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Optimization of the enzymatic conversion of maize stover to bioethanol

By

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ABSTRACT

The severe effects associated with global warming and the rapid increase in oil prices are the driving forces behind the demand for clean carbon-neutral and biofuels such as bioethanol. Research studies are now focusing on using lignocellulosic biomass for bioethanol production due to concerns about food security and inflation. The chosen feedstock for this study was maize stover, given that it is the most abundant agricultural residue in South Africa. Maize stover consists of structural carbohydrates that can be enzymatically converted into fermentable sugars. The major drawback in the production of bioethanol from lignocellulosic biomass has been its high equipment and operational costs due to the use of acids and high enzyme loadings. The aim of this study was to investigate the possibility of optimizing the enzyme hydrolysis of pre-treated maize stover without further increasing the amount of enzymes. The maximum glucose yield attained was 690 ± 35 mg of glucose per gram of substrate which is equivalent to a conversion efficiency of 119%. The preferred pre-treatment method used was 3% sulphuric acid for 60 minutes at 121°C and the enzymatic hydrolysis process was performed at a 5% substrate loading, 50°C and pH 5.0 using 30 FPU per gram of cellulose in the presence of 1.25 $g.L^{-1}$ of Tween 80 for 48 hours. The addition of Tween 80 increased the glucose yields by 23 % and thus, it has the potential of lowering the overall process costs by increasing the glucose yield without further addition of enzymes.

Keywords: Bioethanol, maize stover, lignocellulosic biomass, pre-treatment, enzymatic hydrolysis, Tween 80

OPSOMMING

Die ernstige effekte wat geassosieer word met aardverwarming en die onlangse dramatiese toename in oliepryse is die dryfvere agter die aanvraag vir suiwer koolstofneutrale en herwinbare brandstowwe, soos bioetanol. Navorsing fokus tans op die gebruik van houtagtige sellulose biomassa vir die produksie van bioetanol as gevolg van kommer rondom voedselsekuriteit en inflasie. Die veevoer wat vir die studie gekies is, is mieliestronke, gegewe dat dit die mees oorvloedige landbou-afval in Suid-Afrika is. Mieliestronke bestaan uit strukturele koolhidrate wat ensimaties omgeskakel kan word in gistingsuikers. Die grootste struikelblok in die produksie van bio-etanol vanaf houtagtige sellulose-biomassa is die hoë produksiekoste verbonde daaraan vanweë die duur toerusting en hoë ensiemkostes. Die doel van die studie was om die moontlikhede van die optimalisering van ensiemhidrolise van voorbehandelde mieliestronke sonder die verhoging van die hoeveelheid bygevoegde ensieme, te ondersoek. Die maksimum opbrengs wat bekom is, was 690 ± 35 mg glukose per gram substraat na voorbehandeling wat ekwivalent is aan ‘n omsettingseffektiwiteit van 119%. Die voorbehandelingsmetode gebruik, was 3% swaelsuur vir 60 minute teen 121°C, gevolg deur die ensimatiese hidrolise van ‘n 5% substraat gevul met 30 FPU per gram sellulose teen 50°C en pH 5.0 in die teenwoordigheid van 1.25 $g.L^{-1}$ Tween 80 vir 48 uur. Die byvoeging van Tween 80 het die glukose-opbrengs met 22% verhoog en dus het dit die potensiaal om die kostes in geheel te verlaag deur die vermeerdering van die glukoseproduksie sonder verdere addisionele ensieme.

Sleutelwoorde: Bioetanol, mieliestronke, houtagtige sellulose-biomassa, voorbehandeling, ensiemhidrolise, Tween 80

DECLARATION

I, Nombongo Mabentsela, hereby declare that the dissertation entitled *Optimization of the enzymatic conversion of maize stover to bioethanol,* submitted to the North-West University in completion of the requirements set for the degree of Master of Science, is my own work, has been language edited and has not already been submitted to any other university. I understand and accept that the copies that are submitted for examination are the property of the University.

Signature of student_________________________

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And finally, I am grateful to all my friends for caring, supporting and seeing me through this research, both emotionally and spiritually.

“The Lord, the Lord, the compassionate and gracious God, slow to anger, abounding in love and faithfulness (Exodus 34:6)

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction and motivation

Fossil resources such as crude oil and coal are the major sources of transport fuel and electricity in South Africa. South Africa's economy primarily depends on road transport and, because of the small amount of proven oil reserves, 60% of the diesel and petrol used to sustain this sector is sourced from imported crude oil (Manny, 2006). There has been a rapid increase in oil prices due to the decline of oil reserves and the instability in major oil producing regions (Wabiri and Amusa, 2010). This has raised concerns about the security of oil supplies since it has a major impact on the economic growth of the country (Fofana *et al*., 2009). Another major disadvantage of using fossil fuels is the emission of Greenhouse Gas (GHG) during combustion, which has contributed greatly to air pollution and ultimately to global warming (Silverstein, 2004). Globally, motor vehicles account for more than 70% of carbon monoxide (CO) and 19% of carbon dioxide (CO_2) emissions (Balat and Balat, 2009). Hence, to curb the worldwide crisis of fossil fuel depletion, dependence on expensive oil and global warming, research needs to focus on alternative clean, carbon-neutral and renewable fuels such as biofuels (Chang, 2007). In addition, South Africa is a signatory of the Kyoto protocol and at the Copenhagen climate change conference in 2009 agreed to decrease its GHG emissions by 34% by 2020. Thus, GHG emission mitigation strategies, which include the use of alternative clean-burning energy sources, have to be implemented immediately in order to achieve the targets (EuroActiv, 2009).

The use of bioethanol and bioethanol/petrol blends as alternative transportation fuel goes back to the 1960s, but cheap and plentiful crude oil during the following years rendered the industry uneconomic (Cartwright 2007). Bioethanol can either be used as an additive or a complete replacement for fossil derived transportation fuel (Otero *et al*., 2007). Bioethanol-petrol blends such as E5, E10, and E85, have already been introduced on a large scale in countries like Brazil and the United States of America (USA). The advantages of using bioethanol as a transportation fuel are that it is non-toxic (Galbe and Zacchi, 2007) and has a high octane number. Since bioethanol has a high oxygen content, combustion is more efficient and this reduces carbon monoxide (CO) and nitrous oxide (NOx) emissions (Otero *et al.*, 2007). Thus in theory, using bioethanol as fuel does not contribute to global warming (Sendelius, 2005).

The demand for bioethanol has increased globally, but the production costs have made the use of this fuel less favourable. Bioethanol production represented about 4% of the 1300 billion litres of petrol consumed globally in 2007 (Balat and Balat, 2009). In South Africa, the largest bioethanol producer from a renewable source, sugar cane, is the Illovo Sugar industry. The demand for ethanol is increasing – for instance Sasol now uses synthetic high purity ethanol instead of lead as an additive to increase the octane number of unleaded fuel. This has given the ethanol industry a boost and might be a possible market for bioethanol producers (Mayet, 2006). Bioethanol can also be used to produce ethanol gel fuel. A bioethanol gel fuel is a clean-burning and non-poisonous bio-based fuel used for cooking in specially designed stoves. Unlike coal, charcoal or biomass that is not properly dried, bioethanol gel-fuel provide an extremely safe, smoke free and efficient alternative energy source for rural disadvantaged areas, in particular (Wynne-Jones, 2003). It is estimated that between 80-90% of the people in Sub-Saharan Africa depend on biomass fuels. If well managed, these fuels can effectively improve the rural economic growth in developing countries (Jumbe *et al.*, 2009).

The first generation methods for bioethanol production from biomass sources are based on sugar and starch biomass. In the bioconversion of sugar substrates (e.g. sugar cane) the squeezed juice is directly fermented into bioethanol. For starch substrates, such as maize, a liquefaction step (to make the starch soluble) and a hydrolysis step (to produce glucose) are necessary prior to the fermentation of the available sugars to bioethanol (Hahn-Hägerdal *et al.*, 2007). The major disadvantage of using sugar and starch biomass as feedstock for bioethanol production is that they have a greater value as food sources. This has raised issues about the use of food crops due to concerns about food prices and food security (IFAD, 2008).

The second generation production methods focus on the utilization of lignocellulosic biomass such as agricultural residues (e.g. wheat straw, sugar cane bagasse, and maize stover), forest products (hardwood and softwood) and dedicated crops (switchgrass and salix) for the production of bioethanol. Lignocellulosic biomass is a sufficiently abundant, renewable source of energy and it is not used as a source of food for humans; thus it is regarded as potential feedstock (Kumar *et al*., 2009). However, the conversion of lignocellulosic biomass to bioethanol requires a pre-treatment process prior to hydrolysis due to its recalcitrant nature, and such processes tend to increase the overall cost of lignocellulosic bioethanol production (Silverstein *et al*., 2007). Therefore, there is a need to optimize the existing processes with regard to lowering the production costs while still achieving increased sugar yields (Sánchez and Cardona, 2008).

Maize stover refers to the part of the maize plant that remains on the surface after harvesting the grain. It consists of stalks, leaves, cobs and husks. The crown and the surface roots are not considered part of the stover. Stover is regarded as a promising feedstock for the production of cellulosic bioethanol because like any other lignocellulosic biomass, it contains mainly cellulose and hemicellulose, which can be hydrolysed into fermentable sugars (Chen *et al*., 2007). Maize stover is an abundant agricultural by-product with low commercial value (Ohgren *et al.,* 2006). Kim and Dale (2004) reported that about 203.6 teragram (Tg) of dry maize stover is available globally, which can produce 58.6 gigalitres (GL) of bioethanol, replacing about 42.1 gigalitres (GL) of petrol used in midsize passenger vehicles, fuelled by E85. It has been reported that for every ton of grain, roughly a ton of cellulosic residues, in this case stover, is produced on a dry basis. In South Africa approximately 6.7 metric tons (Mt) of maize stover are produced annually (Lynd *et al*., 2003).

Currently, more than 90% of maize stover that remains after harvesting is ploughed back into the soil for nutrient conservation purposes and its potential to replace fossil fuels is lost. Although crop residue incorporation and mulching play an important role in maintaining soil fertility, complete decomposition of these residues usually takes long. This leads to low ground temperature, decreased nitrogen content in the soil and interference with the germination and growth of seedlings. The ploughed-in crop residues also provides shelter and breeding sites for pests, aggravates many disease problems and lowers the effect of herbicides or pesticides (Glassner *et al*., 1999).

The removal of agricultural residues is beneficial as it eliminates the rigorous consequences of mulching. However, the success of maize stover utilization for bioethanol production depends on the sustainable harvesting of the stover in sufficient quantities without having detrimental consequences on the soil (Glassner *et al*., 1999). It is recommended that at least 30% of the soil surface should be covered by plant residues after harvest for conservation of soil nutrients and water. The rest of the plant residues can be removed and used for industrial purposes (Fowler and Rockstrom, 2001).

1.2 Aims and objectives

The aim of this study was to investigate the possibility of increasing the glucose yields during enzyme hydrolysis of maize stover, at low enzyme loading through the addition of surfactants. The focus was on the simple routes that can be used to process maize stover, which would be cheaper to implement. This study examines (1) the significance of chemical loading during pre-treatment on glucose yields, using dilute sulphuric acid and calcium hydroxide as pre-treatment agents; (2) the effect of pre-treatment time on the glucose yields; and (3) the effect of substrate loading, enzyme loading and the addition of Tween 80 on glucose yields during the enzymatic hydrolysis of maize stover.

1.3 Scope of the investigation

- Chapter 2 is an overview of the literature on the whole process of lignocellulosic bioconversion to bioethanol to give a better understanding of the production of ethanol from lignocellulosic biomass.
- Chapter 3 entails the experimental methods used in this study. It explores the possibility of employing different methods in order to increase the glucose yield during the enzymatic hydrolysis of maize stover.
- All pre-treatment and hydrolysis results are presented and discussed in Chapter 4.
- Chapter 5 offers conclusions arising from this investigation and gives recommendations for further studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

South Africa’s dependency on imported crude oil has a negative impact on the country’s economy due to the soaring oil prices that have led to an increase in petrol prices, and consequently food inflation. In addition, the continued use of fossil fuel has resulted in an increase in Greenhouse Gas (GHG) emissions in the atmosphere. The impact of global warming, due to increased GHG emissions, has globally raised concerns about the use of fossil fuels. South Africa is the 12th highest gas emitter of GHG emissions in the world (Reuters, 2009). In order to lower South Africa’s GHG emissions, alternative fuel sources such as biofuels (e.g. bioethanol, biodiesel and biogas) can be used to blend and eventually substitute fossil fuels (Lal, 2008). This chapter offers an overview on the bioconversion of lignocellulosic biomass into bioethanol and closes off with concluding remarks drawn from the available literature.

2.2 Bioconversion of lignocellulosic biomass

The production of biofuels from the first generation feedstock, such as sugar and starch material, has created challenges in achieving basic food security and economic development, especially in developing countries (Chakauya *et al*., 2009). Recent research efforts have focused on the development of low cost, non-edible and renewable resources for bioethanol production, in order to meet the growing concerns about food security and energy demand (Datar *et al*., 2007). An attractive alternative to using food sources is to convert the lignocellulosic part of the biomass to bioethanol (Galbe and Zacchi, 2007). Lignocellulose is a more complex substrate compared to starch. The cellulose and hemicellulose, which typically comprise two-thirds of the dry mass, are polysaccharides that can be hydrolysed to fermentable sugars and subsequently fermented to ethanol using an appropriate microorganism (Hamelinck *et al*., 2005).

The traditional route applied when processing lignocellulosic biomass to bioethanol consists of different unit operations: pre-treatment (delignification to liberate cellulose and hemicellulose from their complex with lignin), hydrolysis (depolymerisation of the carbohydrate polymers to produce free sugars), fermentation (conversion of sugars to produce bioethanol), and water removal and product separation/purification as shown in Figure 2.1 (Lee, 1997; Mosier *et al.*, 2005).

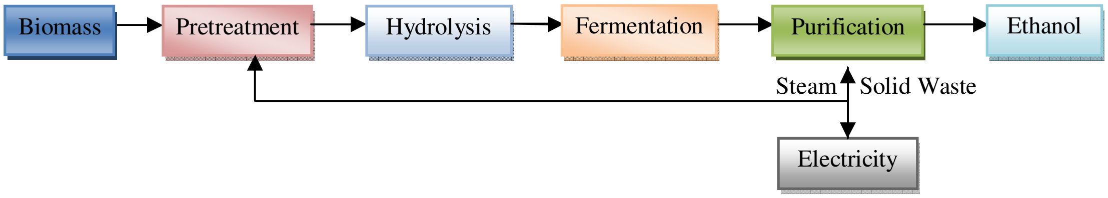

Figure 2.1: Steps involved in the conversion of lignocellulosic biomass to bioethanol

The remaining solid waste material contains by-products, which include lignin, furfurals, xylitol and lactic acid (Pan *et al.*, 2006). Lignin cannot be degraded by cellulases, thus it remains in the solid component after hydrolysis and the solid waste can be used to generate electricity for the process. Furfural released during the breakdown of hemicellulose, can be used to make carpet fibre (Szulczyk *et al.*, 2010). Xylitol is another attractive by-product, it is a five carbon sugar produced during the fermentation of hemicellulose containing hydrolysates. Xylitol has a potential for use as a natural food sweetener, a dental caries reducer or a sugar substitute for diabetics (Saha, 2003). Lactic acid is produced during fermentation, and can be processed into plastics and other valuable products (Balat *et al.*, 2008). Improved ways of purifying these by-products at low costs could make lignocellulosic bioethanol a viable and competent fuel (Szulczyk *et al.*, 2010).

2.3 Chemical composition

2.3.1 Cellulose

Cellulose is the most abundant carbohydrate component found in lignocellulosic biomass. It is found in plant cell walls where it serves as the main source of glucose (Silverstein, 2004). The cellulose content varies in plants due to the great differences in the anatomical structure of cell walls across plant groups and location, but a high cellulose content of about 35-50% of plant dry weight has been reported (Lynd *et al.*, 2002). Cellulose is an unbranched linear homopolymer of glucose molecules (Figure 2.2) joined together through β-(1,4)- glucosidic linkages (Decker *et al.*, 2003).

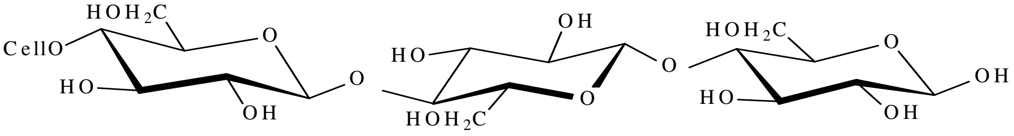

Figure 2.2: Part of the structure of the linear cellulose polymer (Silverstein, 2004)

Cellulose molecules are arranged together to form microfibrils that contain crystalline (less accessible to cellulase enzymes) and amorphous (easily degraded) regions (Decker *et al.*, 2003). The individual polysaccharide chains are bound together in the microfibrils by hydrogen bonds (Heyn, 1966). The microfibrils, in turn, are bundled together to form macrofibrils (Figure 2.3).

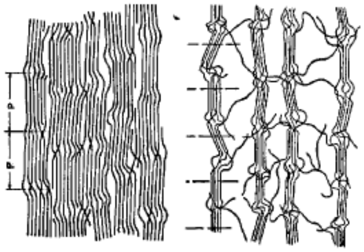

Figure 2.3: Model of microcrystalline structures of cellulose (Heyn, 1966).

2.3.2 Hemicellulose

Hemicellulose is the second most common carbohydrate polymer and it constitutes about 20-35% of lignocellulosic biomass (Lynd *et al.*, 2002). Hemicellulose is a complex polysaccharide that exists in association with cellulose in the cell wall. It is a heterogeneous branched mixture of polysaccharides, composed of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and sugar acids (Saxena *et al.*, 2009). The different main sugar components found in hemicellulose are xylans, mannans, arabinans and galactans (Juház *et al.*, 2005). Xylans have homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, and acetic, ferulic and ρ-coumaric acids. The frequency and composition of branches are dependent on the source of xylan (Saha, 2003). Backbones of mannans consist of randomly distributed β-1,4-linked glucose and mannose units (Juház *et al.*, 2005).

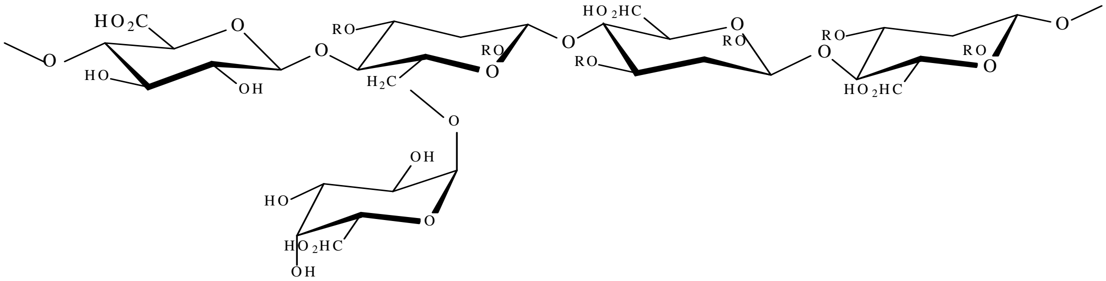

Figure 2.4: Structure of galactoglucomannan (5C sugar) found in softwoods (Silverstein, 2004)

2.3.3 Lignin

Lignin is a highly branched mononuclear aromatic polymer composed of phenyl propane units and it is found in the cell walls of some plants (Figure 2.5). Lignin accounts for approximately 5-30% of lignocellulose (Lynd *et al.*, 2002). The lignified cell wall surrounding the fibre discloses the cementing role of lignin, which binds cellulose and hemicellulose, resulting in the rigidity and the recalcitrance of lignocellulosic biomass (Chandra *et al.*, 2007).

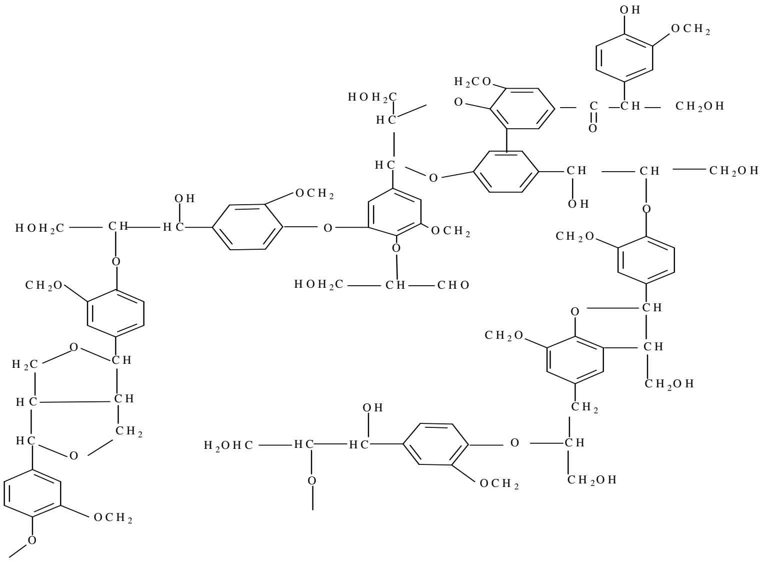

Figure 2.5: Part of the lignin polymer (Silverstein, 2004)

Lignin is also a by-product of photosynthesis; it is converted through a complicated biosynthesis process of enzymatic reactions that include oxidation and reduction steps. The resulting monomeric phenylpropane units have three varieties: coumaryl alcohol, coniferyl alcohol and sinapyl alcohol; each of which has an aromatic ring with different substituents. Lignin is essential for mechanical support, defence and water transport in vascular terrestrial plants (Silverstein, 2004).

2. 4 Pre-treatment

The role of the pre-treatment process is to alter the recalcitrant structure, resulting in increased availability of degradable carbohydrates present in lignocellulosic biomass as represented in the schematic diagram of Figure 2.6 (Mosier *et al.*, 2005).

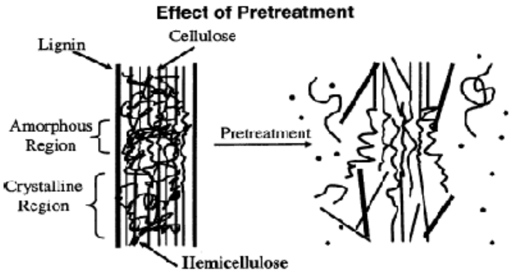

Figure 2.6: Schematic diagram representing the effect of pre-treatment on lignocellulose (Mosier *et al.*, 2005).

A pre-treatment process is regarded as an effective method based on a number of features, such as a high recovery of all carbohydrates, production of no or limited amounts of degradation products, minimum energy consumption and low capital and operational costs (Balat *et al.*, 2008). Severe pre-treatment methods (the use of very high temperatures and chemical doses) lead to the formation of aldehydes, which occurs during the degradation of monosaccharides due to exposure to high temperature. These degradation products are known as furan derivatives and can strongly inhibit microbial growth during fermentation, even at low concentration, such as 0.1% (Mosier *et al.*, 2005). Pre-treatment methods are categorized into different groups, namely: physical, chemical, physicochemical and biological methods (Galbe and Zacchi, 2007). Examples of the most commonly used pre-treatment methods are given below.

2.4.1 Physical pre-treatment methods

A size reduction step is necessary before most chemical and biological pre-treatment processes, since it increases the surface area of the cellulose and thus improves the enzymatic hydrolysis rate (Shi, 2007). Physical pre-treatment methods refer to mechanical and non-mechanical methods. During mechanical pre-treatment, which includes chipping, grinding and milling, physical forces such as shearing or compressive forces are applied to break down lignocellulose into finer particles (Galbe and Zacchi, 2007). Non-mechanical pre-treatment refers to methods like irradiation whereby gamma rays are applied, to make the biomass more accessible so as to increase the hydrolysis rate. The high operational costs associated with this process make it unfavourable (Mousdale, 2008). Energy consumption poses a threat to these methods, since it increases exponentially with decreasing particle size (Silverstein, 2004). These methods have minor effects on the degradation of lignocellulosic polymers but are required prior to other pre-treatment methods (Mousdale, 2008).

Pyrolysis is another physical method being evaluated for the pre-treatment of lignocellulosic biomass due to its ability to decompose cellulose at a very high temperature (Sánchez and Cardona, 2008). This method can be carried out at gas/vapour phase temperatures of approximately 500^{o}C in the absence of oxygen, producing vapours and aerosols and some charcoal. Though the residence time is very short, the high temperatures used and the cooling system required after heating make pyrolysis an extremely costly pre-treatment method (Bridgwater *et al*., 1999).

2.4.2 Chemical pre-treatment methods

Chemical pre-treatment methods use acids, alkalis, ozone or hydrogen peroxide, to list a few, to break down the polysaccharides found in lignocellulosic biomass. Another approach is to use the Organosolv pre-treatment process, which uses an organic solvent mixture in the presence of a catalyst (Wood and Saddler, 1988).

2.4.2.1 Alkaline pre-treatment

The basic procedure for carrying out an alkali pre-treatment method is to soak the biomass in a solution made up of an alkaline chemical such as sodium, calcium, ammonium or potassium hydroxide, for a specific period and at a particular temperature (Kaar and Holtzapple, 2000). Soaking leads to swelling of the plant pores that result in an increase of the internal surface area and a decrease in the degree of crystallinity of cellulose (Galbe and Zacchi, 2007). These pre-treatment methods also act by removing lignin from the complex structure, thereby exposing the cellulose for enzymatic hydrolysis (Mosier *et al.*, 2005). Alkaline pre-treatment methods do not hydrolyse hemicellulose but retain it in the solids, eliminating the need to separately process hemicellulose and cellulose. Hemicellulose and cellulose can be simultaneously hydrolysed using specific commercial cellulases, which often contain enough hemicellulase or xylanase activity to concurrently convert these substrates into xylan and glucan (Wyman *et al.*, 2005). An advantage of alkali pre-treatments is that it also removes acetyl and the various uronic acid substitutions that are formed during the degradation of hemicellulose, which usually lowers the accessibility of the enzyme to the hemicellulose and cellulose surface (Mosier *et al.*, 2005).

Traditional alkaline methods use calcium hydroxide [$Ca(OH)_2$] or aqueous ammonia [NH_4OH]. Calcium hydroxide pre-treatment can be carried out at different temperatures, ranging from 25 to 130^{o}C (Wyman *et al.*, 2005). Calcium hydroxide pre-treatment has been reported to be effective in enhancing the enzymatic hydrolysis of maize stover (Kaar and Holtzapple, 2000). In a study done by Kim and Holtzapple (2005), where only 0.073 g $Ca(OH)_2$ was consumed per gram of raw maize stover for 4 weeks at 55^{o}C and with aeration, the overall yield of glucose was 93.2% at 15 FPU per gram of substrate after hydrolysis of the pre-treated maize stover. Soaking in aqueous ammonia (SAA) is another commonly used alkaline pre-treatment method where the biomass is kept in aqueous ammonia at moderate temperatures (25-80^{o}C) under atmospheric pressure. Kim *et al.* (2008) soaked barley hulls in 15 wt% aqueous ammonia at 75^{o}C. The results showed a glucose yield of 83% after enzyme hydrolysis with 15 FPU per gram of substrate, and 50–66% of the original lignin was removed from the solids without any glucan loss.

2.4.2.2 Dilute acid pre-treatment

The most commonly used and tested approaches are based on dilute sulphuric acid since they are cheap and effective (Galbe and Zacchi, 2007). However, nitric acid, hydrochloric acid and phosphoric acid have been tested as well. Dilute acid pre-treatment acts by solubilising the hemicellulose components into monomeric sugars, making the cellulose more accessible for enzymatic hydrolysis by removing hemicellulose and part of the lignin (Mosier *et al.*, 2005). However, acidic pre-treatment is performed at a low pH and this has an effect on the severity of the method. Severe conditions during pre-treatment cause greater degradation of hemicellulose sugars and this lowers the amount of recoverable sugars and promotes the formation of furfurals (Galbe and Zacchi, 2007). Total sugar yields of up to 93% from maize stover have been reported after pre-treatment at 140^{o}C for 40 minutes using a H_2SO_4 concentration of 0.98% (Lloyd and Wyman 2005).

2.4.2.3 Hydrogen peroxide and ozone pre-treatment

The hydrogen peroxide (H_2O_2) and ozone pre-treatment methods have been examined for the breakdown of lignocellulosic biomass (Kumar *et al.*, 2009). Both these agents attack lignin and hemicellulose in preference to cellulose (Wood and Saddler, 1988). During hydrogen peroxide pre-treatment lignin is solubilised through an oxidative process, thus loosening the lignocellulosic structure, which leads to improved enzymatic conversion. Nevertheless, the decomposition of hydrogen peroxide in the presence of water at high temperatures has been reported, which results in decreased lignin and xylan solubilisation (Silverstein *et al.*, 2007). A total sugar yield of 428 ± 12 $mg.g^{-1}$ which is equivalent to a 90% yield, has been reported after pre-treatment with 7.5% H_2O_2, v/v at 35^{o}C for 24 hours using rice hulls (15%, w/v) as feedstock (Saha and Cotta, 2007).

Ozonation pre-treatment methods have been found to be essentially limited to lignin degradation. Hemicellulose is slightly attacked, while cellulose is hardly affected (Sun and Cheng, 2002). The efficiency of ozone pre-treatment is affected by insufficient time, low ozone concentration or uneven distribution of ozone throughout the sample (Silverstein *et al.*, 2007). García-Cubero *et* *al*. (2009) worked with wheat and rye straw and obtained 88.6% and 57% enzymatic hydrolysis yields, respectively, after ozonolysis pre-treatment.

2.4.3 Physicochemical pre-treatment methods

Physicochemical pre-treatment methods include methods that are a mixture of purely physical and chemical methods. The most commonly used physicochemical methods for the pre-treatment of lignocellulosic materials are steam explosion and ammonia fibre explosion (AFEX) methods (Kumar *et al*., 2009).

2.4.3.1 Steam pre-treatment

In this method, the biomass is heated with high-pressure saturated steam and then the pressure is quickly decreased, which forces the lignocellulosic material to undergo explosive decompression. The role of the steam is to disrupt the hemicellulose, thus improving the accessibility of the cellulose fibrils to the cellulases during hydrolysis (Mosier *et al*., 2005). The addition of catalysts such as H_2SO_4, SO_2 or CO_2 in steam explosion promotes the disintegration of hemicellulose from cellulose and transforms lignin to a much more soluble form, resulting in an increased rate of cellulose hydrolysis. The disadvantage of using steam pre-treatment is its high cost due to the expensive equipment required (Sun and Cheng, 2002). Zimbardi *et al*. (2007) investigated the kinetics of sulphuric acid uptake during the impregnation of maize stover to point out the synergistic effect of the pre-impregnation and steam explosion on the solubilisation of carbohydrates during enzymatic hydrolysis. After a 48 hour hydrolysis period the glucose yield reached a maximum value of 93% of the theoretical after pre-impregnation with 3 wt% H_2SO_4 acid loading followed by treatment with steam explosion at 190°C for 5 minutes.

2.4.3.2 Ammonia fibre explosion pre-treatment

Ammonia fibre explosion (AFEX) is a combination of an alkaline method and steam explosion pre-treatment whereby the lignocellulosic material is soaked in liquid ammonia at a certain temperature and pressure for a period of time, and then the pressure is quickly released (Galbe and Zacchi, 2007). The concept of AFEX is similar to steam explosion, but the pre-treatment conditions (30 - 100°C) are less severe than in steam explosion. An increase in accessible surface area coupled with reduced cellulose crystallinity result in increased enzymatic digestibility, due to the reduction of the lignin content and the de-polymerization of hemicellulose (Mosier *et al.*, 2005).

The hydrolysate does not contain high concentrations of inhibitory compounds; instead the trace amount left after ammonium recovery serves as a nitrogen source for the organisms during fermentation, thus accelerating microbial growth. AFEX treated lignocellulose can be hydrolysed and produce high yields of glucose at low enzyme loadings (Wyman *et al.*, 2005). Teymouri *et al*. (2004) examined the effect of the AFEX pre-treatment on maize stover and a theoretical glucose yield of 98% was observed after treatment with ammonia at 90°C for 5 minutes, using maize stover. However, the use of ammonia, which is an expensive and hazardous chemical, at high pressure leads to high energy consumption and the need to recover ammonia after the process, makes the process economically unfavourable (Mosier *et al.*, 2005).

2.4.4 Biological pre-treatment methods

This category comprises of pre-treatment techniques that exploit the ability of microorganisms such as white and soft-rot fungi to degrade lignin and thus render the lignocellulosic material permeable to enzymes during hydrolysis (Bura, 2004). White-rot fungi are the most effective basidiomycetes for biological pre-treatment of lignocellulosic biomass. Lignin degradation by white-rot fungi, specifically *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor,* is an oxidative process that is catalysed by lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases, which are regarded as the key enzymes for this process (Lee, 1997). Biological pre-treatment methods, unlike most processes, do not require high-energy (steam and electricity), corrosion-resistant and high pressure reactors, which increase the operational cost of pre-treatment processes (Shi, 2007). The rate of biological pre-treatment processes is, however, too slow and some of the material is lost due to the consumption of cellulose and hemicellulose by the microorganism (Galbe and Zacchi, 2007).

2.4.5 Summary of pre-treatment methods

The advantages and disadvantages of all the different pre-treatment methods mentioned previously are highlighted in Table 2.1.

Table 2.1: A summary of all the pre-treatment methods described previously.

Method	Example	Advantages	Disadvantages
Physical pre-treatment	Milling/Grinding	Increases cellulose surface area.	High energy consumption.
	Irradiation	Increases biomass accessibility.	Expensive and has difficulties in industrial application.
	Pyrolysis	Short residence time.	Requires very high temperatures and cooling system, leading to increased operational costs.
Chemical pre-treatment	Alkaline	Uses cheap chemicals, low temperatures. Increases the internal surface area of biomass. Decreases the degree of crystallinity of cellulose.	Does not degrade hemicellulose.
	Dilute acid	Cheap and effective. Removes hemicellulose and part of lignin thus exposing cellulose.	Corrosive. Formation of furfurals.
	Hydrogen peroxide and ozonation	Attack lignin and hemicellulose in preference to cellulose.	Hydrogen peroxide decomposes, causing a decrease in lignin solubilisation. Uneven distribution of ozone through the sample.
Physico - chemical pre-treatment	Steam explosion	Short residence time. Promotes the degradation of hemicellulose. Transforms lignin.	Due to the high temperatures costly equipment is required.
	Ammonia fibre explosion	Increases enzyme digestibility of cellulose by removing hemicellulose.	Ammonia is expensive and hazardous. Uses high pressure systems. Ammonia recovery adds to the operational cost.
Biological pre-treatment	Fungal pre-treatment	Employs low temperatures thus does not require high pressure resistant and expensive equipment.	Slow reaction rate. Loss of substrate (cellulose).

The methods listed in Table 2.1 have one thing in common, i.e. they all increase the hydrolysis rate through the degradation of either hemicellulose or lignin or both, thus improving the accessibility of cellulose, which promotes efficient hydrolysis and results in high sugar yields. The most prominent fact is that the majority of the methods, e.g. steam explosion, pyrolysis and AFEX, require expensive high temperature and pressure systems, thus making them unfavourable for large scale commercial purposes. Mild pre-treatment methods, such as fungal pre-treatment and grinding are inefficient due to their slow rate of reaction. The prevalent challenge when processing lignocellulosic biomass is choosing a method that can effectively solubilise hemicellulose and remove lignin at low temperatures. Chemical pre-treatment methods seem to be promising, particularly the dilute acid and alkaline methods, since they use cheap chemicals and can be carried out at moderate temperatures that do not involve the use of extremely high pressurized systems, thus keeping the operational costs at a minimum. Less expensive methods such as the dilute acid and alkaline pre-treatment methods are more favourable and not complicated and can be easily implemented. Hence, they would be suitable for local and rural bioethanol production plants from lignocellulosic biomass.

2.5 Hydrolysis

The subsequent step after pre-treatment is hydrolysis, whereby cellulose and hemicellulose are split into their component sugars that can then be fermented into bioethanol (Balat *et al.*, 2008). The hydrolysis of cellulose can either be done by means of using acids or enzymes (Demirbas, 2005).

2.5.1 Acid hydrolysis

Acid hydrolysis is conducted using either diluted or concentrated acid solutions, and can be carried out simultaneously with the pre-treatment as a single step (Demirbas, 2005). The dilute acid process uses mild conditions and it only depolymerises the hemicellulose component. More severe conditions are required for cellulose solubilisation and thus, a two stage process is employed in order to achieve high sugar yields (Hamelinck *et al.*, 2005). The combination of acid, high temperature and pressure require unique and expensive reactor materials, thus increasing the cost of the process. The major advantage of dilute acid processes is the fast rate of the reaction (Balat *et al.*, 2008). Recent studies have focused on using the two stage dilute acid method as pre-treatment in combination with the enzymatic hydrolysis. Llyod and Wyman (2005) recovered an overall sugar yield of about 92.5% of the total sugars originally available in the maize stover using the coupled two stage dilute acid pre-treatment and enzymatic hydrolysis method.

The concentrated acid hydrolysis process relies on the decrystallisation of cellulose to sugars. This process uses concentrated acid doses followed by dilution with water to hydrolyse the cellulose into sugars (Demirbas, 2005). The last step is the separation of acid from sugars through acid recovery or acid re-constitution (Saxena *et al.*, 2009). The concentrated acid process uses low temperatures and as a result there is limited degradation of sugars, the only pressures involved are those created by pumping materials from vessel to vessel. This allows for the use of relatively low-cost materials. High yields (such as 90%) have been reported; however, it is a relatively slow process compared to the dilute acid process and the acid recovery systems create additional costs (Balat *et al.*, 2008).

2.5.2 Enzymatic hydrolysis

The enzymatic hydrolysis method is a complex process due to substrate and enzyme-related factors (Yang and Wyman, 2007). The enzymatic hydrolysis route is conducted at mild operating conditions; there is minimal by-product formation, low energy requirement and low chemical disposal costs when compared to the acid route (O'Dwyer *et al.*, 2007). The enzymes used in the process are extracted from bacteria and fungi that can synthesize cellulases under aerobic or anaerobic conditions. The major source of the commercial cellulases commonly used today is the filamentous fungal species, *Trichoderma reesei* and is considered to have the most effective enzyme for the hydrolysis of crystalline cellulose (Sun and Cheng, 2002).

The three major groups of cellulases involved in the hydrolysis of cellulose are: (1) endoglucanase (endo-1,4-β-D-glucanohydrolase) (2) exoglucanase or cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase) and (3) β-glucosidase. Endoglucanases randomly cut at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and as a result new chain ends are produced. Exoglucanases act on the reducing or non-reducing ends of the new cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products of enzymatic hydrolysis. β -Glucosidases are responsible for the hydrolysis of cellobiose to glucose (Figure 2.7) (Lynd *et al.*, 2002).

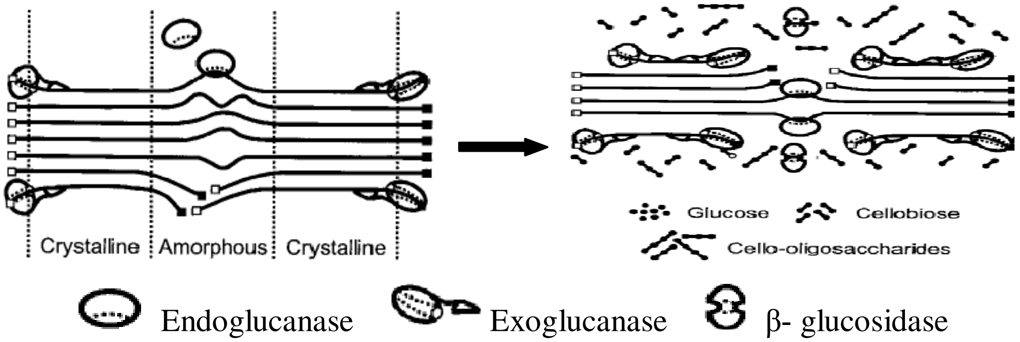

Figure 2.7: Schematic representation of the cellulase system during the hydrolysis of cellulose (Lynd *et al.*, 2002).

Efficient enzymatic hydrolysis of cellulose relies on the adsorption of cellulase on the cellulose surface. The cellulases are then desorbed after the reaction so that they can bind to another site on the cellulose in order for the process to continue and to allow complete hydrolysis of cellulose. The irreversible adsorption of cellulases on cellulose can lead to the deactivation of the enzyme and thus result in low sugar yields (Nag, 2007). The cellulase components that form the cellulase complex act synergistically to efficiently hydrolyse cellulosic substrates. It has been reported that the hydrolysis of lignocellulosic biomass by cellulases improves in the presence of β-glucosidase (cellobiase). β-Glucosidase hydrolyses cellobiose, one of the products formed during enzyme hydrolysis, which tends to inhibit the cellulase activity when present at high concentrations in the hydrolysate (Sun and Cheng, 2002).

The hydrolysis rate of cellulose by cellulases is greatly affected by hemicellulose and lignin. Within the cell wall structure hemicellulose coats the cellulose-fibrils resulting in decreased accessibility of the cellulose. Therefore, it is essential to hydrolyse hemicellulose during pre-treatment in order to expose cellulose and to facilitate its complete degradation during hydrolysis. Lignin acts as an attractant of cellulases, thus preventing the enzyme from binding to cellulose, resulting in non-productive binding and decreased enzyme activity. The adsorption of cellulases to lignin has been reported to slow down the rate of enzymatic hydrolysis due to the deactivation of the enzyme caused by the phenolic groups, which are released during the solubilisation of lignin (Eriksson *et al.*, 2002). Additional factors that affect the enzymatic hydrolysis include end product inhibition and substrate concentration (Sun and Cheng, 2002). End product inhibition occurs when high concentrations of cellobiose are present in the hydrolysate; hence β-glucosidase from *Aspergillus niger* is usually added to complement the activity of the cellulases by immediately breaking down cellobiose into glucose, which has no effect on cellulase activity (Murray, 2005). It has been observed that the activity of cellulases decreases at high substrate loadings, probably due to the inhibition of adsorption of the cellulase enzymes (Kristensen *et al.*, 2009).

The rate of cellulose conversion to glucose decreases as time progresses due to rapid hydrolysis of the readily accessible fraction of cellulose, product inhibition, and slow inactivation of absorbed enzyme molecules (Balat *et al.*, 2008). To overcome this, an option would be to increase the enzyme loading to obtain high yields of fermentable sugars from cellulose. However, a high enzyme loading has a negative impact on the overall process cost. Therefore, methods that enhance enzyme activity are necessary for the reduction of enzyme loading while improving glucose yields (Eriksson *et al.*, 2002).

Hemicellulose holds a strong potential as a source of value-added useful products, such as bioethanol, xylitol and 2, 3-butanediol, through enzyme conversion. Since hemicellulose is a highly substituted carbohydrate with different components, its enzymatic degradation requires the use of xylanases and several accessory enzymes to achieve complete hydrolysis (Saha, 2003). Hemicellulose bioconversion involves mannanase and xylanase enzymes for the degradation of the hemicellulose backbone components, while β-xylosidases and β-mannosidases degrade the xylo-oligosaccharides further. α-Arabinosidases and α-galactosidases release arabinose and galactose molecules, whereas acetyl xylan esterase, ferulic acid esterase and ρ-coumaric acid esterase cleave the ester linkages between xylose units and acetic acid and between arabinose side chain residues and ferulic acid and ρ-coumaric acid groups from the sugar units of xylan, respectively (Juhász *et al*., 2005; Saha, 2003).

2.5.3 Summary of hydrolysis methods

There are several advantages and disadvantages of acid and enzymatic hydrolysis methods that are shown in Table 2.2. A summary of the different hydrolysis methods discussed in literature is given.

Table 2.2: Advantages and disadvantages of the different hydrolysis methods.

Method	Advantage	Disadvantage
Dilute acid hydrolysis	Fast reaction rate.	Uses high temperature and pressure systems. Only the hemicellulose component is depolymerised. Requires a two stage process in order to hydrolyse the cellulose component as well. Low sugar yields.
Concentrated acid hydrolysis	Decrystallises cellulose to sugars. Low temperatures are used. No pressure systems are required.	High water use due to the need to dilute the hydrolysate prior to fermentation. Separation of acid from sugars and acid reconstitution increase the operational costs.
Enzymatic hydrolysis	No formation of degradation components. Conducted at mild temperatures Low utility costs. Does not use corrosive chemicals. Produces higher sugar yields.	Slow. Very sensitive. Unproductive binding to lignin. Enzymes are expensive.

The objective of lignocellulosic biomass hydrolysis is to break down cellulose and the remaining hemicellulose into their constituent sugars. The hydrolysis process determines the success of achieving improved ethanol yield; the more efficient the hydrolysis rate and the recovery of fermentable sugars, the higher the ethanol yield. The best hydrolysis method would have higher hydrolysis yields without increasing the process costs. Acid hydrolysis methods, though they take minutes to hours to be completed, have additional costs due to the use of high temperatures, and the lower yields make these methods unfavourable. Both the dilute and concentrated acid hydrolysis methods have the potential to produce high sugar yields, but due to the loss of a considerable amount of the produced sugars caused by the use of high temperatures or the need to recover or separate the acid from the hydrolysate, the sugar yields decrease. The main advantage of using the enzymatic hydrolysis method is that it is conducted at mild conditions – as a result there are no additional costs and though it is slow, the sugar yields are higher. The inactivation of cellulase attributable to unproductive binding of the enzyme to lignin can be prevented by using additives. Even though the high cost of enzymes is still a challenge, progress has been made in reducing the amount of enzymes added during the hydrolysis of lignocellulosic biomass while maintaining high sugar yields. Thus, the enzymatic process is the most preferable method for the hydrolysis of biomass.

2.6 The effect of surfactants during enzyme hydrolysis

The addition of surfactants such as non-ionic detergents has been reported to be effective in increasing enzyme activity. Nonionic surfactants include berol, polyoxyethylene, Tween 80 and Tween 20 (Kristensen *et al.*, 2007). Alkasrawi *et al.* (2003) reported that, with the addition of a surfactant such as Tween 20, enzyme loading can be reduced by as much as 50% and still obtain the same ethanol yield. The addition of surfactants during hydrolysis improves the hydrolysis rate by (1) adsorbing at the air-liquid interface and thus prevents enzyme denaturation during hydrolysis, (2) promoting the availability of reaction sites and (3) preventing irreversible adsorption of enzymes to lignin, thus eliminating enzyme inactivation (Eriksson *et al.*, 2002). Chen *et al.* (2008) reported an increase in the hydrolysis yield from 81.2 to 87.3% after the addition of 5 g.L^{-1} of Tween 80 using 80 g.L^{-1} substrate concentration and an enzyme dosage of 20 FPU per gram of substrate for 48 hours. The addition of surfactants during fermentation has also been reported to have some positive effect on the ethanol yields (Lee *et al.*, 1996). However, compatibility of the surfactant and the microorganism to be used for fermentation is important for the application of surfactants, because some surfactants can inhibit cell growth, thus resulting in a lower ethanol yield (Wu and Ju, 1998). For instance, Tween 20 has been reported to be highly inhibitory even at a low concentration of 0.1%, to some microorganisms such as *Dekkera clausenii* (Sun and Cheng, 2002).

2.7 Fermentation

Fermentation of lignocellulosic biomass hydrolysates is one of the most challenging biotechnological processes. The productivity of the fermentation process is greatly dependent on environmental factors, which include pH, temperature, the chemical composition of the fermentation medium, the concentration of essential nutrients or inhibitory compounds present in the hydrolysate (Silverstein, 2004) and, most important, the efficiency of the microorganism to ferment all the available monosaccharides, such as glucose, xylose, mannose, galactose, arabinose and oligosaccharides (Balat *et al.*, 2008).

The most commonly used microorganism for industrial production of ethanol is the yeast species called *Saccharomyces cerevisiae*, because of its high resistance to inhibitory compounds that might be present in the hydrolysate, as well as its high ethanol tolerance. A major drawback is that *S. cerevisiae* cannot utilize xylose and other pentose sugars. *S. cerevisiae* can, however, ferment the xylose isomer xylulose (Eliasson *et al.*, 2000).

The reported theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide per kg of xylose (eq. 1) and glucose (eq. 2), according to the following overall reaction (Balat *et al.*, 2008):

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2, \qquad (1)$$

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \qquad (2)$$

Fermentation can be operated as a batch, fed-batch or a continuous process. In a batch process a closed system is used where nothing is added after inoculation except possibly acid or alkali for pH control or air during aerobic fermentations. Fermentation stops when the limiting nutrient is exhausted and products, which usually have inhibitory effects, accumulate. This is the most commonly used process for bioethanol production due to low investment costs, does not require much control, can be accomplished with unskilled labour and complete sterilization and management of feedstocks are easier than in the other processes (Prasad *et al.*, 2007).

Fed-batch fermentation is a process whereby the major substrate component is added to the growth medium at intervals during fermentation. The process is first started as a batch process, but it is prevented from reaching the steady state by adding the substrate feed once the initial substrate is consumed. The fermentation is continued at a certain growth rate until a practical limitation inhibits the cell growth (Saarela *et al.*, 2003). This intermittent feeding of the substrate achieves higher volumetric productivities as a result this process has gained popularity over the past several years in the ethanol industry (Sánchez and Cardona, 2008).

The continuous fermentation process makes use of an open system where fresh medium is continuously added and the product is removed at the same rate, thus the reaction volume is kept constant. Advantages of continuous systems compared to conventional batch systems mainly include: decrease of product inhibition effect, lower maintenance and operation requirements, better system control and higher productivities (Sánchez and Cardona, 2008). However, the high risk of contamination and mutation due to long cultivation periods and periodic handling, and the use of larger and expensive reactor volumes make this system less favourable (Prasad *et al.*, 2007).

There are four ethanol fermentation processes that can be used: direct microbial conversion (DMC), separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) (Nag, 2007) and simultaneous saccharification and co-fermentation (SSCF) (Balat *et al.*, 2008).

2.7.1 Direct microbial conversion (DMC)

Lignocellulosic biomass can be directly converted into ethanol using microorganisms that have the potential to delignify the biomass and convert the resulting cellulose into ethanol, usually using co-cultures of different microbial strains. Direct microbial conversion combines cellulase production, hydrolysis and fermentation into a single step (Nag 2007). This is usually accomplished in two ways, namely a solid state fermentation process, which is defined as the microbiological transformation of biological materials in their natural state in the absence of any free water, and a liquid state fermentation process, also known as submerged fermentation, which is carried out in dilute solutions or slurries (Kumar *et al*., 2006). Microorganisms that can hydrolyse cellulose and ferment the produced sugar components rapidly and efficiently do not occur naturally. Hence, research is focusing on constructing organisms into which genes encoding for all the required enzymes are cloned (Lynd, 1996). The process is attractive in that it reduces the overall operation cost since only one reactor is needed and no chemicals are added. However, the ethanol yield is low and the organisms used usually have low ethanol tolerance (Silverstein, 2004). In addition, it is not easy to mimic the natural habitat whilst still maintaining a contaminant - free environment. The development of the feasible biological delignification process would be possible if the ecophysiological requirements and optimal bioreactor designs of the lignin-degrading microorganisms are effectively coordinated (Lee, 1997).

2.7.2 Separate hydrolysis and fermentation (SHF)

Separate hydrolysis and fermentation (SHF) is a process whereby a sequential route is employed. The solid fraction of the pre-treated lignocellulosic biomass, which mainly contains cellulose, is enzymatically hydrolysed and the resulting hydrolysate is then fermented to ethanol in a separate step (Linde *et al*., 2008). One of the main advantages of the SHF process is that each step can be performed at its optimal operating conditions, thus promoting complete hydrolysis of cellulose. The SHF process is however, prone to end product inhibition as discussed previously (see Section 2.5.2) (Sun and Cheng, 2002).

2.7.3 Simultaneous saccharification and fermentation (SSF)

In this method, both the enzymatic hydrolysis and fermentation unit processes are performed in a single step. The SSF process allows for the immediate conversion of the fermentable sugars formed during the enzymatic hydrolysis to be converted into ethanol (Hahn-Hägerdal *et al*., 2007). The advantages of the SSF process compared to the SHF, include: (1) an increase in the hydrolysis rate since the sugars that decrease the cellulase activity are quickly converted into ethanol, (2) higher product yields, (3) lower requirements for sterile conditions since glucose is removed immediately and the ethanol produced inhibits bacterial growth, and (4) a shorter process time (Sun and Cheng, 2002). However, this process operates at non-optimal conditions regarding hydrolysis. Yeasts operate optimally at temperatures below 37°C for example, while optimal enzymatic hydrolysis temperature is 50°C (Sánchez and Cardona, 2008).

2.7.4 Simultaneous saccharification and co-fermentation (SSCF)

The simultaneous saccharification and co-fermentation (SSCF) method is an improved version of the SSF technology (Balat *et al*., 2008). Research is now focusing on techniques that promote the fermentation of all the available sugars in lignocellulosic biomass with the intention of achieving high ethanol yield. This method is based on the co-fermentation of hexose and pentose sugars. The SSCF is carried out by genetically engineered microbes that ferment xylose and glucose in the same process unit concurrently with the enzymatic hydrolysis of cellulose and hemicellulose (Lynd *et al*., 2002). The SSCF process can only be accomplished by inserting genes that either encode the production of xylose reductase (XR) and xylitol dehydrogenase (XDH) or the xylose isomerase (XI) into industrial strains such as *S. cerevisiae* (Eliasson *et al*., 2000). These enzymes enable *S. cerevisiae* to also ferment xylose to bioethanol using different pathways, where xylose is first isomerised to xylulose prior to fermentation (Chu and Lee, 2007). Ohgren *et al*. (2006) were able to obtain an increased overall ethanol yield from 52 to 64% (theoretical) during the co-fermentation of pre-treated maize stover, using TMB3400 - a strain that had been transformed with the plasmid YIpXR/XDH/XK, which gave it the ability to co-ferment glucose and xylose during the SSCF process.

2.7.5 Summary of the fermentation processes

The distinctive features of each fermentation process, in terms of advantages and disadvantages, are listed in Table 2.3.

Table 2.3: Summary of the advantages and disadvantages of each fermentation process

Process	Advantages	Disadvantages
DMC	Requires one reactor. Cost effective.	Very slow. It is not easy to achieve a natural habitat in a laboratory setting.
SHF	Operates at optimal conditions.	End product inhibition. Lower yields.
SSF	Higher ethanol yields due to increased hydrolysis rate. Shorter process time.	Non-optimal process conditions.
SSCF	Ferments both hexose and pentose sugars.	Requires genetically engineered microbial strains.

The common challenge in the different fermentation processes is obtaining an organism that can ferment both hexose and pentose sugars in order to achieve a high ethanol yield and still be able to grow in the presence of ethanol. The SSF process is the most preferred method since it produces higher ethanol yield but the organism used, *S. cerevisae*, cannot ferment pentose sugars. With improved recent technologies organisms that can utilize all the sugars present in lignocellulosic biomass have been constructed and tested in a process called the SSCF. The SSCF process is promising since it has the potential to increase the overall ethanol yield by using organisms that can convert both hexose and pentose sugars into ethanol (Lynd *et al.*, 2002). The difficulty with inserting genes from a different organism into another is that the inserted genes can fail to be expressed in the new host, resulting in low ethanol yield.

2.8 Concluding remarks

The reliance on imported crude oil has resulted in food inflation due to high oil prices and the increase in GHG emissions has increased the demand for alternative clean burning energy sources such as biofuels. The use of biofuels (bioethanol, biodiesel and biogas) has the potential to reduce the amount of imported crude oil and the GHG emissions, whilst improving the economy (Manny, 2006). For instance, if each country produces its own transportation biofuels, it would not solely rely on imported crude oil and its economy would improve because there will be more jobs created and less money spent on import markets. In addition, biofuels are clean burning fuels, thus they have the potential to contribute towards achieving the targeted 34% GHG emission reduction by 2020.

Bioethanol can be used as a sole transport fuel (e.g. E85), a petrol enhancer (e.g. E10), for the production of gel fuels and for industrial purposes (Balat *et al.*, 2008). The success of bioethanol production depends on availability of the appropriate feedstock and the operational cost of the overall process. Second generation (lignocellulosic feedstock) technologies are more beneficial because they do not pose a threat to food security (Glassner *et al.*, 1999). However, due to the complex structure of lignocellulosic biomass the process is very costly. The most influential factors during this process are the pre-treatment and the cost of enzymes used during the enzymatic hydrolysis of lignocellulose (Gray *et al.*, 2006).

The pre-treatment method is crucial for the subsequent steps involved, since its role is to open up the lignocellulosic structure such that all the present structural carbohydrates are available, thus facilitating a high recovery of cellulose and hemicellulose without the formation of fufurals, and at low costs (Galbe and Zacchi, 2007). Mild pre-treatment methods such as the dilute acid and calcium hydroxide methods that are affordable and non-complicated, therefore, are more favourable for the pre-treatment of lignocellulosic biomass, since they would be easy to implement, particularly in rural settings. The most preferable method for the hydrolysis of cellulose to fermentable sugars is the enzymatic route because it produces higher yields and is conducted under mild conditions. The addition of surfactants during enzyme hydrolysis has the potential to improve sugar yields at low costs, thus making the process economically viable. The

SSF process, though it does not utilize all the available sugars, is still regarded as the suitable method, since it produces higher ethanol yield. In addition, the SSF process is not affected by end product inhibition and does not require genetically engineered microbial strains, which are difficult to work with due to their instability. Finally, the production of bioethanol from lignocellulosic biomass depends on the progress of optimizing each process unit, thus improving the yields of the product at affordable costs.

CHAPTER 3
EXPERIMENTAL

3.1 Introduction

This Chapter describes the experimental methods and also states all the materials and equipment used in this study. The details about the feedstock and its composition are given in Section 3.2. The experimental procedure is elaborated in Section 3.3. The two different methods used to pretreat maize stover, which are calcium hydroxide and dilute sulphuric acid methods, are discussed in Section 3.4. Section 3.5 deals with the optimization of the enzymatic hydrolysis of maize stover. Lastly, in Section 3.6 the analysis method used in this study is given.

3.2 Feedstock

Air dried and shredded maize stover was received in 2008 from a local farm in the North-West Province. In this study maize stover was milled to pass through a 1.5 mm screen using a hammer mill and then stored in sealed glass bottles at room temperature. The biomass primarily consisted of stalks, leaves and cobs. Different images of shredded and milled maize stover are shown in Figure 3.1.


(a) (b)

Figure 3.1: Images of (a) shredded and (b) milled stover

3.2.1 Composition of maize stover

The composition of the maize stover used in this study was determined by the Agricultural Research Centre (ARC) at the Irene Analytical Services Laboratory in Pretoria. The results from the analysis are shown in Table 3.1.

Table 3.1: Composition of maize stover

Component	**%**
Cellulose	31.45
Hemicellulose	26.02
Lignin	17.62
Protein	2.490
Fat	1.220
Moisture	7.450
Unknown	13.75

These results were obtained from the ARC laboratory.

The compositional analysis results reflect that cellulose, hemicellulose and lignin are the major constituents of maize stover at 31.45%, 26.02% and 17.62%, respectively, and the total carbohydrate content is 57.47%. According to these results an overall theoretical sugar yield of 0.58 gram glucose per gram biomass ($g.g^{-1}$) can be obtained.

3.3 Experimental procedure

The first step prior to chemical processing of lignocellulosic biomass is to mill the feedstock. Milling physically breaks down the feedstock, thus increasing its surface area and allowing easy accessibility to the substrate. The milled stover was pre-treated with calcium hydroxide or sulphuric acid at a constant temperature using an autoclave. After autoclaving the samples were cooled at room temperature and immediately hydrolysed with Celluclast ® 1.5 L (a cellulase enzyme) supplemented with Novozyme ® 188 (a β-glucosidase) and the produced glucose was calculated based on HPLC analysis. An illustration of the experimental procedure is shown in Figure 3.2. Chemical dose and time were varied during pre-treatment and for the enzymatic hydrolysis the variables were enzyme, substrate and Tween 80 loadings. The measured variable was the glucose yield, for all the experiments.

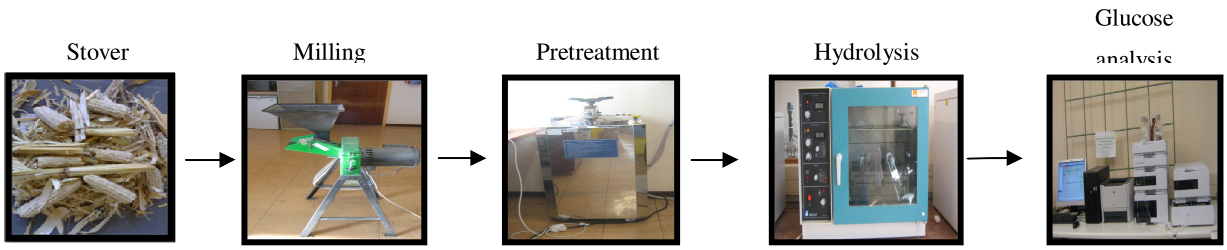

Figure 3.2: A schematic representation of the experimental procedure.

3.4 Pre-treatment

The pre-treatment of stover was performed at a moderate temperature using less expensive chemicals in order to reduce the process costs, as discussed in Section 2.5.5. The focus was on the success of calcium hydroxide and sulphuric acid to alter the lignocellulosic structure such that the carbohydrates present in the stover are available for enzymatic hydrolysis. The initial experiment performed was the determination of the effect of chemical dose during pre-treatment for both calcium hydroxide and dilute acid methods. The chemical dose was varied and the pre-treatment time was chosen, based on findings from literature with minor modifications. Then the pre-treatment time was varied in order to establish the best residence time at a specific chemical loading. The pre-treatment temperature was kept at 121°C. The pre-treated solids were not washed, in all the pre-treatment experiments, because it has been reported that water washing the pre-treated stover reduces the amount of recovered sugar by 10% (Kaar and Holtzapple, 2000).

3.4.1 Calcium hydroxide pre-treatment

Calcium hydroxide [$Ca(OH)_2$] loadings of 0.1, 0.2, 0.3, 0.4, and 0.5 g of $Ca(OH)_2$ per gram of dry biomass were used to pretreat 10 g of stover in 100 mL of distilled water for 180 minutes at 121°C in an autoclave (Kaar and Holtzapple, 2000; Silverstein *et al.*, 2007). The samples were cooled to room temperature and then hydrolysed using an enzyme loading of 30 FPU per gram of substrate. After enzyme hydrolysis for 24 hours the samples were filtered and analysed for glucose content using the HPLC in order to determine which calcium hydroxide loading would produce the highest glucose yield after pre-treatment and enzyme hydrolysis.

3.4.2 Dilute sulphuric acid pre-treatment

Sulphuric acid [H_2SO_4] pre-treatment was done at various acid dosages of 0.5, 1.0, 2.0, 3.0 and 4.5 wt% H_2SO_4 using 100 mL of distilled water to pretreat 10 g of stover for 60 minutes in an autoclave at 121ºC (Saha *et al*., 2005). The samples were cooled to room temperature and then hydrolysed with an enzyme loading of 30 FPU per gram of cellulose. After enzyme hydrolysis for 24 hours the samples were filtered and analysed for glucose content using the HPLC in order to determine which sulphuric acid loading would result in the highest glucose yield after pre-treatment and enzyme hydrolysis.

3.4.3 Pre-treatment time

The first experiments were conducted for 180 minutes for calcium hydroxide and 60 minutes for the sulphuric acid pre-treatment method. For this study, the effect of time during pre-treatment was determined by treating 10 g of maize stover with 0.2 g of $Ca(OH)_2$ per gram of dry biomass and 3 wt% H_2SO_4 at different residence times of 30, 60, and 120 minutes using an autoclave at 121ºC (Silverstein *et al*., 2007). After enzyme hydrolysis for 24 hours the samples were filtered and analysed for the glucose content using the high performance liquid chromatography (HPLC). The aim was to determine a residence time that produced the maximum amount of glucose after pre-treatment of maize stover.

3.5 Hydrolysis

The acid hydrolysis process requires severe conditions such as excessive high temperature or acid concentrations which result in the loss of sugars, hence the lower yields. Therefore, the enzymatic route was chosen for the hydrolysis of maize stover in preference to acid hydrolysis, given that the enzymatic hydrolysis is conducted at mild conditions and it produces higher sugar yields.

3.5.1 Enzymes

The cellulase used in this study was Celluclast ® 1.5 L isolated from *Trichoderma reesei*. Its activity was determined to be 74 FPU (mL enzyme)$^{-1}$ in solution, as measured by the filter paper assay (see Appendix A). The cellulase was supplemented with Novozyme ® 188 from *Aspergillus niger*, a β-glucosidase enzyme that degrades cellobiose, at a ratio of 1:1.75 (i.e. Celluclast ® 1.5 L: β-glucosidase). The enzymes used in this study were acquired from Novozyme Co., in South Africa. The Filter Paper Unit (FPU) is used to define enzyme activity. A quantity of 0.1875 FPU is the enzyme activity that will produce a reducing sugar equivalent to 2.0 mg of glucose (Adney and Baker, 1996). The protein contents of Celluclast ®1.5 L and Novozyme ® 188 have been reported to be 191 and 143 mg.mL^{-1}, respectively (Silverstein *et al.*, 2007).

3.5.2 Enzyme hydrolysis

Maize stover samples, pre-treated according to the procedure described in Section 3.4, were used for enzyme hydrolysis. For the preliminary hydrolysis experiments a solid loading of 5% and an enzyme loading of 30 FPU per gram of cellulose were used. The medium used for enzyme hydrolysis contained 5 mL of 10 g.L^{-1} of sodium azide, added to avoid microbial contamination in a total of 100 mL of 0.05 M citrate buffer solution (pH 4.8). Hydrolysis was performed in an incubator shaker at 50^{o}C, 150 rpm and the pH was adjusted to 5.0. Controls (non-pre-treated samples) were included to highlight the importance of pre-treatment prior to hydrolysis (Silverstein *et al.*, 2007).

The substrate concentration and cellulase activity tend to restrict the rate of enzymatic hydrolysis of lignocellulosic biomass. The effect of substrate loading on enzymatic hydrolysis was determined at a fixed enzyme loading of 30 FPU per gram of cellulose by varying the substrate loading from 5 to 15%. The effect of enzyme loading was investigated by adding different amounts of Celluclast ® 1.5 L and Novozyme ® 188 at a constant substrate loading of 5%. The different enzyme loadings used were 7.5, 15 and 30 FPU per gram of cellulose. The glucose yield was measured after 48 hours (Kristensen *et al.*, 2009).

The ability of the non–ionic surfactant to enhance the enzymatic hydrolysis of maize stover was determined by monitoring the glucose yield during enzyme hydrolysis after adding Tween 80 for samples pre-treated with 3 wt% sulphuric acid for 60 minutes. The various concentrations of Tween 80 used were 0 (control), 1.25, 2.5 and 5 g.L^{-1}, hydrolysed for 48 hours (Chen *et al.*, 2008).

3.6 Analysis

The samples were filtered after hydrolysis prior to analysis through 0.2 µm pore size Acrodisc ® syringe filters using GHP membrane (25 mm). The analysis of glucose concentration present in the hydrolysate was done using an Agilent 1200 series High Performance Liquid Chromatography (HPLC) machine with an auto sampler, quaternary pump and refractive index detector (RID). The HPLC was also equipped with a Shodex sugar SP0810 column (8.0 mm ID*300mm). The injection sample volume was 5 µl using water as the mobile phase at a flow rate of 1 mL.min^{-1}. The column temperature was 80^{o}C and the RID temperature 55^{o}C. The run time for each sample was 20 minutes. A glucose calibration curve was constructed using standards prior to analysis of the hydrolysates. The standards were prepared by running samples with known concentrations of glucose through the HPLC system. The linear equation established between the response (peak areas) and the concentration of the standards was then used to calculate the unknown glucose concentration in the hydrolysates (see Appendix B).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Introduction

In this Chapter, the results obtained from the experimental methods described in Chapter 3, are discussed. The major sugar component found in the maize stover was glucose with xylose as the second highest sugar constituent and the other sugars such as arabinose and galactose were present in minute amounts. Thus, glucose was used as an indicative measure for all the experiments done in this study. The experimental error is shown in Section 4.1. The results of the two different pre-treatment methods used, are presented in Section 4.3, while the effect of different variables on the final glucose yields during enzyme hydrolysis is analysed in Section 4.4. And finally, the results are discussed in Section 4.5.

4.2 Experimental error and repeatability

The experimental error was calculated to determine the reproducibility of the hydrolysis experiments. The experimental error was found to be 5% during a 48 hour enzyme hydrolysis with the addition of Tween 80 (Appendix C). The confidence interval was calculated at 95% using Eq. 1 and the experimental error was calculated using Eq. 2 (Vardeman, 1994):

$$CI = \bar{x} + z.\frac{\sigma}{\sqrt{n}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

$$Error\,\% = \frac{z.\frac{\sigma}{\sqrt{n}}}{\bar{x}}.100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

4.3 Pre-treatment

Lignocellulosic biomass is naturally complex and recalcitrant; therefore, pre-treatment is crucial if efficient enzymatic hydrolysis is to be achieved. In this study the effect of pre-treatment on the hydrolysis yield was determined by comparing the glucose yield after the enzymatic hydrolysis of all un-treated and pre-treated samples, and the conversion efficiency of the methods. Various chemical doses and pre-treatment times were examined for each method used in order to establish the optimum chemical loading that produces the highest glucose yield.

4.3.1 Results of calcium hydroxide pre-treatment

Maize stover was pre-treated with calcium hydroxide according to the procedure described in Section 3.4.1. The effect of varying the calcium hydroxide loading on the glucose yield is illustrated in Figure 4.1. The conversion efficiency was determined based the glucose yield obtained per gram of biomass used as a ratio of the maximum theoretical sugar yield which was 0.58 g.g^{-1}, based on the composition of the maize stover used in this study (see Section 3.2). The values for the glucose yield and conversion efficiency are tabulated under the Appendix B Section in Table B.1.

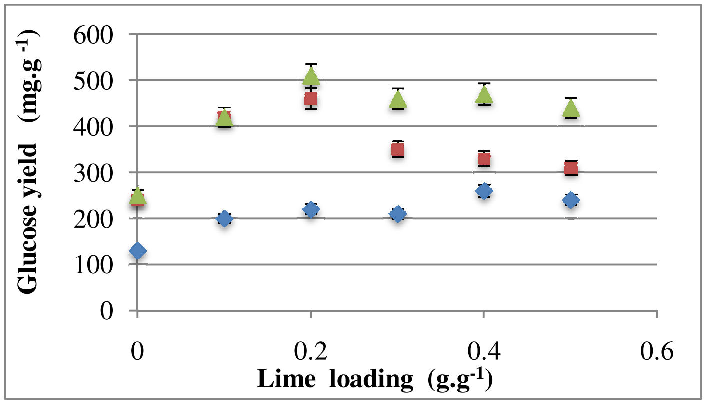

Figure 4.1: Effect of calcium hydroxide loading on glucose yields after pre-treatment and enzyme hydrolysis of maize stover (◆ 0 hour, ■ 8 hours and ▲ 24 hours). Pre-treatment conditions: 0.1, 0.2, 0.3, 0.4, and 0.5 g of $Ca(OH)_2$ per gram of dry biomass at 121˚C for 180 minutes. Enzyme hydrolysis: 50˚C, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ and at a 5% solid loading.

The glucose yield in the control sample (no calcium hydroxide added) increased from an initial yield of 130 ± 7 to 250 ± 13 $mg.g^{-1}$ (for conversions 1 $g.g^{-1} \approx 1000$ $mg.g^{-1}$ this applies to all the results in this study). Although there was an increase in glucose yield in the control sample, the conversion efficiency of the hydrolysis method was low, only 43% of the theoretical carbohydrate content was converted to glucose after enzyme hydrolysis for 24 hours. In the presence of calcium hydroxide, the glucose yield increased to a maximum of 510 ± 26 $mg.g^{-1}$ after pre-treatment at a calcium hydroxide loading of 0.2 g of $Ca(OH)_2$ per gram of dry biomass, which resulted in a 88% glucose recovery during enzyme hydrolysis. At higher calcium hydroxide loadings, such as 0.5 g of $Ca(OH)_2$ per gram of dry biomass, the glucose yield decreased to as low as 440 ± 22 $mg.g^{-1}$ after a 24 hour enzyme hydrolysis period.

The increase in glucose yield during the hydrolysis of the control sample (i.e. the un-pre-treated sample) indicated that the enzymes were able to convert some of the cellulose to glucose but the conversion was limited because the cellulosic structure was still intact – that is the enzymes had low access to the cellulose due to the bound lignin and hemicellulose. The high glucose yield obtained when the stover was hydrolysed after pre-treatment with calcium hydroxide at 0.2 g of $Ca(OH)_2$ per gram of dry biomass, showed that calcium hydroxide does have the ability to improve enzyme activity. This means that calcium hydroxide can solubilise the lignocellulose matrix of maize stover thus allowing more access of the enzyme to cellulose. The more accessible the cellulose to the enzymes, the easier it is for the enzyme to hydrolyse it into glucose; hence, the increased yield after pre-treatment.

The decrease in glucose yield at higher calcium hydroxide loadings such as 0.5 g of $Ca(OH)_2$ per gram of dry biomass is due to inactivation of cellulase at high calcium hydroxide concentrations. At high $Ca(OH)_2$ concentrations, the reaction rate is lowered and less glucose is thus produced in the same amount of time (Stutzenberger and Sterpu, 1978). Therefore, a calcium hydroxide loading of 0.2 g of $Ca(OH)_2$ per gram of dry biomass was used for the subsequent calcium hydroxide pre-treatment experiments in this study.

4.3.2 Results of sulphuric acid pre-treatment

The procedure for pre-treatment with sulphuric acid (H_2SO_4) is described in Section 3.4.2. The glucose yields obtained, after sulphuric acid pre-treatment and enzyme hydrolysis of maize stover and also the conversion efficiency of the hydrolysis method used in this study are given in Table B.2 (see Appendix B Section). Figure 4.2 shows the effect of using different sulphuric acid dosages during pre-treatment.

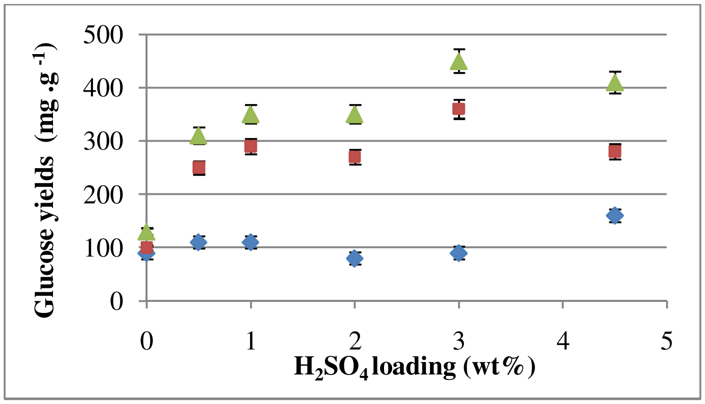

Figure 4.2: Effect of sulphuric acid dosage on glucose yields after pre-treatment and enzyme hydrolysis of maize stover (◆ 0 hour, ■ 8 hours, ▲ 24 hours). Pre-treatment conditions: 0.5, 1.0, 2.0, 3.0 and 4.5 wt% of H_2SO_4 at 121˚C for 60 minutes. Enzyme hydrolysis: at 50˚C, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ and at a 5% solid loading.

There was a marked increase in glucose yield after hydrolysis for 24 hours, from 130 ± 7 mg.g^{-1} in the control sample to a maximum of 450 ± 23 mg.g^{-1}, which translates into a 22% and 78% glucose recovery after enzyme hydrolysis, for the control and the sample that was pre-treated with 3 wt% sulphuric acid, respectively. After the enzymatic hydrolysis of the samples pre-treated with 4.5 wt% sulphuric acid the conversion efficiency (71%) was slightly lower, hence the glucose yield decreased to 410 ± 21 mg.g^{-1}. Dilute sulphuric acid pre-treatment enhances enzyme hydrolysis by solubilising hemicellulose, leading to increased availability of cellulose, which is easily converted to glucose during enzyme hydrolysis, hence the increase in glucose yield after pre-treatment with sulphuric acid. The decrease in glucose yield, at 4.5 wt% acid loading, falls within the experimental error of this study.

4.3.3 Results of pre-treatment time

The effect of pre-treatment time was studied using 3 wt% H_2SO_4 and 0.2 $Ca(OH)_2$ per gram of biomass as pre-treatment agents, at different autoclave residence periods. The results obtained are illustrated Figure 4.3. A detailed illustration for the glucose yield and conversion efficiency is in Table B.3 under the Appendix B Section.

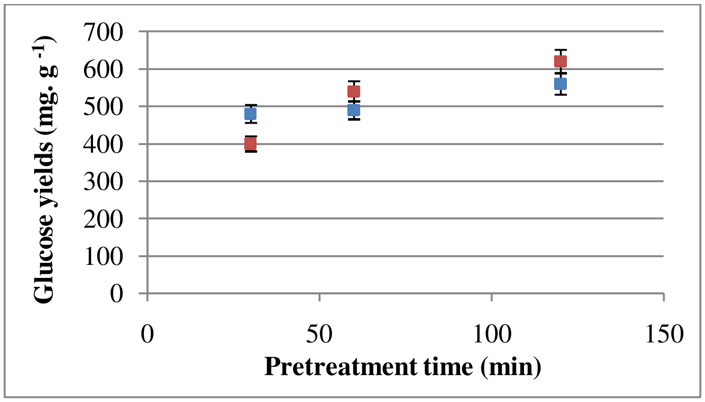

Figure 4.3: Effect of pre-treatment time on glucose yields after pre-treatment and enzyme hydrolysis of maize stover [🟦 0.2 g of $Ca(OH)_2$. (g of dry biomass)$^{-1}$, 🟥 3 wt% H_2SO_4]. Pre-treatment conditions: at 121°C for 60 minutes. Enzymatic hydrolysis: 50°C, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ and at a 5% solid loading for 48 hours.

The results revealed that the enzymatic hydrolysis of maize stover after prolonged pre-treatment time increases the glucose yield. The glucose yield improved from 480 ± 24 to 560 ± 28 mg.g^{-1} and 400 ± 20 to 620 ± 31 mg.g^{-1}, as the pre-treatment time increased for samples treated with calcium hydroxide and sulphuric acid, respectively. The highest conversion efficiency was 107% which was obtained after a 48 hours hydrolysis period for the sample treated with 3 wt% sulphuric acid. The conversion efficiency significantly increased when the hydrolysis period was extended from 24 to 48 hours. The reason for improved glucose yields, was that prolonged pre-treatment and hydrolysis time allows for more exposure of the lignocellulosic biomass to the chemical agent used, resulting in an increased solubilisation of hemicellulose and lignin removal. This improves the accessibility of cellulose to cellulase during enzyme hydrolysis; hence the high glucose yields after longer pre-treatment time. However, longer pre-treatment periods are associated with the formation of inhibitory products and low carbohydrate (i.e. cellulose and hemicellulose) recovery (Saha *et al*., 2005).

4.4 Enzymatic hydrolysis

The goal of enzyme hydrolysis is to degrade the carbohydrates recovered from the pre-treatment process into monomeric sugars that can be easily fermented to bioethanol. Optimization of the enzymatic hydrolysis method was done by investigating various substrate loadings, enzyme loadings and also Tween 80 was added so as to enhance the hydrolysis of cellulose and improve the glucose yields.

4.4.1 Effect of substrate loading

The effect of substrate loading was investigated in order to establish the optimal ratio of substrate to enzyme loading during the enzymatic hydrolysis of maize stover. The procedure is outlined in Section 3.5. The aim was to determine if the glucose yield would improve at high substrate loadings. The glucose yields and the conversion efficiency achieved during enzyme hydrolysis at higher substrate loadings of pre-treated maize stover are shown in Table B.4 (see Appendix B). The effect of substrate loading on the glucose yield during hydrolysis is demonstrated in Figure 4.4.

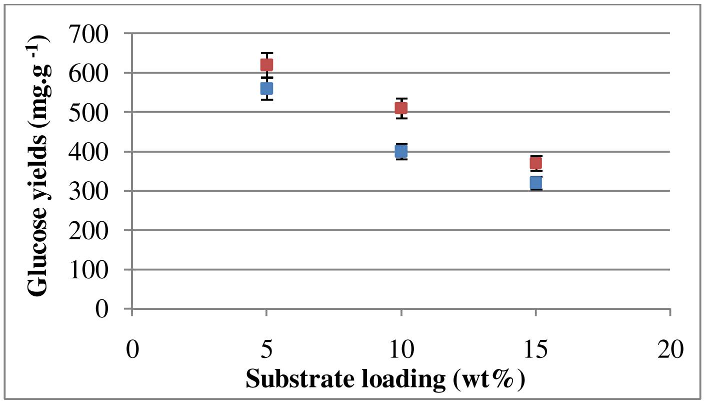

Figure 4.4: Effect of substrate loading on glucose yields after enzyme hydrolysis of pre-treated maize stover [🟦 0.2 g of $Ca(OH)_2$. (g of dry biomass)$^{-1}$, 🟥 3 wt% H_2SO_4]. Pre-treatment conditions: at 121°C for 60 minutes. Enzymatic hydrolysis: solid loading at 5, 10 and 15%, 50°C, pH 5.0 and 30 FPU. (g of cellulose)$^{-1}$ for 48 hours.

According to the results obtained in this study (Figure 4.4) an increase in substrate loading leads to reduced glucose yield. At higher solid loadings such as 15% the efficiency was approximately 40% lower when compared to 5% solid loading; as a result the amount of recoverable glucose decreased from 560 ± 28 mg.g^{-1} and 620 ± 31 mg.g^{-1} to 320 ± 16 mg.g^{-1} and 370 ± 19 mg.g^{-1} after the enzymatic hydrolysis of the samples pre-treated with calcium hydroxide and sulphuric acid, respectively. The decrease in glucose yield at high substrate loadings is caused by inhibition of cellulase adsorption onto cellulose during enzyme hydrolysis (Kristensen *et al.*, 2009). The enzymatic hydrolysis of cellulose depends on the ability of the enzyme to adsorb to cellulose, thus allowing maximal proximity between the enzyme active site and the substrate which in turn leads to an efficient hydrolysis reaction rate. Therefore, for the sample with the higher substrate loading there was inefficient binding between the cellulase and cellulose which resulted in reduced enzyme activity, thus the low glucose yield. The exact mechanism involved in the inhibition of cellulase adsorption at high substrate loadings is still unknown.

The high glucose yield obtained at a 5% substrate loading indicates that the substrate to enzyme ratio was sufficient for the hydrolysis of maize stover and there was no enzyme inhibition. The result obtained in this study point out that high substrate loading of 10% and above, lowers the enzyme activity; hence the decrease in glucose yield. Thus a 5% substrate loading was used for all the subsequent hydrolysis experiments.

4.4.2 Effect of enzyme loading

The enzymatic hydrolysis of pre-treated maize stover was performed according to the procedure described in Section 3.5. The use of low enzyme loadings is necessary for reducing the hydrolysis process costs because of high enzyme prices. Thus, the aim of this experiment was to investigate the effect of using enzyme loadings below 30 FPU per gram of cellulose during the enzyme hydrolysis of maize stover. The glucose yield obtained and the efficiency of the enzyme hydrolysis method, using lower enzyme loadings are shown in Table B.5 (see Appendix B). The effect of using various enzyme loadings on the glucose yield during hydrolysis is demonstrated in Figure 4.5.

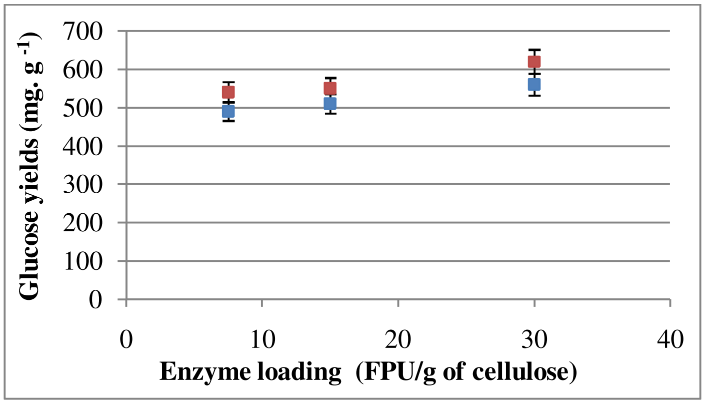

Figure 4.5: Effect of enzyme loading on glucose yields after enzyme hydrolysis of pre-treated maize stover [🟦 0.2 g of $Ca(OH)_2$. (g of dry biomass)$^{-1}$, 🟥 3 wt% H_2SO_4]. Pre-treatment conditions: at 121°C for 60 minutes. Enzymatic hydrolysis: at 7.5, 15, 30 FPU. (g of cellulose)$^{-1}$, 50°C, pH 5.0, and 5% substrate loading for 48 hours.

The glucose yield slightly decreased when enzyme loadings lower than 30 FPU per gram of cellulose were used during the enzymatic hydrolysis of maize stover. The glucose yield decreased from 620 ± 31 to 540 ± 27 mg.g^{-1} for the samples pre-treated with 3 wt% sulphuric acid and from 560 ± 28 to 490 ± 25 mg.g^{-1} of biomass for samples treated with 0.2 g of $Ca(OH)_2$ per gram dry biomass, when the enzyme loading was reduced to 7.5 FPU per gram of cellulose. The explanation for the decline in glucose yields is that the ratio between the enzyme and substrate decreased making the cellulase enzyme complex less efficient (Yang *et al.*, 2009). This means that the amount of the enzyme was not enough to hydrolyse all the available substrate; hence the low glucose yield at reduced enzyme loadings. However, the glucose yield and the conversion efficiency achieved only decreased by 13% even though the enzyme loading had been reduced by 75% (i.e. from 30 to 7.5 FPU per gram of cellulose). Thus, enzyme loading can be reduced without significantly affecting the efficiency of hydrolysis. This has important economical implications since it has the potential to reduce the amount of enzymes added during hydrolysis which would ultimately lower the costs of the overall process.

4.4.3 Effect of Tween 80 during enzymatic hydrolysis

The addition of non-ionic surfactants during enzyme hydrolysis has been proven to increase the hydrolysis efficiency of cellulose containing substrates, and ultimately the glucose yield (Alkasrawi *et al.*, 2003). In this study Tween 80 was evaluated for its ability to enhance the enzymatic hydrolysis of maize stover as discussed in Section 3.5. The glucose yield and the conversion efficiency during the enzymatic hydrolysis of pre-treated maize stover are shown in Table B.6 (see Appendix B) and Figure 4.6 illustrates the effect of adding Tween 80.

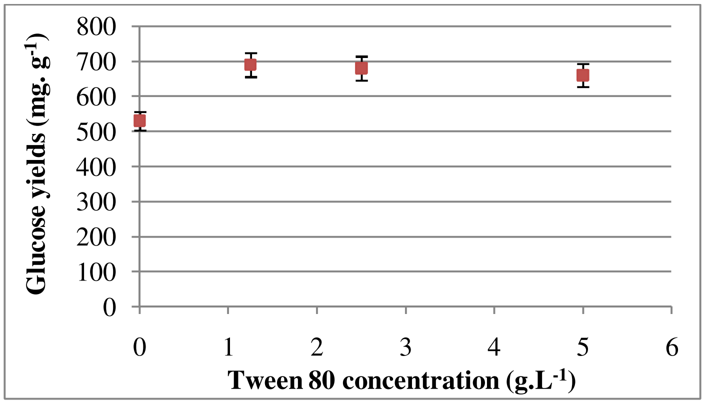

Figure 4.6: Effect of Tween 80 during enzyme hydrolysis of pre-treated maize stover. Pre-treatment conditions: 3 wt% H_2SO_4 at 121˚C for 60 minutes. Enzymatic hydrolysis: at 50˚C, Tween 80 at 0, 1.25, 2.5 and 5 g.L^{-1}, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ and at 5% substrate loading for 48 hours.

When Tween 80 was added during the enzymatic hydrolysis of maize stover the conversion efficiency greatly improved to a maximum of 119%, thus producing the highest glucose yield even though both the enzyme and substrate loadings were kept constant. The glucose yield increased from 530 ± 27 $mg.g^{-1}$, in the control samples (hydrolysed without Tween 80), to a maximum of 690 ± 35 $mg.g^{-1}$ for samples hydrolysed in the presence of Tween 80 at a concentration of 1.25 $g.L^{-1}$. This means that the glucose yield increased by 23% when Tween 80 was added during enzymatic hydrolysis of pre-treated maize stover. An explanation for the improved glucose yield was that Tween 80 reduced the adsorption of the enzyme onto lignin and this enabled the enzyme to only react with cellulose (Chen *et al.*, 2008).

Lignin acts as an attractant of cellulases causing unproductive binding of the cellulase to lignin, which results in decreased enzyme activity and low glucose yield (see Section 2.5.2). In this instance, Tween 80 reduced the unproductive binding by interacting with lignin such that the non-specifically bound enzyme was released and available for cellulose hydrolysis. Hence, the glucose yield increased during enzymatic hydrolysis when Tween 80 was added (Eriksson *et al.*, 2002). Higher concentrations of Tween 80 did not have a positive effect on the hydrolysis yield and, therefore, 1.25 $g.L^{-1}$ is regarded as an optimal Tween 80 concentration to be used during the enzymatic hydrolysis of maize stover.

4.5 Discussion

The results obtained from the ARC laboratory demonstrate the composition of the maize stover used in this investigation. The chemical compositional analysis was required because it reflects the theoretical carbohydrate amount that is available in the maize stover used in this study. The most essential component in lignocellulosic biomass is cellulose, since it can be easily degraded into glucose after the pre-treatment of feedstock and then fermented into bioethanol. Hemicellulose is another degradable component present in maize stover that can be hydrolysed into five carbon sugars such as xylose and arabinose and subsequently converted to ethanol when an appropriate microbial strain is used. Lignin is the complex component substituted with phenyl propane units and, therefore, cannot be degraded nor fermented into bioethanol.

The major components present in the maize stover used in this investigation were cellulose, hemicellulose and lignin at 31.45%, 26.02% and 17.62%, respectively and the total carbohydrate content was 57.47% (see Section 3.3). The cellulose and hemicellulose content obtained in this study is slightly below the average reported values of 38% cellulose and 32% hemicellulose from maize stover (Glassner *et al.*, 1999). Values of about 38.7% cellulose, 21.7% hemicellulose and 19.3% lignin have been reported from maize straw (Chen *et al.*, 2008). For other lignocellulosic feedstock such as wheat straw, higher cellulose content has been reported. Saha *et al.* (2005) obtained 48.57 ± 0.30% cellulose, 27.70 ± 0.12% hemicellulose and 8.17 ± 0.90 lignin which make up the total carbohydrate content of 76.27 ± 0.42% on dry solid basis from wheat straw. The chemical composition of maize stover has been demonstrated to be influenced by the difference in maize hybrids, location, crop maturity and storage method. Therefore, variation in compositional analysis was expected, given that the location of the maize stover used in this study and that from the comparative studies were completely different (Chen *et al.*, 2007).

Many previous studies have been carried out on the enzymatic hydrolysis of maize stover after pre-treatment with dilute sulphuric acid or calcium hydroxide (lime). Most dilute sulphuric acid pre-treatment methods use elevated temperatures such as 130 - 200^{o}C and the residence time required is usually short (2-15 minutes). These conditions can be severe and lead to the loss of sugars due to degradation resulting in the formation of furfurals (Wayman *et al.*, 2005). Lime pre-treatment is usually carried out at temperatures between 25 - 140^{o}C (Kim and Holtzapple, 2006; Kaar and Holtzapple, 2000). Though pre-treatment at low temperatures would be favourable due to low energy consumption and reductions of furfural production, these methods tend to be inefficient and thus increase the amount of enzymes required during hydrolysis. Therefore, pre-treatment methods that employ moderate temperatures that are efficient enough to solubilise the lignocellulosic structure without denaturing the sugars are of preference (Galbe and Zacchi, 2007). Thus, for this study a moderate temperature of 121^{o}C was used for both methods during the pre-treatment of maize stover.

The results were analysed using the glucose yield (glucose produced per gram of biomass) obtained after enzymatic hydrolysis as a measuring variable and the conversion efficiency of the process, which was defined as the amount of glucose that could be recovered during the process, based on the theoretical yield (0.58 $g.g^{-1}$) of the maize stover used in this study (see Section 3.2). The conversion efficiency determines the performance of the hydrolysis method and it also reflects on the pre-treatment process – the higher the glucose yield, the more efficient the method that was used for the enzymatic hydrolysis and during pre-treatment of the feedstock. This trend was identified in this study in all the experiments.

Pre-treatment prior to hydrolysis of maize stover has a huge impact on the glucose yield. For instance, the glucose yield increased by 51% and 71% after enzyme hydrolysis for 24 hours of maize stover pre-treated with lime at 0.2 g of $Ca(OH)_2$. (g of biomass)$^{-1}$ and sulphuric acid at 3 wt%, respectively. Further increases were obtained when the pre-treatment time was longer, though the extent of the effect was not the same for the two different pre-treatment methods. Pre-treatment with calcium hydroxide for a longer period increased the yield by 14% and sulphuric acid pre-treatment method by 35%. Thus, sulphuric acid is a better pre-treatment method when compared to the calcium hydroxide method.

The maximum glucose yield obtained, after calcium hydroxide pre-treatment and enzyme hydrolysis, was 510 ± 26 mg.g^{-1} with a conversion efficiency of 89%, after pre-treatment at 0.2 g of $Ca(OH)_2$ per g of biomass at 121˚C for 120 minutes and enzyme hydrolysis at 50˚C, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ for 48 hours. Kaar and Holtzapple (2000) reported a yield of approximately 450 mg.g^{-1}at pre-treatment conditions of 0.075 g $Ca(OH)_2$ (g dry biomass)$^{-1}$ at 120˚C for 5 h and hydrolysis conditions at 5 FPU cellulase (g dry biomass)$^{-1}$ for 72 h at 50 ˚C, using maize stover as feedstock. The maximum glucose conversion they obtained was 88% after a hydrolysis period of 7 days. The increased glucose yield achieved in this study, after calcium hydroxide pre-treatment and enzyme hydrolysis of maize stover was a result of the higher chemical and enzyme loading used. However, the advantage was that the pre-treatment and hydrolysis periods were shorter, yet high glucose yields were achieved, compared to the 5 hours of pre-treatment and a hydrolysis period of 72 hours to 7 days that Kaar and Holtzapple (2000) used. Shorter process periods are more favourable since they consume less energy and time and thus, would result in reduced process costs.

The dilute sulphuric acid method yielded a maximum of 620 ± 31 mg.g^{-1} which was equivalent to 107% conversion efficiency, after pre-treatment with 3 wt% H_2SO_4 of sulphuric acid at 121˚C for 120 minutes and enzyme hydrolysis at 50˚C, pH 5.0, 30 FPU. (g of cellulose)$^{-1}$ for 48 hours.

Linde *et al*. (2008) also observed a high glucose recovery of 102% after steam pre-treatment at 190°C for 10 minutes using dilute H_2SO_4 – impregnated wheat straw as feedstock. The results obtained for the dilute sulphuric acid method revealed that the conditions used in this study can enhance the glucose yield and that sulphuric acid dose is indirectly proportional to the temperature used for pre-treatment. For instance, Saha *et al.* (2005) observed a maximum glucose of approximately 313 ± 8 $mg.g^{-1}$ after pre-treatment of wheat straw at a low acid dose of 0.5% (v/v) of H_2SO_4 at 140°C for 60 minutes. The temperature they used was higher; hence the shorter pre-treatment time and the acid dose was very low. However, they recorded a lower glucose yield which was 50% less than that obtained in this study. The higher the temperature, the lower the acid dose and the shorter the pre-treatment time required during pre-treatment. In addition, high temperatures such as 200°C have been used whereby pre-treatment conditions involved a low acid dose of sulphuric acid of about 0.22% for 5 minutes and produced a 57.7% glucose yield after the enzymatic hydrolysis maize stover (Loyd and Wayman, 2005).

The enzymatic route was used to hydrolyse the pre-treated samples in an attempt to degrade the complex carbohydrates, present in the hydrolysate, into monomeric sugars. A couple of factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, reaction conditions (temperature, pH, as well as other parameters), and product inhibition. The major obstacle in achieving an economically viable commercial operation of enzymatic hydrolysis of cellulose has been identified to be the slow reaction rate, which leads to poor process efficiency and thus low glucose yield, coupled with product inhibition (Yang *et al*., 2009). Hence, this investigation focused on improving enzyme hydrolysis efficiency by examining different substrate, enzyme and Tween 80 loadings so as to improve the glucose yield thus, optimizing the enzymatic method.

During the optimization of enzymatic hydrolysis of pre-treated stover, it was observed that an increase in substrate loading resulted in reduced glucose yield. Hydrolysis at a high substrate loading, such as 15%, resulted in low conversion efficiencies and decreased the glucose yield by approximately 40% after pre-treatment with both methods. The decrease in glucose yield at high substrate loadings is a known phenomenon. Research studies reveal that it is a consequence of either product inhibition, insufficient hydrolysis time or inhibition of the binding of the cellulases to cellulose which often leads to slow reaction rates, poor efficiency and ultimately reduced glucose yields (Yang *et al.*, 2009; Kristensen *et al.*, 2009). Although high substrate loadings have the potential to increase the glucose concentration, poor hydrolysis efficiency coupled with rising raw material prices, which largely determine the final price of ethanol, are the major challenges facing the use of high substrate loadings, thus making low substrate loadings more favourable.

A 75% decrease in enzyme loading (30 to 7. 5 FPU per gram of cellulose) resulted in 11% and 13% decrease in glucose yield for the samples pre-treated with calcium hydroxide and sulphuric acid, respectively. Therefore, enzyme loading can be reduced without significantly affecting the efficiency of hydrolysis, an important economical aspect. Higher enzyme loadings have been reported to effectively enhance efficiency and the glucose yield during the enzymatic hydrolysis of maize stover. However, the high enzyme costs make high enzyme loadings impractical for commercial application of bioethanol production from lignocellulosic biomass (Yang *et al.*, 2009). Instead of increasing the enzyme loading above 30 FPU per gram of cellulose, the effect of adding Tween 80 during the hydrolysis process was investigated. The glucose yield increased from 530 mg. g^{-1} in the control sample (i.e. the sample without Tween 80) to 690 mg. g^{-1} for the sample hydrolysed in the presence of 1.25 g.L^{-1} Tween 80, the conversion was calculated to be 92 to 119%, respectively. The efficiency was enhanced by 23%. This has significant economical implications because the glucose yield increased without further addition of enzymes, which tends to increase the process cost. Chen *et al.* (2008) reported an increase in glucose yield of 7.5% after adding 5 g.L^{-1} of Tween 80, which is 50% more than the amount of Tween 80 used in this study, in their enzyme hydrolysis method. Therefore, the method used, achieved the main objective of this study which was to improve the enzymatic conversion of maize stover for bioethanol production. The recommended method for the efficient processing of maize stover is, therefore, pre-treatment with 3 wt% sulphuric acid for 60 minutes at 121°C, followed by the enzymatic hydrolysis at a 5% substrate loading, using 30 FPU per gram of cellulose at 50°C and pH 5.0 in the presence of 1.25 g.L^{-1} of Tween 80 for 48 hours.

Recent developments in enzymatic hydrolysis of lignocellulosic biomass using methods that involve hollow fibre ultra-filtration (UF) membrane reactors have been reported to increase the hydrolysis efficiency and reducing sugar yield to 200% and 206%, respectively, after pre-treatment with steam explosion of maize stalk (Yang *et al*., 2009). Specialised equipment required for such methods is expensive and thus would increase the process costs for bioethanol production from lignocellulose. The addition of Tween 80 has the potential to lower the overall process costs. Tween 80 costs 125 R/kg, Celluclast ® 1.5 L is 172.20 R/kg and Novozyme ® 188 is 420 R/kg. Taking into consideration the fact that the price of Tween 80 is much lower than the price of enzymes, the method used in this study to improve the glucose yield during the enzymatic hydrolysis of maize stover is cost effective. Therefore, the addition of Tween 80 during the enzymatic hydrolysis of maize stover has the potential to enhance the process efficiency and ultimately the glucose yields while keeping the process costs low.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

This study has formed a baseline for further studies on the enzyme hydrolysis of maize stover from a South African perspective. The conclusions drawn from this study are as follows:

- Calcium hydroxide and sulphuric acid pre-treatment methods, using moderate temperatures, had a significant effect on the solubilisation of the lignocellulosic structure during the pre-treatment of maize stover. Sulphuric acid (3 wt%) produced the highest glucose yield; hence it is considered the best method for the pre-treatment of maize stover used in this study.
- The effect of pre-treatment time was different for the two methods used. Sulphuric acid pre-treatment improved significantly at longer periods by 35%, whereas with calcium hydroxide the glucose yield increased by 14%.
- The maximum glucose yield of 620 mg.g^{-1} was obtained at a substrate loading of 5%, after pre-treatment with 3 wt% of sulphuric acid. Increasing the substrate loading to 15% reduced the glucose yield by 40%, for both pre-treatment methods.
- The reduction of the enzyme loading slightly reduced the glucose yield, implying that lower enzyme loadings can be utilized during the hydrolysis of maize stover.
- The high enzyme loadings used during enzyme hydrolysis can be reduced by using Tween 80, thus reducing the process costs. The glucose yield increased by 23% after adding 1.25 g.L^{-1} of Tween 80.

In conclusion, the maximum glucose yield achieved in this study was 690 mg. g^{-1} for the sample hydrolysed in the presence of 1.25 g.L^{-1} Tween 80, the conversion efficiency was calculated to be 119% which is lower than what Yang *et al*. (2009) obtained in their study where the hydrolysis efficiency and reducing sugar yield was 200% and 206%, respectively, after pre-treatment with steam explosion of maize stalk. Therefore, there is still a shortfall in the methods used in this study and more work needs to be done for the pre-treatment and enzymatic hydrolysis of maize stover with the ultimate goal of converting all the recovered sugars to bioethanol.

5.2 Recommendations

There is still a need for further investigations concerning the bioconversion of maize stover. Future studies should focus on the following areas:

- The planning phase of any bioethanol producing plant should consider the type of storage of the biomass to be used. A storage technique that enhances sugar recovery would be preferable. The focus should be on whether the biomass is best stored in its wet or dry form.
- The use of a minimal amount of water during each production unit process is preferable since South Africa is already experiencing a crisis due to limited water resources.
- It would be advisable to run the whole bioethanol production plant using renewable energy because producing a carbon neutral fuel using fossil fuels that are responsible for the increase of greenhouse gas emitted into the atmosphere, is a futile exercise.
- The presence of sugars, other than glucose i.e. xylose, arabinose and galactose in maize stover suggest that this feedstock has the potential to be used as a preferred lignocellulosic biomass for bioethanol production. Therefore, more research in the bioconversion of hemicellulose constituents (such as xylose, arabinose and galactose) into useful products, using efficient and cost competitive methods would make the overall bioethanol production process economically viable.
- A quantitative analysis of ethanol that can be produced after fermentation, in the presence of Tween 80 should be done and the focus must not only be on glucose but on all the available sugars present in lignocellulosic biomass.

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APPENDICES

APPENDIX A

Determination of cellulase activity in Celluclast 1.5 L

Introduction

This method describes a procedure for the measurement of cellulase activity in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The value of 2.0 mg of reducing sugar such as glucose from 50 mg of Whatman No. 1 filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC. The assay procedure involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes and then calculating the activity (in FPU/mL) of the original stock from the dilution required (Adney and Baker, 1996). The dilutions used to calculate the activity of Celluclast ® 1.5 L enzyme used in this study are given in Table 1. The starting concentration is 1:20 with an enzyme activity of 0.05. The term concentration is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture.

Table A 1: Enzyme dilutions

Dilution #	Citrate buffer (mL)	1:20 Enzyme (mL)	Concentration
1	16	4	0.0100
2	17	3	0.0075
3	18	2	0.0050
4	19	1	0.0025

A glucose standard curve was constructed by measuring the absorbance of the prepared glucose standards using a spectrophotometer at 540 nm. The glucose concentration in mg. $(0.5\ \text{mL})^{-1}$ of the standards was then plotted as a function of absorbance.

Table A 2: Dilution of glucose standards and construction of standard curve

Glucose stock (mL)	Citrate buffer (mL)	Concentration (mg/0.5 mL)	Absorbance (540 nm)
1.0	4.0	1.00	0.254
1.0	2.0	1.65	0.422
1.0	1.0	2.50	0.667
1.0	0.5	3.35	0.868

Note: Glucose stock solution = 10 mg/mL

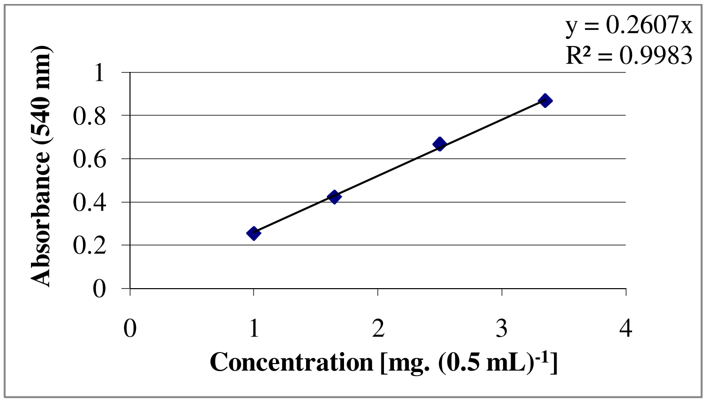

Figure A 1: Glucose concentration as a function of absorbance

The standard curve was then used to calculate the glucose concentrations in the diluted enzyme mixtures after hydrolysis. In other words, the different samples containing 50 mg filter paper were hydrolysed with the different enzyme dilutions and then the resulting glucose concentrations were measured using a spectrophotometer at 540 nm.

Table A 3: Glucose Concentration of samples as determined from standard curve

Dilution	Absorbance (540 nm)	Glucose [mg.$(0.5mL)^{-1}$]
1	0.931	3.57
2	0.617	2.37
3	0.518	1.99
4	0.348	1.33

The principle of this method is that at a specific enzyme concentration (dilution) 2 mg of glucose will be released and this concentration is used to estimate the activity of the original enzyme solution. To find the required enzyme concentration a straight line is drawn between two data points that are very close to 2.0 mg and this line is used to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar. In this plot, and in the equation below used for calculating FPU, the term "enzyme concentration" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of mL of the original solution present in each mL of the dilution).

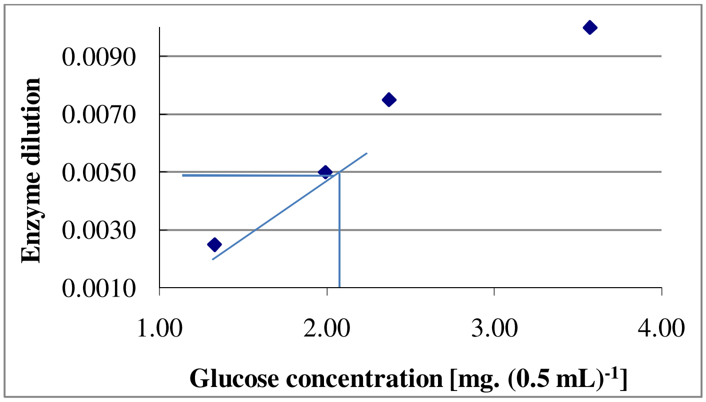

Figure A 2: Determination of enzyme activity for Celluclast ® 1.5 L

The enzyme activity was calculated to be 74 FPU/mL using Equation A.1

$$Enzyme\ activity = \frac{C}{D} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(A. 1)}$$

where

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

$$\frac{(2.0mg\ glucose/0.18016mg\ glucose\ /\mu moL)}{(0.5mL\ enzyme\ dilution\ x\ 60\ minutes)} = 0.37\mu mol/minute - mL \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(A.2)}$$

D is the enzyme concentration that corresponds with 2.0 mg of glucose released after adding 0.5 mL of the diluted cellulase ® 1.5 L enzyme.

APPENDIX B

HPLC glucose analysis

ChemSoft ® software for HPLC's from Agilent Technologies was used to quantify the peaks obtained from the HPLC instrument. A calibration curve for glucose analysis was done by injecting glucose standards. Concentrations of 0.2, 1, 3, 6, and 12 g.L^{-1} were used to prepare the standards and the peak area was recorded. The calibration curve required for the determination of glucose concentration in an unknown sample was constructed and is presented in Figure B 1. The linear equation that was fitted to the experimental data (R = 0.999) was used to calculate the glucose concentration of experimental samples in this study. All the standards were injected three times to ensure accuracy of the instrument.

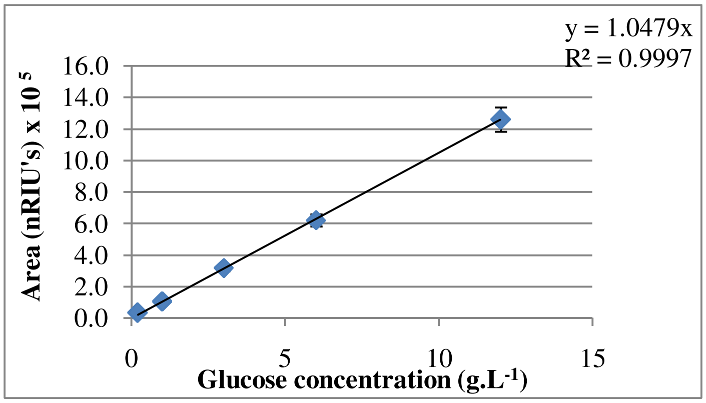

Figure B 1: Calibration curve for glucose

Calculations:

Glucose concentration

The concentration of glucose present in the samples was calculated using the linear curve equation:

$y = m.x$

Thus: $x = \frac{y}{m}$

where

x is the unknown concentration

y is the area of the glucose peak from the HPLC

m is the gradient from the linear curve

Grams of glucose produced (g) = glucose concentration * 0.1L

Glucose yields (mg of glucose/g of biomass) $= \frac{Glucose\ (g)}{Substrate\ (g)} . 1000\ mg$

Conversion efficiency ($\%$) = $\frac{grams\ of\ glucose\ produced\ (g)}{Theoretical\ grams\ of\ carbohydrate\ \ (g)} . 100$

Note: The substrate amount used during the enzyme hydrolysis is 5 g of the pre-treated maize stover. Theoretically the maize stover used in this study has a total carbohydrate content of 57.47% (≈ 0.58 g) (see Section 3.3).

The glucose concentrations obtained through analysis using the calibration curve (Figure B 1), as well as the calculated glucose yield and conversion efficiency obtained from the experiments done in this study, are given in Table B 1 to B 6.

Table B 1: Calcium hydroxide pre-treatment

Lime loading [g $Ca(OH)_2$]	**Time (h)**	**Area (RIU*s)**	**Concentration (g.L^{-1})**	**Glucose yield (g.g^{-1})**	**Conversion efficiency (%)**
0	0	7.06E+04	6.74	0.13	22
	8	1.27E+05	12.16	0.24	41
	24	1.31E+05	12.54	0.25	43
0.1	0	1.05E+05	9.99	0.20	34
	8	2.20E+05	20.10	0.42	72
	24	2.18E+05	20.83	0.42	72
0.2	0	1.15E+05	11.01	0.22	38
	8	2.40E+05	22.86	0.46	79
	24	2.69E+05	25.71	0.51	88
0.3	0	1.11E+05	10.57	0.21	36
	8	1.83E+05	17.45	0.35	60
	24	2.39E+05	22.81	0.46	79
0.4	0	1.34E+05	12.82	0.26	45
	8	1.75E+05	16.71	0.33	57
	24	2.45E+05	23.37	0.47	81
0.5	0	1.26E+05	12.06	0.24	41
	8	1.60E+05	15.28	0.31	53
	24	2.28E+05	21.76	0.44	76

Table B 2: Sulphuric acid pre-treatment

Acid loading (wt%)	**Time (h)**	**Area (RIU*s)**	**Concentration (g.L^{-1})**	**Glucose yield (g.g^{-1})**	**Conversion efficiency (%)**
0	0	4.68E+04	4.47	0.09	16
	8	5.29E+04	5.05	0.10	17
	24	7.05E+04	6.73	0.13	22
0.5	0	5.61E+04	5.35	0.11	19
	8	1.30E+05	12.41	0.25	43
	24	1.65E+05	15.75	0.31	53
1	0	5.87E+04	5.60	0.11	19
	8	1.52E+05	14.51	0.29	50
	24	1.84E+05	17.56	0.35	60
2	0	4.29E+04	4.09	0.08	14
	8	1.44E+05	13.74	0.27	47
	24	1.82E+05	17.37	0.35	60
3	0	4.87E+04	4.65	0.09	16
	8	1.91E+05	18.23	0.36	62
	24	2.34E+05	22.33	0.45	78
4.5	0	8.27E+04	7.89	0.16	28
	8	1.47E+05	14.04	0.28	48
	24	2.13E+05	20.28	0.41	71

Table B 3: Effect of time after pre-treatment

Method	**Time (h)**	**Area (RIU*s)**	**Concentration (g/L)**	**Glucose yield ($g.g^{-1}$)**	**Conversion efficiency (%)**
0.2g $Ca(OH)_2$	30	2.51E+05	23.91	0.48	83
	60	2.59E+05	24.74	0.40	69
	120	2.93E+05	28.00	0.49	84
3% H_2SO_4	30	2.11E+05	20.16	0.54	93
	60	2.82E+05	26.92	0.56	97
	120	3.26E+05	31.07	0.62	107

Table B 4: Effect of substrate loading after enzyme hydrolysis

Method	**Substrate ($g.L^{-1}$)**	**Area (RIU*s)**	**Concentration ($g.L^{-1}$)**	**Glucose yield ($g.g^{-1}$)**	**Conversion efficiency (%)**
0.2g $Ca(OH)_2$	5	2.93E+05	28.00	0.56	97
	10	4.19E+05	39.99	0.62	107
	15	4.98E+05	47.51	0.40	69
3% H_2SO_4	5	3.26E+05	31.07	0.51	88
	10	5.40E+05	51.48	0.32	55
	15	5.74E+05	54.79	0.37	64

Table B 5: Effect of enzyme loading after enzyme hydrolysis

Method	**Enzyme (FPU. g^{-1})**	**Area (RIU*s)**	**Concentration ($g.L^{-1}$)**	**Glucose yield ($g.g^{-1}$)**	**Conversion efficiency (%)**
0.2g $Ca(OH)_2$	7.5	2.58E+05	24.63	0.49	84
	15	2.67E+05	25.51	0.54	93
	30	2.93E+05	28.00	0.51	88
3% H_2SO_4	7.5	2.83E+05	27.05	0.55	95
	15	2.91E+05	27.74	0.56	97
	30	3.26E+05	31.07	0.62	107

Table B 6: Effect of Tween 80 after enzyme hydrolysis

Method	**Tween 80 (g.L^{-1})**	**Area (RIU*s)**	**Concentration (g.L^{-1})**	**Glucose yield (g.g^{-1})**	**Conversion efficiency (%)**
3% H_2SO_4	0	2.645E+01	26.45	0.53	91
	1.25	3.59E+05	34.29	0.69	119
	2.5	3.55E+05	33.90	0.68	117
	5	3.45E+05	32.88	0.66	114

Appendix C

Experimental error

The experimental error was calculated using three samples pre-treated with 3 wt% H_2SO_4 for 60 minutes at 121°C and the enzymatic hydrolysis was done using 5% substrate loading, 30 FPU per gram of substrate at 50°C, pH 5.0 in the presence of 1.25 $g.L^{-1}$ of Tween 80 for 48 hours. The analysis of these samples was done using a Zorbax carbohydrate column (Agilent system) connected to a refractive index detector using 75% Acetonitrile as a mobile phase at a flow rate of 1 mL/min, a 10 µl injection volume and at a column temperature of 35°C. The calculations for the error percentage are in Table C 1.

Table C 1: Experimental error calculations

Time (hours)	**Exp. 1 Yield ($g.g^{-1}$)**	**Exp. 2 Yield ($g.g^{-1}$)**	**Exp. 3 Yield ($g.g^{-1}$)**	**Average**	**STDEV**	**95% Confidence (±)**	**Error (%)**
0	0.13	0.16	0.13	0.14	0.01	0.03	18
4	0.14	0.14	0.14	0.14	0.00	0.00	2
6	0.19	0.14	0.15	0.16	0.02	0.04	26
12	0.20	0.18	0.19	0.19	0.01	0.02	9
24	0.19	0.18	0.20	0.19	0.01	0.01	6
48	0.18	0.18	0.18	0.18	0.00	0.01	3

The points used to determine the probability of the experimental work are shown in Figure C 1.

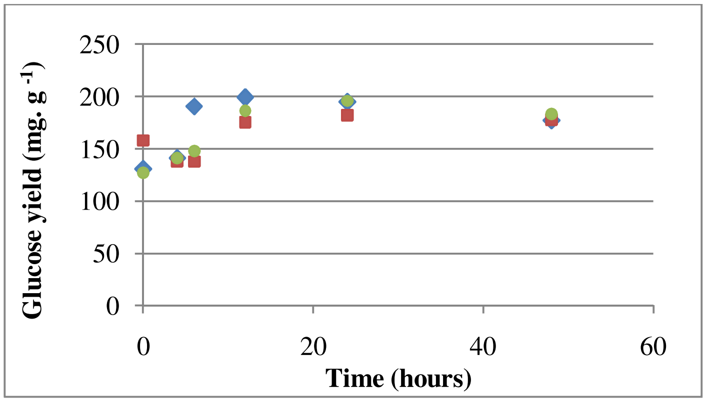

Figure C 1: Probability of the experimental error

(◆ Experiment 1, ■ Experiment 2, ● Experiment 3)

The experimental error of the study was calculated as the average error percent (%) of the points at the stabilized phase i.e. 24 and 48 hours, and it was 5%. These were the same number of hours as used for hydrolysis as described in Chapter 3. Saha *et al.*, (2005) reported an error of 1.7% during the enzymatic hydrolysis of wheat straw to monomeric sugars after pre-treatment with dilute H_2SO_4. The error of this study is regarded as an acceptable percentage taking into consideration that the feedstock used, was maize stover, which consists of different plant components (stalk, cobs, leaves and tassels) with various characteristics making it difficult to work with.