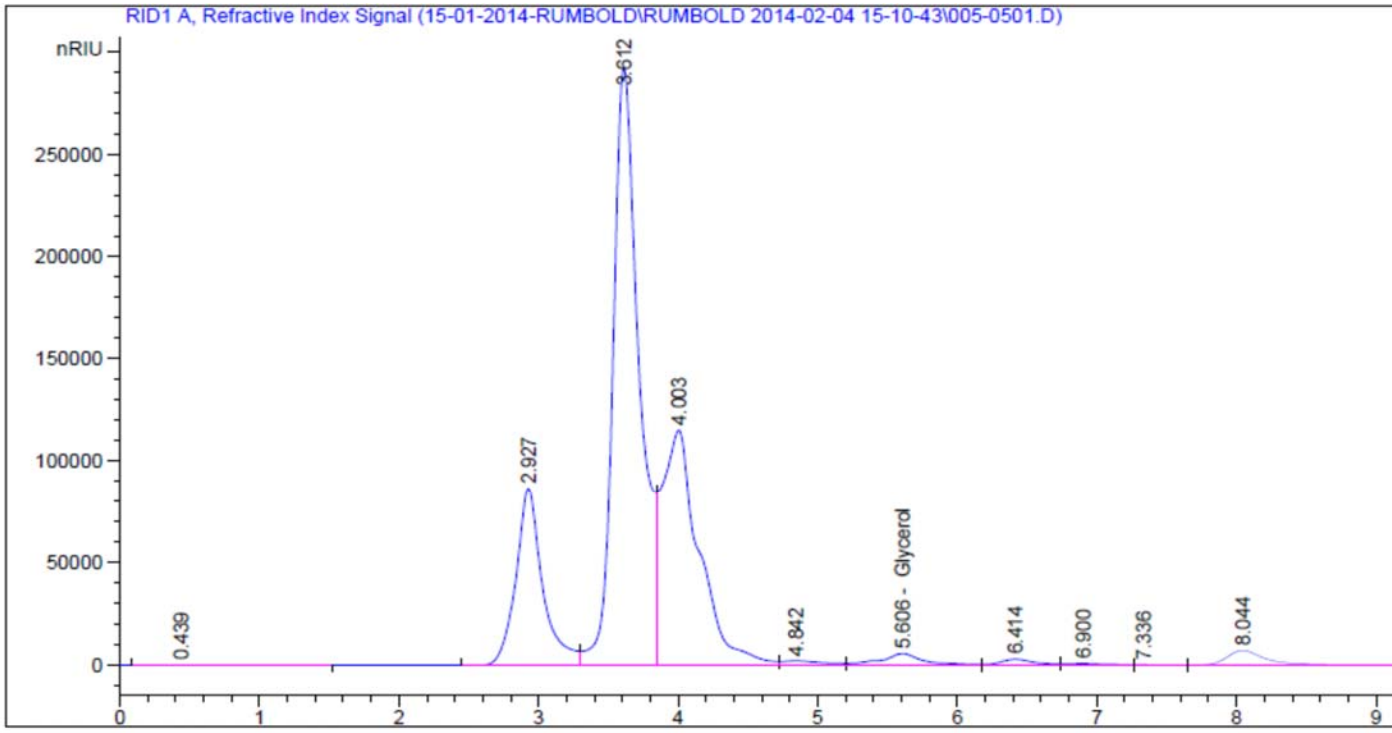
Calib. Data Modified  :      Friday, October 04, 2013 12:39
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area     Amount    Grp    Name
 [min]          [nRIU*s]                 [mg/ml]
-------|------|----------|----------|----------|--|-------------------
  0.484 BV      1238.09424     0.00000     0.00000     ?
  1.436 VV      1121.69299     0.00000     0.00000     ?
  2.946 VV      2.38951e6      0.00000     0.00000     ?
  3.614 VV      8.11257e6      0.00000     0.00000     ?
  3.997 VV      1.27786e6      0.00000     0.00000     ?
  4.156 VV      9.09518e5   4.06016e-6     3.69279     Glucose
  4.446 VV      2.11257e5   4.30599e-6 9.09670e-1     Xylose
  4.835 VV      1.01125e5      0.00000     0.00000     ?
```

Figure A10: Chromatogram of AMD treated switch grass digested by 2,5% enzyme

Appendix A11

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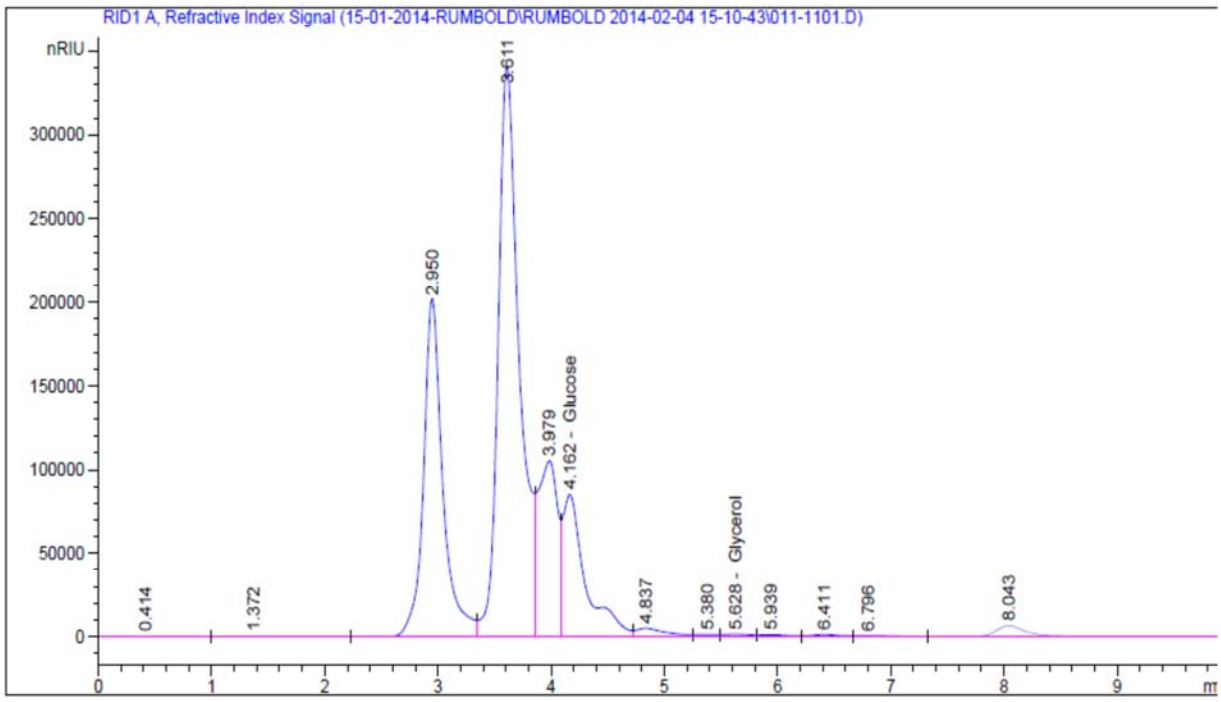
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area    Amount    Grp   Name
 [min]          [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|------------------
  0.439 BV       840.20807    0.00000     0.00000    ?
  2.927 BV      1.15421e6     0.00000     0.00000    ?
  3.612 VV      3.80977e6     0.00000     0.00000    ?
  4.003 VV      2.15642e6     0.00000     0.00000    ?
  4.168             -           -           -        Glucose
  4.432             -           -           -        Xylose
  4.842 VV      4.57367e4     0.00000     0.00000    ?
```

Figure A11: Chromatogram of water treated switch grass digested with 5% enzyme

Appendix 12

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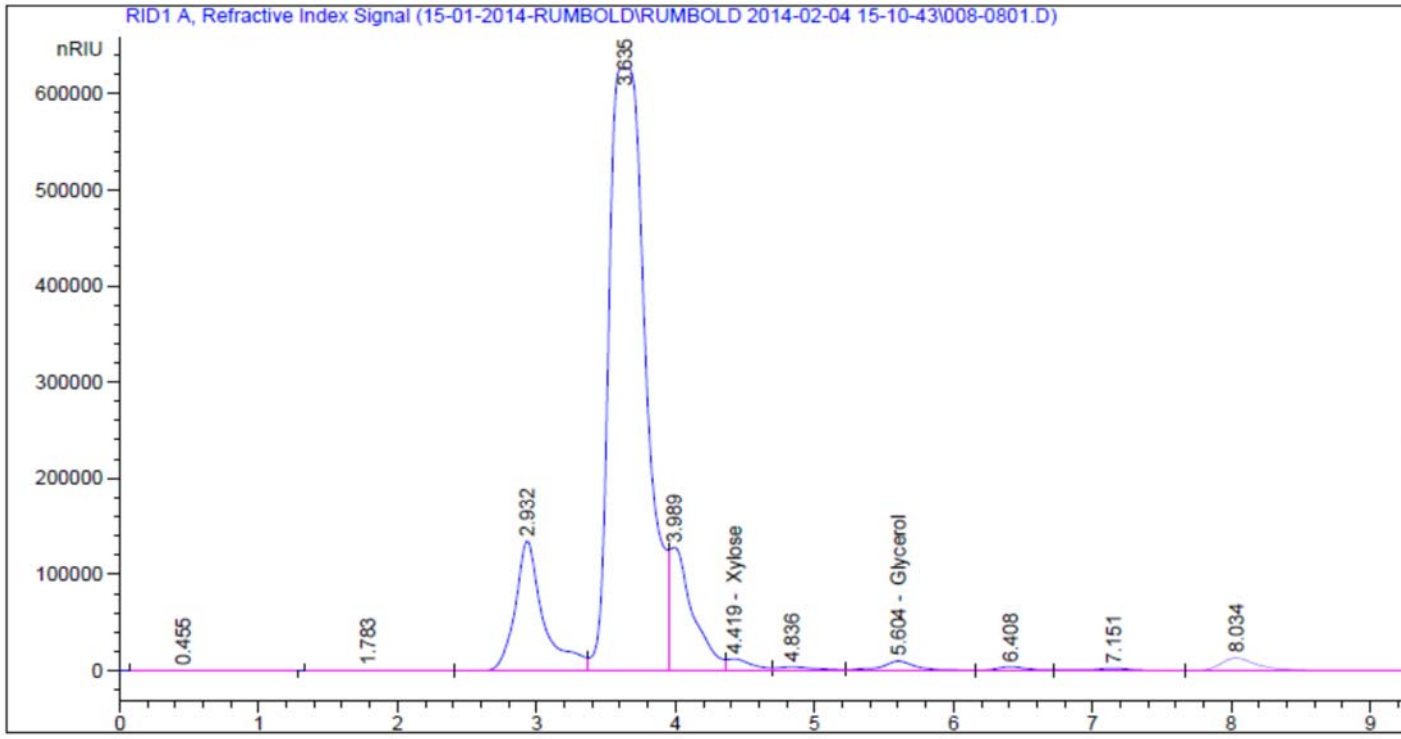
Calib. Data Modified  :      Friday, October 04, 2013 12:39
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type     Area      Amt/Area    Amount   Grp   Name
 [min]          [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|-----------------
  0.414 BV     1510.00513    0.00000    0.00000    ?
  1.372 VV     1412.60474    0.00000    0.00000    ?
  2.950 VV     2.39703e6     0.00000    0.00000    ?
  3.611 VV     4.42765e6     0.00000    0.00000    ?
  3.979 VV     1.29308e6     0.00000    0.00000    ?
  4.162 VV     1.17901e6   4.09262e-6   4.82525    Glucose
  4.432            -           -          -       Xylose
  4.837 VV     9.37726e4     0.00000    0.00000    ?
```

Figure A12: Chromatogram of AMD treated switch grass digested with 5% enzyme

Appendix A13

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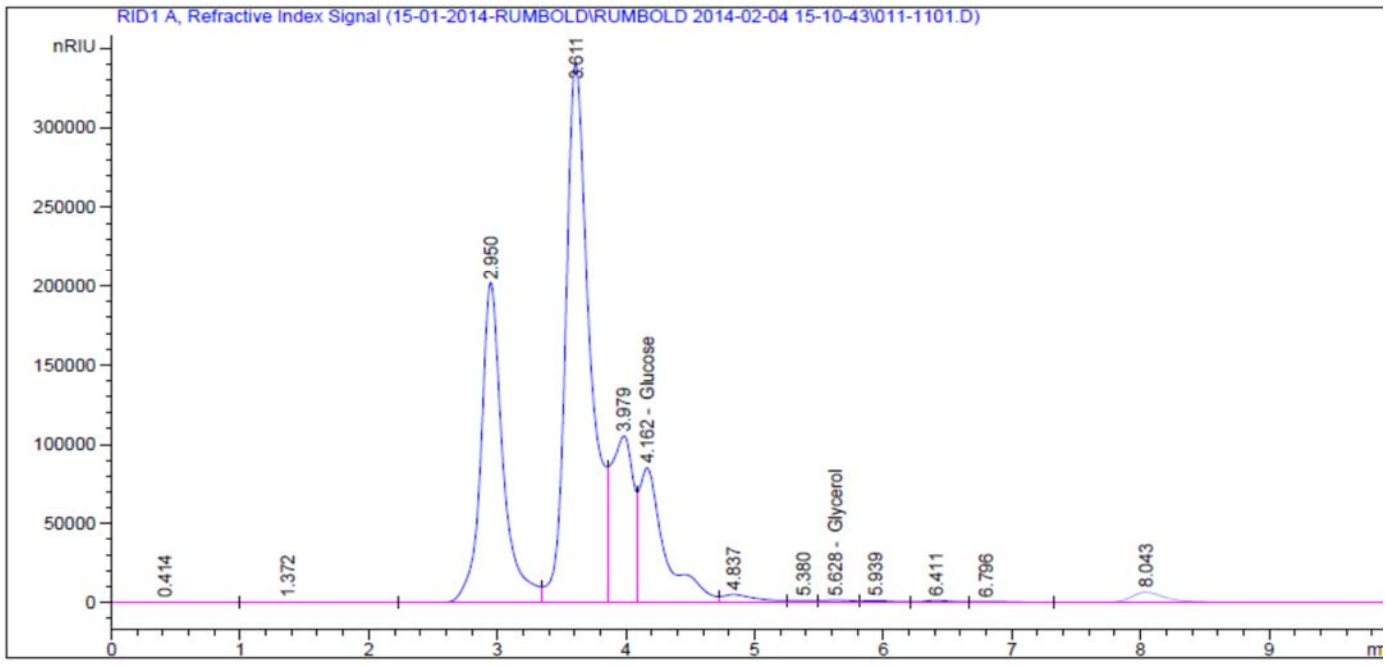
Calib. Data Modified  :      Friday, October 04, 2013 12:39
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area    Amount    Grp   Name
 [min]           [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|------------------
  0.455 BB      1090.95496    0.00000    0.00000    ?
  1.783 BV       804.61267    0.00000    0.00000    ?
  2.932 VV      1.91229e6     0.00000    0.00000    ?
  3.635 VV      1.14322e7     0.00000    0.00000    ?
  3.989 VV      1.52559e6     0.00000    0.00000    ?
  4.168             -           -          -        Glucose
  4.419 VV      1.53343e5   4.17004e-6 6.39447e-1    Xylose
  4.836 VV      8.32491e4     0.00000    0.00000    ?
```

Figure A13 Chromatogram of water treated switch grass digested with 10% enzyme

Appendix A14

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=====================================================================

Sorted By             :      Signal
Calib. Data Modified  :      Friday, October 04, 2013 12:39
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal
Uncalibrated Peaks    :      compound name not specified


RetTime  Type      Area      Amt/Area     Amount    Grp   Name
 [min]           [nRIU*s]                [mg/ml]
-------|------|----------|----------|----------|--|-----------------
  0.414 BV     1510.00513     0.00000     0.00000    ?
  1.372 VV     1412.60474     0.00000     0.00000    ?
  2.950 VV     2.39703e6      0.00000     0.00000    ?
  3.611 VV     4.42765e6      0.00000     0.00000    ?
  3.979 VV     1.29308e6      0.00000     0.00000    ?
  4.162 VV     1.17901e6   4.09262e-6     4.82525    Glucose
  4.432           -            -           -        Xylose
  4.837 VV     9.37726e4      0.00000     0.00000    ?
```

Figure 14: Chromatogram of AMD treated switch grass digested with 10% enzyme