Bioconversion of corn fibre to ethanol

by

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ABSTRACT

Corn fibre, due to its chemical and structural properties, was evaluated as a potential technically viable feedstock that might be used to demonstrate the effectiveness of a biomass to ethanol bioconversion process. One of the major goals of the thesis was to see if we could define the optimum pretreatment conditions which would result in maximal sugar recovery and ethanol production, with a minimum number of subprocess steps.

Corn fibre was pretreated using SO₂-catalysed steam explosion at thirteen different severity conditions, chosen based on response surface modeling, with the goal of finding optimal pretreatment conditions. These conditions were defined to allow us to recover most of the hexose hemicellulose and cellulose derived sugars in an hydrolysable and fermentable form. The chosen severity conditions had a pronounced effect on the total amount of sugars recovered from corn fibre. Due to the chemical and biological nature of corn fibre (low lignin and high carbohydrate content), we were able to establish optimum, mild steam pretreatment conditions – 190°C for 5 minutes after exposure to 3% SO₂ – which yielded 93% of the total sugars in a hemicellulose-rich water soluble fraction, and a readily hydrolysable cellulose fraction by enzymes. The optimum pretreatment conditions also resulted in the production of a limited amount of sugar decomposition products, which allowed us to effectively ferment hexose sugars to ethanol using Saccharomyces cerevisiae.

Efforts to increase the low sugar concentrations in the water soluble fraction were evaluated in an attempt to increase the final ethanol concentrations recovered following fermentation. Increasing the sugar concentration by using a high consistency (12% w/v) cellulose-rich water insoluble fraction in acetate buffer resulted in the generation of a glucose stream of 55 g L⁻¹. However, mixing this higher concentration water insoluble,
cellulose stream was problematic, as evident by incomplete hydrolysis. As an alternative strategy, the hemicellulose-rich water soluble fraction was added to the cellulose-rich stream, effectively increasing the sugar concentration while reducing the number of subprocess steps to pretreatment, hydrolysis and fermentation. Although combining the hemicellulose and cellulose streams increased the overall sugar concentration, cellulose hydrolysis was inhibited due to end-product inhibition. Increased hydrolysis time, and enzyme loadings were able to further increase cellulose hydrolysis yields.

In an effort to increase overall cellulose conversion, minimize end-product inhibition, and simplify the overall process, the simultaneous saccharification and fermentation of the entire slurry after steam explosion was investigated. This permitted a simplified two-stage bioconversion process, i.e., pretreatment and SSF, while alleviating the problem of end-product inhibition, as shown by good (86%) hexose to ethanol conversion at 8% solids consistency with minimum enzyme loading (10 FPU g cellulose⁻¹). The overall efficiency of the process was also improved, as shown by the fact that ethanol productivity was approximately five times greater for an SSF when compared to the SHF approach.

This study elucidated the technical merits of process integration during the bioconversion of corn fibre to ethanol when using steam explosion of corn fibre, followed by simultaneous saccharification and fermentation of the pretreated slurry. A preliminary techno-economic modeling study indicated that the contribution of each of the subprocess steps to the final total cost was highly dependant on the nature of the feedstock. For example, during the bioconversion of softwoods to ethanol, the delignification process was shown to be the main cost driver of the process, while for corn fibre, the model pointed out that the hydrolysis step was the main contributor to the total production cost for all the cases.
analysed in this thesis. It also indicated the greater economic viability of the proposed process options (SO$_2$-catalysed steam explosion and SSF), compared with the bioconversion of softwoods to ethanol, or compared to the separate hydrolysis and fermentation option for corn fibre (SHF), as determined by reductions in relative costs by 52% and 21%, respectively.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>AD</td>
<td>absorbance detector</td>
</tr>
<tr>
<td>AFEX</td>
<td>ammonia fibre explosion treatment</td>
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<tr>
<td>anti</td>
<td>antibiotic</td>
</tr>
<tr>
<td>Ara</td>
<td>arabinose</td>
</tr>
<tr>
<td>CD</td>
<td>catalytic domain</td>
</tr>
<tr>
<td>CDM</td>
<td>carbohydrate-binding modules</td>
</tr>
<tr>
<td>CFX</td>
<td>corn fibre xylan</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CS</td>
<td>combined severity</td>
</tr>
<tr>
<td>DDGS</td>
<td>distiller dried grains with solubles</td>
</tr>
<tr>
<td>DNS</td>
<td>dinitrosalicylic acid</td>
</tr>
<tr>
<td>FPB</td>
<td>Forest Products Biotechnology</td>
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<tr>
<td>FPU</td>
<td>filter paper units</td>
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<tr>
<td>EC</td>
<td>enzyme commission</td>
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<tr>
<td>ED</td>
<td>electrochemical detector</td>
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<tr>
<td>ETBE</td>
<td>ethyl tertiary butyl ether</td>
</tr>
<tr>
<td>ETOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>g</td>
<td>acceleration due to gravity (i.e., 2000 x g)</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>GMYP</td>
<td>culture media (1% glucose, 0.5% malt extract, 0.3% yeast extract and 0.5% peptone)</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
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<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IU</td>
<td>international units</td>
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<td>L</td>
<td>litre</td>
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<tr>
<td>Log Ro</td>
<td>severity factor</td>
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<tr>
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<tr>
<td>MTBE</td>
<td>methyl tertiary butyl ether</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
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</table>
nm  nanometer
NREL National Renewable Energy Laboratory
OPEC Oil Producing and Exporting Countries
OD optical density
ODW oven–dried weight
ODT oven-dried ton
p para
PAD pulsed amperometric detector
PDU plant development unit
rpm revolutions per minute
RSM response surface methodology
s second
SHF separate hydrolysis and fermentation
SO₂ sulphur dioxide
SSF simultaneous saccharification and fermentation
SSL spent sulphite liquor
t time
T temperature
TAPPI Technical Association of the Pulp and Paper Industry
TCA trichloroacetic acid
μL microlitre
μm micrometre
v/v volume per volume
UV ultraviolet light
w/v weight per volume
Xyl xylose
Y_{E_{BOH}}^{y_{ETOH}} relative ethanol yield
YP culture media (1% yeast extract, 1% peptone)
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1.0 INTRODUCTION

1.1 Background

Over the last fourteen years, the Forest Products Biotechnology group at UBC has been investigating the pretreatment and enzymatic hydrolysis of biomass as a means of converting wood derived sugars to ethanol. As will be discussed in more detail within this thesis, the pretreatment and hydrolysis of softwood residues is a complex, multi-component process that, despite significant progress, has proven difficult to establish in an economically viable fashion. At the same time government and industry groups in the U.S. have continued to invest in corn-to-ethanol plants, with ethanol production increasing significantly over the last 2–3 years (Dien et al., 2002). Various groups have suggested that the first biomass-to-ethanol plants should make use of the infrastructure and experience that already exists at the current corn-to-ethanol facilities, and that there are enough supplies of corn stalks and corn stover available to more than double the amount of ethanol that could be obtained from the annual corn harvest (Dien et al., 2002; Grohmann and Bothast, 1997; Leathers, 2003). About the same time as this strategy was being advocated we and other groups suggested that a lignocellulosic feedstock such as corn fibre, which was available in significant amounts and which would probably be more amenable to bioconversion was more likely to be an “ideal feedstock”. What we mean by this is a lignocellulosic substrate which could be pretreated in mild enough conditions that most of the carbohydrate could be readily recovered, either during pretreatment or subsequent enzymatic hydrolysis, without the need for extensive post-treatment such as alkali extraction to remove lignin. This thesis examines the potential of corn fibre to demonstrate the technical and economic viability of a
steam explosion/enzymatic hydrolysis process, and the conditions and processes that might be used in the bioconversion of corn fibre to ethanol. Before discussing the previous research in the overall pretreatment and bioconversion area it is worthwhile discussing why we should be interested in the potential of supplementing and possibly replacing oil based gasoline by biomass based ethanol.

1.2 Overview

During the past few decades, global warming has emerged as a major political and scientific issue. It is likely that this is due primarily to increased emissions of greenhouse gases formed by burning fossil fuels, concurrent with increased energy consumption (Dickerson and Johnson, 2004; Sun and Cheng, 2002). Various studies have shown that ethanol or ethanol-blended transportation fuels produce less harmful emissions, while the production of ethanol from biomass has the advantage of displacing transportation fuels derived from oil with a fuel obtained from a renewable resource (Bergeron, 1996; Galbe and Zacchi, 2002a; Tyson, 1993; von Sivers and Zacchi, 1995). In addition to environmental reasons, other motivations, such as fossil fuel exhaustion, geopolitical concerns about reliance on foreign fuel supplies, and the case of adaptation to a carbon-based fuel are some of the factors influencing the emergence of ethanol as a viable fuel supplement and/or alternative to gasoline. Although the major ethanol producers currently use either sugar cane (Brazil) or corn (North America) as the substrate for the "sugar to ethanol process", various lignocellulosic biomass feedstocks, including: agricultural residues, wood residues, food-processing waste, municipal solid wastes, herbaceous energy crops and pulp and paper industry wastes have the potential to serve as low-cost, abundant feedstocks for the production of fuel ethanol (Wiselogel et al., 1996).
It is recognized that softwoods are the dominant source of lignocellulosic materials in British Columbia (2,249,000 dry tonnes of residues available annually), and this is the primary reason why the Forest Products Biotechnology group at the University of British Columbia has investigated the potential for generating ethanol from softwood-derived lignocellulosic substrates for the last fourteen years (McCloy and Associates, 2003). Due primarily to the recalcitrant nature of the lignin in softwoods and the need for a high degree of utilisation of all carbohydrate components in this feedstock, the production of ethanol thus far involves numerous costly steps, including pretreatment, fractionation, delignification, hydrolysis and fermentation (Gregg et al., 1998). The multicomponent nature of the overall process combined with the relatively high contribution of the lignocellulosic feedstock to overall production costs (Nguyen and Saddler, 1991; Zacchi and Axelsson, 1989), and the need for a relatively low selling price for fuel ethanol, has resulted in both technical and economical challenges.

As predicted by many techno-economic models, the cost of the raw material is a major contributor (about 25-40%) to the total production cost of ethanol from lignocellulosics (Galbe and Zacchi, 2002a; Gregg et al., 1998; Nguyen and Saddler, 1991; Wingren et al., 2003). In order to decrease the overall cost of biomass to ethanol, it has been suggested that advocates of the first bioconversion plants might initially look at low value products which already exist at starch to ethanol plants, thus saving transportation costs. One feedstock which is currently available is corn fibre, a renewable resource that is available in significant quantities from the corn wet milling industry which could potentially serve as a low-cost feedstock for the production of fuel-grade alcohol. Why target corn fibre as a potential feedstock for the commercial biomass to ethanol process? Reasons include its
very high carbohydrate (~76%) and low lignin content (~7%), its availability in large quantities in most ethanol and starch processing plants, and the fact that it has few competing uses resulting in its relatively low cost (Grohmann and Bothast, 1997). Moreover, it has been predicted that the utilisation of the corn fibre fraction in the conversion process could potentially increase the overall ethanol yield of a plant by approximately 10% (Grohmann and Bothast, 1997).

1.3 Why bioethanol?

Energy consumption has increased steadily over the last century as the world’s population has grown and more countries have become industrialized. Fossil fuels have been the major resource used to meet increased energy demands. According to Campbell and Laherrere (1998), the global demand for oil is currently rising at more than 2 percent per year. From 1985 to 1998, energy use is up by about 30 percent in Latin America, 40 percent in Africa and 50 percent in Asia (Campbell and Laherrere, 1998). Moreover, it has been predicted that the annual global oil production will decline from 25 billion barrels to approximately 5 billion barrels by 2050 (Campbell and Laherrere, 1998).

With the inevitable depletion of the world’s oil supply, there has been an increasing worldwide interest in alternative, non-petroleum-based energy sources (Kerr, 1998). As oil based fuels such as petroleum and diesel currently supply 97% of all the energy consumed for transportation purposes, both industry and governments worldwide have been actively identifying, developing and commercializing technology for alternative fuels over the past 20 years (Putsche and Sandor, 1996). One fuel that has the potential to match the convenient features of petroleum, at a reasonable price, is ethanol produced from lignocellulosic biomass resources, commonly referred to as bioethanol.
The unique advantage of bioethanol is that it does not contribute net CO₂ to the atmosphere (Mielenz, 2001). The elimination of net CO₂ emissions is achieved because it does not consume fossil fuels, but rather uses plant byproducts to create the energy to run processes (leading to a net zero greenhouse gas emissions profile) (Wyman, 1994). In addition, oxygenates such as ethanol and methyl tertiary butyl ether (MTBE) are known to reduce carbon monoxide levels by improving overall fuel combustion (oxidation) (Lynd, 1996). However, keeping in mind that MTBE is being phased out of use in North America for health and environmental concerns, ethanol is its main replacement (Mielenz, 2001). Bioethanol technology is also attractive in that its raw materials do not compete as a food source for humans and that residues are readily available, based upon existing farm and forestry practices (Wiselogel et al., 1996). Cellulosic biomass for the production of bioethanol is abundant, with prominent examples including agricultural residues (e.g., corn stover, fibre, rice straw, and sugarcane bagasse), forestry residues (e.g., forest thinnings, waste wood), industrial wastes (e.g., sawdust and paper sludge), portions of municipal solid waste (e.g., waste paper and yard waste), herbaceous plants (e.g., switchgrass and elephant grass), and various woody species (e.g., poplar and willow) (Wiselogel et al., 1996). Finally, because ethanol can be made from domestic biomass resources, it is an important alternative for fueling a transportation sector that is currently near-totally dependent on economically, politically, and strategically volatile sources of oil that continually climb and fluctuate at the whim of the Oil Producing and Exporting Countries (OPEC) (Putsche and Sandor, 1996).
1.4 Sugar to ethanol industry

World ethanol production (all categories, fuel, industrial and potable) reached an estimated 31.4 billion litres in 2001, up from the previous year's 29.9 billion, and if all recently announced ethanol projects are implemented, total fuel ethanol production worldwide could grow to 36 billion litres by 2005 (Berg, 2001). Although Brazil and the U.S. are the main producers of ethanol, other North American and European countries with a significant agronomic-based economy are considering using current technology for fuel bioethanol production.

Brazil is the leading producer of fuel ethanol in the world producing 12.5 billion litres of ethanol commercially from sugarcane each year, to be used either in 22% ethanol/gasoline blends or as a neat fuel (Mielenz, 2001). However, because price-controlled U.S. sugar costs of $0.40 to $0.44 kg\(^{-1}\) correspond to a bioethanol cost of approximately $0.70 L\(^{-1}\), sugar cane is too expensive a source for fuel production in North America. As a result, corn starch is typically used as the feedstock for most U.S. commercial ethanol production. In the United States, fuel ethanol production grew from virtually nil in 1980 to approximately 6.63 billion litres in 2001 (Berg, 2001). Almost all of this ethanol is produced from corn starch and used in 10% ethanol blends, consequently reducing gasoline consumption and extending petroleum supplies (Berg, 2001).

Although the leading producers are currently Brazil and the United States, other countries have begun or are about to begin ethanol production. For example, Canada produced about 238 million litres of ethanol in 2001, and plans are in place to more than double current output within the next few years (CRFA, 2001). Although most Canadian ethanol is derived from wheat and corn starch, Canada has the distinction of having
produced about 17 million litres of ethanol from wood processing waste, spent sulphite liquor (at the Tembec plant in Quebec) (McCloy and Associates, 2003). However, most of this ethanol is sold for industrial uses such as food processing and pharmaceutical industry (McCloy and Associates, 2003).

France is one of the largest ethanol producing country in Europe, producing about 115 million litres in 2000, mostly from sugar beet for conversion to ethyl tertiary butyl ether (ETBE) for use as an oxygenate in gasoline (Galbe and Zacchi, 2002b; Mielenz, 2001). Additionally, Spain now has two plants that produce ethanol from grain with a total capacity of about 226 million litres per year. A third plant is scheduled, and will facilitate a total capacity of 426 million litres (Mielenz, 2001). Finally, Sweden made about 25 million litres of ethanol in 2001 from wheat, to be blended with gasoline up to a level of 5% ethanol (Galbe and Zacchi, 2002b; Wingren et al., 2003). Recently, the European Commission published an action plan and directives to encourage increased use of ethanol and biodiesel in the transportation sector with the goal of substituting 20% of diesel and gasoline with alternative fuels, and thus reducing greenhouse gas emissions by 2020 (Galbe and Zacchi, 2002b). It is apparent that the implementation of these measures will undoubtedly promote greater fuel ethanol industry growth in Europe.

1.5 Starch to ethanol industry in North America

As mentioned previously, to lower the overall cost of the biomass to ethanol, it is probably advisable to first evaluate the bioproducts that might already exist at current starch to ethanol plants to see if these lignocellulosic residues could be used as a feedstocks. Most fuel ethanol in North America is produced from corn, either by dry grinding or wet milling processes. Since 2001, the growth of the dry grind mill capacity has exceeded that of wet
mills in the U.S., primarily due to the lower capital costs involved in building a dry mill (Dien et al., 2002). As such, in recent times, no new wet mills have been built; instead producers have relied on expanding existing facilities (Dien et al., 2002). Wet mills are typically larger than dry mills and can produce a variety of products including ethanol.

1.5.1 Corn kernel structure and chemical composition

As noted earlier, most domestic fuel ethanol is produced from corn, which is composed of starch, sugar, fibre, protein, and ash. Starch and sugar constitute the greatest portion (70-75%) of the corn kernel, most of which is in a starch form (Watson and Ramstad, 1987). Protein or gluten is the next largest component, comprising about 10% of the corn dry weight; the germ of the kernel comprises about 4-5%; and finally, the fibre constitutes about 3-5% (Watson and Ramstad, 1987). The fibre contains cellulosic carbohydrates that are not currently converted into ethanol by any conventional technology. Ash comprises the remaining 2% of the kernel (Watson and Ramstad, 1987).

There are four major parts of the corn kernel: the seed coat (pericarp), germ (embryo), the tip cap and the endosperm (Figure 1). The seed coat is a layer of fibre that protects the kernel from attack by microorganisms and insects, comprising up to 5% of the dry corn kernel. It is mainly composed of cellulose and hemicellulose. The pericarp thickness ranges from 25 to 140 μm among genotypes (Watson and Ramstad, 1987). Corn fibre, a bioproduct of the wet milling process, is a mixture of the pericarp (corn hull, seed coat) and residual starch which is not extracted during the procedure.

The germ is the only living part of the corn kernel, comprising about 10% of the kernel weight and is the major depository of lipids (83% of the total kernel lipids). It also
Figure 1 Corn kernel.
contains 70% of the kernel sugars and 26% of the kernel protein (Watson and Ramstad, 1987).

The tip cap is the point at which the kernel is attached to the cob and is the major site of entry for food and water into the kernel. The endosperm compromises about 80-85% of the kernel and is composed primarily of starch (86-89% of the total kernel starch) and also contains 74% of the kernel protein, of which the majority is in the form of insoluble storage proteins (Watson and Ramstad, 1987).

1.5.2 Corn dry grind process

The two main technologies used in the corn to ethanol industry are the corn dry grinding and wet milling processes. Wet milling produces corn fibre or corn gluten feed, which is sold as a low-value cattle feed and can be a potential source of biomass for ethanol production at the existing starch to ethanol plants. The largest dry grinding facilities in the United States are: the New Energy Company of Indiana (South Bend, IN), South Point Ethanol (South Point, OH) and Chief Ethanol Fuels, Inc. (Hastings, NE) (Elander and Putsche, 1996).

A schematic representation of the dry grind process is shown in Figure 2. After cleaning, corn is hammer milled to pass through a 0.32-0.48 cm screen (Dien et al., 2002). Once milled, the meal is mixed with water to form a mash, and the pH of the mash adjusted to 6.0 with ammonia and α-amylase is added. The primary enzymatic activity of α-amylase is to cleave α-1,4 linkages in amylose and amylopectin to produce dextrins of varying chain lengths. The viscosity of the slurry decreases as the chain lengths are shortened (Elander and Putsche, 1996). Heat (110°C) is applied at this stage to enable liquefaction of mash using a jet cooker. The corn mash is kept at an elevated temperature for several minutes,
and then it flows from the holding tube into a flash tank where the temperature falls to 80-90°C, and the level of bacteria in the mash declines. Finally, the remaining two thirds of the \( \alpha \)-amylase is added and the mash is liquefied for at least 30 minutes (Dien et al., 2002).

Simultaneous saccharification and fermentation (SSF) of the liquefied mash takes place at 32°C, pH 4.5-5, by the use of glucoamylase, to liberate glucose molecules from the nonreducing end of dextrins, and yeast (Saccharomyces cerevisiae) to ferment the glucose to ethanol (Elander and Putsche, 1996). Fermentation usually lasts 48-72 hours and ethanol is produced at a concentration of 10-12% (v/v) (Dien et al., 2002). The ethanol is brought up to 100% (v/v) concentration using distillation and molecular sieve bed (Dien et al., 2002).

Other products obtained during the corn dry grind process are Distiller Dried Grains with Solubles (DDGS) and carbon dioxide (CO\(_2\)) (Dien et al., 2002). During the dry grinding process, fermentation byproducts are formed, such as glycerol, succinate, and acetate (Elander and Putsche, 1996). In addition, low-level contamination by Lactobacillus bacteria, that produce lactic acid from glucose, is common in most industrial-scale processes (Elander and Putsche, 1996).
Figure 2 Corn dry grind process for production of ethanol from corn (Watson and Ramstad, 1987).
Figure 3 Corn wet milling process (Watson and Ramstad, 1987).
1.5.3 Corn wet milling process

Corn wet milling is an industrial process which separates corn kernels into the endosperm (the source of gluten and starch), germ (containing most of the oil) and pericarp (e.g., corn fibre) (Dien et al., 2002). The largest wet mill plants in the United States are: Archer Daniels Midland (Decatur, IL, and Clinton, IA), Perkin Energy (Perkin, IL) and A.E. Stanley Manufacturing Co. (Loudon, TN) (Elander and Putsche, 1996). A schematic representation of the wet milling process is shown in Figure 3.

The initial process step is cleaning. Following cleaning is steeping, during which corn is soaked in water and treated with sulphur dioxide (1600 ppm) at 52°C for 20-40 hours. The corn is further acidified by the growth of lactic acid bacteria at later stages during the steep. Steeping swells the kernel and loosens the bonds between the protein and starch. According to MacMasters (1962) and Pérez et al., (2001) the presence of lactic acid in the steeping water makes the cell walls easier to break down.

Following steeping, the kernels are shredded in a mill designed to dislodge the germ. The germ, which contains about 30% oil, is then separated in hydrocyclones, based on its lower density (Dien et al., 2002). The oil is then extracted from the recovered, washed and dried germ, and the residual meal is blended into corn gluten feed (Dien et al., 2002). Next, the remaining mixture (protein and starch) is separated from the corn fibre by passing the slurry along metal screens. The fibre is further washed to recover additional starch and finally used as corn gluten feed or corn fibre. The gluten is then separated from the starch by centrifugation, and the recovered protein (gluten) is sold as gluten meal.

The remaining material, mostly starch, moves to starch washing filters to further remove residual protein (99.5% pure) (Dien et al., 2002). Across the corn wet milling
industry, about 80% of starch slurry goes to corn syrup, sugars, and alcohol production, with the relative amounts of each of the streams varying among plants (Watson and Ramstad, 1987). Some of the starch is dried and marketed as unmodified starch, some is modified into specialty starches, but most is converted into corn syrups and dextrose. Corn syrup and corn sugars are formed by hydrolysing the starch, either by partial or complete hydrolysis (using mineral acids, enzymes, or a combination) respectively.

The cleaned starch, if used for ethanol, is enzymatically hydrolysed to glucose using α-amylase followed by glucoamylase with pullanase (Dien et al., 2002). Separate saccharification and fermentations by S. cerevisiae are carried out continuously to reduce the amount of dried yeast needed. After about 2 days approximately 90% of the starch is converted to ethanol and then the fermentation broth is transferred to a still where the ethanol is distilled. Subsequent distillation and treatment steps produce 95%, absolute or denatured ethanol (Watson and Ramstad, 1987).

1.6 Biomass sources for bioconversion purposes

Various lignocellulosic biomass feedstocks, including: agricultural residues (corn stover, corn fibre, sugar cane bagasse and wheat rice straw); wood residues (hardwood and softwood sawdust, slabs and mill shavings); food-processing waste and municipal solid wastes (building materials, waste paper) have the potential to serve as low-cost, abundant feedstocks for the production of fuel ethanol. For example, in British Columbia, forest-derived wastes and residues (2,249,000 dry tonnes of residues available annually, of which one-half is estimated to be whitewood) are a very attractive feedstock for the production of ethanol (McCloy and Associates, 2003).
For the past 14 years, the Forest Products Biotechnology group (FPB) at UBC has focused on commercialization of the bioconversion process of softwood and hardwood residues to ethanol. In general, softwoods, due to their anatomical structure and chemical composition, have proven to be more recalcitrant to bioconversion (including pretreatment and hydrolysis) than hardwoods or agricultural residues. The FPB group has used a techno-economic model of biomass to both elucidate the level of maturity of each of the process steps and to identify which of the components contributes the most to the overall cost of the process (Gregg et al., 1998).

Ideally, the pretreatment of softwood residues should lead to the optimal recovery of hemicellulose sugars in a monomeric form, while producing an easily hydrolysable water insoluble cellulosic fraction with minimal formation of inhibitory compounds. However, this is difficult to achieve due to the fact that the optimum severities for maximum recovery of hemicellulose, lignin and cellulose are not all the same (Heitz et al., 1991). Therefore, in optimizing pretreatment conditions there is always a compromise between maximum hemicellulose recovery in a fermentable form, and complete enzymatic digestability of solids, as a function of severity. This has been accomplished through the use of a less severe, single-stage pretreatment step as practiced at the FPB group (195°C, 4.5 min and 4.5% SO₂) (Boussaid et al., 1999; Wu et al., 1999) and/or a two-stage pretreatment process (Nguyen et al., 2000; Söderström et al., 2002). As a consequence of using milder conditions during pretreatment to ensure a high degree of softwood carbohydrate component utilisation, a delignification process has been shown to be necessary in addition to other costly steps, including pretreatment, fractionation, hydrolysis and fermentation (Yang et al., 2002).
Another major disadvantage of SO₂-catalysed steam explosion and enzymatic hydrolysis of softwood residues is the generation of dilute process streams, particularly with regard to the water soluble, hemicellulose fraction. This is because high yield recovery of hemicellulose sugars requires a significant water-wash, further diluting the stream. The economic consequences of using a dilute sugar stream or losing a fraction of the hemicellulose due to the post-delignification water-wash pose significant problems for the commercialization of biomass to ethanol processes.

An additional problematic issue is that during the bioconversion of softwood to ethanol, two types of inhibitors are generated. These include process-derived inhibitors, created during pretreatment (e.g., lignin and sugar degradation products), and naturally-occurring inhibitors, produced from the feedstock and recovered in the hemicellulose fraction (e.g., sterols, acetic and uronic acids and resin/fatty acids) (Söderström et al., 2003). These compounds may cause inhibition in the fermentation step, and an additional detoxification step such as overliming is often required.

Although there is considerable debate about the significant cost driver of the bioconversion of softwood to ethanol (Galbe and Zacchi, 2002a; Zacchi and Axelsson, 1989), the FPB group found that the delignification of pretreated substrate contributes the most to the overall price of ethanol (Gregg et al., 2004). At this time, the group is exploring organosolv pulping (ethanol, acetic acid) as an alternative pretreatment option for softwoods. However, earlier work on hardwoods and agricultural residues has indicated that SO₂-catalysed steam explosion could be a technologically and economically feasible pretreatment choice for feedstocks such as corn fibre. It may also be possible to identify compromise conditions for easily converted feedstocks using steam explosion to recover
maximum sugars and minimum amounts of inhibitors, in a very simple two-step process, including pretreatment and simultaneous saccharification and fermentation (SSF).

1.7 Why is corn fibre a potential feedstock for commercial biomass to ethanol plants?

Corn fibre is exported primarily to Europe as corn gluten feed after combining it with corn steep liquor. However, because of uncertain markets for corn gluten feed and a relatively low price for wet corn fibre ($27 CAN/ton), there has long been a strong interest in finding more valuable products from corn fibre (Archer-Daniels-Midland, 2003). Currently, in Canada over 9 billion kilograms of corn are processed annually by the corn wet milling industry in the production of syrups, starch, oil and alcohol fuel (O'Connor, 2000). Although substantial, this is only a fraction (4%) of the material processed in the United States, which consumes ~229 billion kilograms of corn annually (O'Connor, 2000). Corn fibre is a mixture of corn hulls and residual starch not extracted during the milling process, and comprises up to 9% of the dry weight of the corn kernel (Anderson and Watson, 1982).

In addition, it has been predicted that the utilisation of the corn fibre fraction in the conversion process could potentially increase the overall ethanol yield by approximately 10% (Grohmann and Bothast, 1997).

1.8 Chemical composition of lignocellulosics

The major component of lignocellulosic biomass is cellulose, which can comprise as little as 25% of some agricultural residues (corn fibre) and as much as 43% of some softwoods (Douglas-fir Pseudotsuga menzeisii) and hardwoods (trembling aspen Populus tremuloides) (Table 1) (Bothast and Saha, 1997; Elander and Putsche, 1996; Ramos et al., 1992; Robinson et al., 2002). Hemicellulose, a second structural polymer composed of carbohydrate, accounts for 30% of corn fibre biomass, and 20 and 21% of aspen and
Douglas-fir, respectively. Lignin accounts for 29 and 23% of softwood and hardwood biomass, respectively. In contrast, agricultural residues generally contain lower amounts of lignin, with corn fibre containing about 7% (Bothast and Saha, 1997). Proteins, oils, extractives and ash in widely varying ratios make up the remaining fractions of lignocellulosic biomass (Elander and Putsche, 1996). Thus, although the major components of the various forms of biomass used for the bioconversion process are similar (cellulose, hemicellulose and lignin), each type of feedstock has different processing requirements, and corresponding potential ethanol yields, as a result of chemical and structural variations.
Table 1 Chemical composition of lignocellulosic biomass (% weight)) (Douglas-fir, aspen and corn fibre; carbohydrates and lignin) (Bothast and Saha, 1997; Bura et al., 2003; Elander and Putsche, 1996; Grohmann and Bothast, 1997; Ramos et al., 1992; Robinson et al., 2002).

<table>
<thead>
<tr>
<th></th>
<th>Douglas-fir</th>
<th>Aspen</th>
<th>Corn fibre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>43**</td>
<td>48**</td>
<td>46*</td>
</tr>
<tr>
<td>Lignin</td>
<td>29</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Galactose</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Xylose</td>
<td>4</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Mannose</td>
<td>13</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* glucose from cellulose and starch
**glucose from cellulose and hemicellulose
1.8.1 Chemical composition of corn fibre

It is apparent that the structural features and chemical composition (i.e. carbohydrate and lignin content) of a feedstock have a direct impact on its susceptibility to processing during bioconversion processes. Thus, a better understanding of the building blocks of corn fibre is essential in helping to define an efficient bioconversion process utilizing the feedstock. Obtainable from the wet milling industry, corn fibre is primarily composed of the outer kernel covering or seed pericarp, along with adherent starch. The compositional analysis of corn fibre can vary considerably, according to the source of the material and analytic methods used to quantify the material. Generally, corn fibre has been estimated to include up to 35% arabinoxylan and 15-25% cellulose (Leathers, 2003). Cellulose is a linear homopolymer of D-glucose linked by β-(1-4) bonds (Bothast and Saha, 1997). Adherent starch levels vary by production facility and on a day-to-day basis, but can be 15-20% or greater (Leathers, 2003). The starch granule is composed of two glucan polymers, amylose and amylopectin (Watson and Ramstad, 1987). Amylose, which makes up 25-30% of the starch, is an essentially linear structure of glucose units linked by α-(1-4) bonds (Grohmann and Bothast, 1997). Corn amylose has a degree of polymerization of 100-1,000 glucose units (Watson and Ramstad, 1987). Amylopectin, composing 70-75% of the starch, is a branched molecule with α-(1-6)-linked branch points and linear regions of α-(1-4)-linked glucose units (Leathers, 2003).

Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids and comprise up to 35% of the corn fibre. Xylose is the major component, making up 15-17% (Whistler and BeMiller, 1956). Unlike cellulose, hemicelluloses are not chemically homogenous. Corn fibre hemicelluloses have
been structurally and chemically analysed and, as previously shown, are mainly arabinoglucuronoxylans (Montgomery and Smith, 1957; Whistler and BeMiller, 1956). Corn fibre xylan (CFX) is a low molecular weight heteroxylan, composed of β-(1,4)-linked xylose residues extensively substituted (about 80%) with side chains of 4-O-methylglucuronic acid, arabinose, and a trisaccharide composed of arabinose, xylose, and galactose (Figure 4) (Hespel, 1998). It contains 48-54% xylose, 33-35% arabinose, 5-11% galactose, and 3-6% glucuronic acid (Doneer and Hicks, 1997). The oligomers have the same basic unit composed of one arabinofuranose residue esterified on position O-5 by ferulic acid (Figure 4). This type of structure has been found in all feruloylated oligomers isolated from monocots (Ishii, 1997). From this unit, the other oligomers are built by adding one xylose residue on position 2 of the arabinose, and one galactose residue on the O-4 of the xylose residue (Figure 4). As can be seen from Figure 5, which shows the possible association of polysaccharides in the corn fibre cell wall, the heteroxylans that are probably highly cross-linked through diferulic bridges, creating a network in which the cellullosic microfibrils may be embedded (Saulnier and Thibault, 1999). Apart from the diferulic bridges, structural wall proteins, which are present in the pericarp, may be cross-linked together by isodityrosine bridges and with feruloylated heteroxylans, thus forming an insoluble network (Saulnier and Thibault, 1999).

Lignin is also present and comprises up to 7% of the dry weight of corn fibre (Grohmann and Bothast, 1997). A relatively large amount of phenolic acids (~5%), mainly ferulic acid, as well as small amounts of p-coumaric acid, are present in corn fibre (Saulnier and Thibault, 1999). The high amount of ferulic acid linked to heteroxylan though arabinofuranose, suggests that corn fibre may contain a high level of phenolic dimers
(Saulnier and Thibault, 1999). If all these dimers cross-link heteroxylan chains, each heteroxylan molecule should be cross-linked, on average, through 30 diferulic bridges (Saulnier and Thibault, 1999).

Protein, oils and ash make up the remainder of corn fibre, in varying ratios, due to differences in the efficiency of milling operations. Corn fibre contains 11-12% protein (albumins, globulins, and free amino acids), 3-4% fat and less than 1% ash (Watson and Ramstad, 1987). Triglycerides are the predominant storage lipids in corn fibre of which the amount depends on the germ separation process and comprise up to 2.5% (Moreau et al., 1996; Wu and Norton, 2001). Sterol esters, sterol ferulates, sterols, free fatty acids, phytosterols, tocopherols and waxes are also found in corn fibre in minimal quantities (Moreau et al., 1996; Wu and Norton, 2001).
Figure 4 Schematic structure of corn fibre heteroxylan (Saulnier and Thibault, 1999).

A: Arabinose
X: Xylose
G: Galactose
GlcA: Glucuronic Acid
FeA: Ferulic Acid
Figure 5 Model of the corn fibre cell walls (Saulnier and Thibault, 1999).
Bioconversion of lignocellulosic residues to ethanol includes various process steps: feedstock preparation, pretreatment, fractionation, post-treatment (delignification), enzymatic hydrolysis, fermentation, product recovery and waste treatment. Pretreatment of the lignocellulosic substrates is essential for efficient enzymatic hydrolysis because of the various physical and chemical barriers that inhibit the accessibility of cellulase enzymes to the cellulose substrate. To be fully effective, it should provide maximum recovery of the lignin, hemicellulose-derived sugars, and cellulosic residues, with the latter component being readily hydrolysed by enzymes and all of the derived sugars fermented to ethanol. Each feedstock requires different processing requirements due to chemical and structural variations. Likewise, it has been suggested that during hydrolysis of steam-treated substrates, lignin acts as a physical barrier that hinders contact between the substrate and enzymes. Therefore, a delignification process is required during the bioconversion of softwood residues to ethanol (Figure 6) (Boussaid et al., 2000; Robinson, 2003; Yang et al., 2002). The need for lignin removal during the bioconversion of softwood to ethanol makes this process economically challenging, by increasing process complexity, decreasing overall hemicellulose recovery (as it requires a water-wash step after delignification) and increasing the problems associated with dilute sugar stream.
Figure 6 Schematic representation of bioconversion of: softwood (Douglas-fir), hardwood (aspen) and agricultural residues (corn fibre) to ethanol.
Results are equivocal when evaluating the relative influence of delignification of hardwood residues on enzyme digestability. Schwald et al., (1988) reported a beneficial effect of lignin removal on enzymatic hydrolysis of wood pulps, whereas Ramos et al., (1992) obtained little or no increase in enzymatic digestability after extraction of lignin by dilute alkali from pretreated hardwood substrate. However, after a post-treatment with hydrogen peroxide (H$_2$O$_2$), both hydrolysis rate and glucose yield improved significantly (Ramos et al., 1992).

As shown in Figure 6 and in subsequent sections, the conversion of corn fibre to ethanol is a relatively simple process. The chemical and structural characteristics of corn fibre make this feedstock more amenable to SO$_2$-catalysed steam explosion when compared to softwood and hardwood residues. Thus, it is possible to select the optimum pretreatment conditions (time, temperature and SO$_2$ concentration) and, concurrently obtain maximal hemicellulose and cellulose recovery. After pretreatment, the hydrolysate (pH adjusted—water insoluble and soluble fractions) can be enzymatically digested and fermented in one reactor (simultaneous saccharification and fermentation; SSF). This process does not require fractionation, delignification, water-wash with separate hydrolysis (of the water insoluble stream) and fermentation (water soluble fraction). Good carbohydrate recovery and high ethanol concentrations may be obtained in a single reactor with minimal enzyme and yeast supplementation. This substantially reduces the complexity of the overall corn to ethanol bioconversion, while simultaneously lowering the cost and time associated with the need for separate processes.
1.9.1 Pretreatment

Various pretreatment options have been used to fractionate, solubilise, hydrolyse, and separate cellulose, hemicellulose and lignin moieties. These different processes usually exploit a combination of chemical, physical and mechanical treatments that serve to render the lignocellulosics more receptive to subsequent enzymatic hydrolysis (McMillan, 1992). The physical pretreatment techniques do not involve chemical application, and typical examples are milling, irradiation, steam explosion, and hydrothermolysis (high-temperature cooking) (Hsu, 1996). Chemical pretreatment techniques have received the most attention by far among all the categories of pretreatment methods and typical examples include dilute acid, alkali, solvent, ammonia, SO$_2$, CO$_2$, other chemicals, steam explosion and pH-controlled hydrothermolysis (McMillan, 1993). The last category of pretreatment methods, facing major techno-economic challenges, is biological pretreatment. This group comprises pretreatment techniques of applying lignin-solubilising microorganisms to render lignocellulosic materials amenable to enzymatic digestion (Hsu, 1996). The difficulty in implementing this method at an industrial scale is primarily due to the relatively slow rate of the process and inability of the microorganisms to specifically solubilise and consume lignin in a distinct fashion from hemicellulose and cellulose (McMillan, 1993).

1.9.1.1 Steam explosion

As mentioned earlier, steam explosion is a method which converts biomass into useful chemicals and has been the topic of much research (Boussaid et al., 2000; Eklund et al., 1995; Glasser and Wright, 1998; Overend and Chornet, 1987; Schwald et al., 1989a; Söderström et al., 2002; Toussaint et al., 1991). It has only been in the last two decades that steam explosion has been considered as a potential process for the economical production of
chemicals, feed, fuel and polymers (Avellar and Glasser, 1998). During the 1970's and 1980's, several patents were issued advocating steam explosion for ruminant feed (Alegro, 1974; Brown, 1984). In the 1970's, the potential of the steam explosion technique as a defibrillation and fractionation process was more fully realized and since then this process has been widely utilized in biomass conversion (Avellar and Glasser, 1998). Steam explosion is a fusion of various pretreatment processes, combining both physical and chemical elements, causing the rupture of the wood cell wall structure, and hydrolysis and solubilisation of biomass components (Ramos and Saddler, 1994).

Previous work has shown that SO$_2$-catalysed steam explosion can successfully pretreat softwood (Boussaid et al., 2000; Carrasco, 1992; Clark et al., 1989; Clark and Mackie, 1987; Schwald et al., 1989b; Söderström et al., 2002; Stenberg et al., 1998; Tengborg et al., 1998) and hardwood residues (Eklund et al., 1995; Mackie et al., 1985; Schwald et al., 1989a) as part of the overall bioconversion process. In addition, steam explosion is recognized as one the most cost effective pretreatments for lignocellulosic residues prior to enzymatic saccharification (Clark and Mackie, 1987). The impregnation of SO$_2$ allows lower temperatures and shorter reaction times, and thereby reduces the formation of degradation products (Excoffier et al., 1991). The use of SO$_2$ as a catalyst results in improved enzymatic accessibility to cellulose and enhanced recovery of the hemicellulose-derived sugars (Boussaid et al., 1999; Boussaid et al., 2000). It was reported that more than 75% of the original hemicellulose-derived sugars can be recovered in the water soluble fraction after steam explosion of Douglas-fir (Pseudotsuga menziesii) wood chips at relatively mild conditions (Boussaid et al., 2000). It has been shown previously that adding SO$_2$ prior to steam explosion enhanced the carbohydrate hydrolysis rate, while reducing the
degree of polymerization of the oligomers and increasing the proportion of monomers in the water soluble stream (Clark et al., 1989). In addition, a combination of steam explosion with acid hydrolysis increases pore volume and enzyme accessibility, reduces particle size (Boussaid et al., 2000) and increases the available surface area (Michalowicz et al., 1991).

One of the possible explanations for the role of SO$_2$ in the catalysed steam explosion is that SO$_2$ is not the actual catalyst; it is converted into sulphuric acid, preferably inside wood cells (Brownell et al., 1988). The conversion of SO$_2$ occurs either by oxidation or disproportionation, or both. The oxidation of SO$_2$ occurs readily in the presence of air or oxygen at temperatures below 100°C. Disproportionation occurs at higher steam temperatures, in the absence of oxygen. In the case of disproportionation, the oxidation of SO$_2$ to H$_2$SO$_4$ is balanced by an equivalent proportionation, the reduction of SO$_2$ to thiosulphate or, more likely, to elemental sulphur (Brownell et al., 1988). Other possible explanations suggest that SO$_2$ may act as a weak acid by simply dissolving in water inherent in the green wood chips to form sulphurous acid, and it may selectively attack the wood lignin to form lignosulphonates which are very strong acids; or it may act in both these ways depending upon the level of SO$_2$ impregnation (Mackie et al., 1985).

1.9.1.1.1 Severity of pretreatment

During SO$_2$-steam explosion pretreatment, there are three main process variables: temperature, time and SO$_2$ level. In order to maximize recovery yields for each fraction of pretreated wood (cellulose, hemicellulose and lignin) Overend and Chornet (1987) have proposed the introduction of a single factor, the reaction ordinate $R_0$ which allows for the evaluation of the explosion process. It is based on the assumption that the overall process follows first-order reaction kinetics. The severity factor was developed based on H-factor
(kraft pulping), which combined temperature and cooking time into a single variable (Overend and Chornet, 1987). This factor, increases as a function of time $t$ (min) and temperature $T$ (°C), as follows:

$$R_o = te^{(T-100)/14.75}$$  \hspace{1cm} (Eq.1.1)

Chum et al. (1990) introduced a third parameter, the environmental pH, into the equation above to describe the combined severity (CS):

$$CS = \log R_o - \text{pH}*$$  \hspace{1cm} (Eq.1.2)

*where pH is calculated from the amount of sulphuric acid added.

By application of the CS, it is possible to compare different experiments and processes on an equal footing.

By employing a low pretreatment severity, only limited chemical and physical changes occur in the biomass. As the severity of pretreatment increases, first the hydrolysis of hemicellulose occurs, releasing oligomeric and monomeric sugars. Eventually, under sufficiently severe pretreatment conditions, the cellulose will start to hydrolyse. One of the main issues associated with optimizing pretreatment conditions for any feedstock is finding conditions at which hydrolysis of oligomeric sugars to monomers takes place, with a minimal formation of sugar degradation products. This is even further complicated due to the fact that the optimum treatment severities for maximum recovery of hemicellulose, lignin and cellulose are all different (Heitz et al., 1991). Cellulose and lignin require higher treatment severities to be recovered in high yield. However, these are obtained at the expense of the hemicellulose, with sugar decomposition resulting in inhibitor production which limits the effectiveness of the fermentation stage. Thus, obtaining maximum yields of each of the polymeric fractions would probably require a two-stage treatment cycle, with
removal of the bulk of the hemicellulose after pretreatment at low severity (while generating
the minimum amount of inhibitors) followed by a more severe post-treatment to obtain a
cellulose which can be readily hydrolysed (Heitz et al., 1991). Although this option for
softwoods proved to increase the overall carbohydrate recovery, due to the high cost of the
delignification process, it has been shown to be economically challenging (Gregg et al.,
2004).

A similar approach was used by Söderström et al., (2002) and Nguyen et al., (2000)
where they accommodated the different severity requirements for each of the biomass
components by performing a two-step pretreatment process, in which the first step is
optimised for the recovery of hemicellulose-derived sugars and the second step is effective
at degrading the cellulose and making it hydrolysable by cellulases. However, further
evaluation is needed to determine whether the advantages of obtaining higher overall sugar
yield outweigh the cost of additional pretreatment processes (Nguyen et al., 2000;
Söderström et al., 2002; Söderström et al., 2003).

1.9.1.2 Pretreatments of corn fibre

As discussed previously, pretreatment of lignocellulosic biomass is crucial to
enzymatic hydrolysis. Ideally, the pretreatment of lignocellulosic biomass should lead to a
reduction in particle size of the biomass, optimal recovery of hemicellulose-derived sugars
in a monomeric form, and the production of an easily-hydrolysable water insoluble,
cellulosic fraction. While size reduction is not required for the treatment of corn fibre, a
successful pretreatment for this feedstock should include converting the xylan fractions to
monosaccharides in high yields, minimal inhibitor formation and a process that produces a
cellulose fraction amenable to enzymatic hydrolysis.
Although several pretreatment options for corn fibre have been evaluated for their efficacy in fractionating, solubilising, hydrolysing, and separating the major compositional constituents, no single process to-date has proven superior.

Osborn and Chen (1984) evaluated the use of acid hydrolysis at various temperatures for its potential in converting corn fibre, and demonstrated that partial hydrolysis could be readily accomplished by treatment with dilute sulphuric acid at 135°C. Employing a similar process, it was also shown that corn fibre could be converted effectively when treated with dilute sulphuric acid at temperatures ranging from 140°C to 160°C, detoxified with calcium hydroxide, and the solubilised sugar fermented to ethanol using recombinant *Escherichia coli* KO11 (Beall et al., 1992). However, neither group achieved complete hydrolysis of the polysaccharides in the water insoluble fraction of the corn fibre, and all observed significant inhibitor formation, even under relatively mild conditions.

Grohmann and Bothast (1997) investigated the saccharification of polysaccharides in corn fibre by sequential treatment with dilute sulphuric acid at 100-160°C followed by partial neutralization and enzymatic hydrolysis with mixed cellulase and amyloglucosidase at 45°C. Although this process showed that enzymatic hydrolysis achieved an 85% conversion of all polysaccharides in the corn fibre, it was also apparent that significant inhibitor formation occurred at all pretreatment conditions tested between 140 and 160°C.

An alternative method has also been developed in an attempt to overcome the toxicity problems associated with dilute acid pretreatment and neutralization. Moniruzzaman *et al.*, (1997) established and optimised the ammonia fibre explosion treatment (AFEX) of corn fibre. While this research indicated that the AFEX process circumvented inhibitory by-product formation, it did point to a need for better xylan-
degrading enzymes, as the hemicellulose remained in a polymeric form after processing (Moniruzzaman et al., 1997). Similar problems were observed when using alkaline peroxide and hot water pretreatments which, while effectively preparing cellulose for enzymatic hydrolysis, did not completely hydrolyze the xylan (Allen et al., 2001; Leathers and Gupta, 1996; Weil et al., 1998).

Although many corn fibre pretreatment methods have been investigated, none has been proven to be completely effective. Therefore, in an attempt to establish and optimise the bioconversion of corn fibre to ethanol, an alternate pretreatment method involving SO$_2$-catalysed steam explosion was assessed and is described within this thesis.

1.9.2 Delignification

As shown previously, there are several benefits to lignin removal prior to enzymatic hydrolysis, including: the ease of enzymatic hydrolysis, improvements in further enzyme recycling steps and an increase in the relative cellulose content of the steam-treated material. However, these benefits may be diminished by the high cost of the delignification process, as well as the loss of significant amounts of hemicellulose during the additional wash after lignin removal.

Many lignocellulosic substrates behave differently with regard to delignification properties. Although it was found that sequential steam explosion and alkali-peroxide washing of aspen wood improved the rate and yield of enzymatic hydrolysis by about 10% (Ramos et al., 1992), the same procedure carried out with softwoods reduced the hydrolysability of these pretreated substrates (Ramos and Saddler, 1994). However, it has been reported that the recalcitrant lignin remaining after Douglas-fir wood had been steam exploded at medium severity, was significantly reduced by post-treatment with 1%
hydrogen peroxide at pH 11 and 80°C for 45 minutes. Eighty-two percent cellulose conversion at 10 FPU g\(^{-1}\) cellulose was achieved after 48 hours of reaction (Yang et al., 2002). It is probable that the differences observed for hardwoods and softwoods can be explained by differences in cell morphology and lignin composition. Generally, guaiacyl lignins are more resistant to chemical modification than are syringyl based lignins, which are found primarily in hardwood and grasses (Ramos et al., 1992).

Corn fibre, due to its low lignin content (7%), mostly in the form of phenolic acids, (mainly ferulic acid, as well as a small amount of \(p\)-coumaric acid) will probably allow greater use of process options, such as leaving the lignin with the water insoluble cellulose stream until cellulose hydrolysis is complete.

1.9.3 Hydrolysis

The pretreatment processes are designed only to initiate the breakdown of the biomass structure and partially hydrolyse the carbohydrate polymers, making them accessible to enzymatic attack. There is obviously a great deal of interest in obtaining fermentable sugars from corn fibre during the saccharification process for conversion to ethanol and value-added co-products.

Hydrolysis of cellulose to glucose can be achieved using either inorganic acids or cellulolytic enzymes. As mentioned earlier, chemical hydrolysis of biomass is relatively efficient and inexpensive, however, it generates fermentation inhibitors (Leathers, 2003). On the other hand, enzymatic hydrolysis, despite its relatively slow rate, is a biocompatible and environmentally friendly option (as it avoids the use of corrosive chemicals).

As shown previously, steam exploded softwood residues need additional post-treatment to produce a cellulose-rich, water insoluble fraction amenable to complete
enzymatic hydrolysis in 48 hours using an enzyme loading of 10 FPU g\(^{-1}\) cellulose (Yang et al., 2002). With regard to corn, although the cellulose and starch associated with corn fibre may be readily hydrolysable by conventional enzymes, corn fibre xylan has proven to be a recalcitrant substrate (Leathers, 2003). The highly branched nature of maize bran heteroxylan, together with the interconnection with a cell wall protein network which is reinforced by diferulic bridges between polysaccharide chains, probably explains the extreme resistance of corn fibre tissue to enzymatic attack by pure xylanase and ferulic esterase or mixtures of cell wall degrading enzymes (Saulnier et al., 1995).

1.9.3.1 Cellulolytic enzymes

Cellulases, perform a crucial task during saccharification by catalysing the hydrolysis of cellulose to soluble and fermentable carbohydrates. They are synthesized mainly by fungi and bacteria and are produced both aerobically and anaerobically. The aerobic mesophilic fungus *Trichoderma reesei* and its mutants have been the most intensively studied source of cellulases (Philippidis, 1996). The enzyme system for the conversion of cellulose to glucose generally comprises three distinct classes of enzyme (Lynd et al., 2002):

- endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4),
- exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as celloextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (also known as cellobiohydrolases) (EC 3.2.1.91), and
- β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21).

Endoglucanases cut at random, at internal amorphous sites in the cellulose polysaccharide chain, and generate oligosaccharides of varying lengths and consequently
new chain ends (Mansfield et al., 1999). Exoglucanases act on the reducing and nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products (Mansfield et al., 1999). Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Lynd et al., 2002). β-Glucosidases hydrolyse soluble cellodextrins and cellobiose to glucose (Bothast and Saha, 1997).

Cellulase systems exhibit higher collective activity than the sum of the activities of the individual enzymes, a phenomenon known as synergism. Five forms of synergism have been reported:

- endo-exo synergy between endoglucanases and exoglucanases (Lynd et al., 2002),
- exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains (Fägerstam and Pettersson, 1980),
- synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes (Lynd et al., 2002),
- intramolecular synergy between catalytic domains (CDs) and carbohydrate-binding modules (CDMs) (Lynd et al., 2002; Teeri, 1997), and
- endo-endo synergy between endoglucanases (Mansfield et al., 1998)

1.9.3.2 Hydrolysis of high concentration sugars

Economic studies have also shown substrate concentration to have a major impact on process costs (Nguyen and Saddler, 1991). Substrate loading in hydrolysis reactors affects production costs in several ways. High substrate concentration results in smaller volumes required for hydrolysis reactors and ethanol fermenters, thus lowering capital costs. Moreover, high substrate concentration yields a higher concentration of ethanol, which can
lower the steam requirement in the ethanol stripping tower (Nguyen, 1993b). For example, by doubling the ethanol concentration from 2.5 to 5%, the energy required to distill a fermentation broth to high-purity ethanol (93.5%) using conventional distillation techniques can be reduced by one third (Zacchi and Axelsson, 1989).

To increase the concentration of sugars available for saccharification and further fermentation, one can use a high concentration of solids; combine the water soluble and insoluble streams during hydrolysis; physically concentrate the water soluble fraction or supplement the water soluble fraction with glucose derived from other processes, such as enzymatic hydrolysis.

1.9.3.2.1 High consistency solids

It has been shown previously that batch enzymatic hydrolysis yield and, to a lesser extent, initial hydrolysis rate are influenced by the concentration of the substrate-pretreated softwoods and hardwoods (Gregg and Saddler, 1996; Schwald et al., 1989a). An inverse relationship was found between the concentration of substrate and the enzymatic hydrolysis yield. In an industrial process, it is preferable to hydrolyse high dry matter content, for example 10% (w/v) without separation of the water soluble and insoluble streams after the pretreatment process. In employing this approach, there is no need for filtration and washing steps, and thus facilitating capital cost reduction concurrently with the benefits of obtaining the abovementioned concentrated fermentable sugar streams.

One of the major factors affecting the hydrolysis of high sugar concentrations is end-product inhibition. This is due to the release of cellobiose and glucose sequentially, which can result in the inhibition of both the endo- and exo-cellulases and β-glucosidase (Breuil et al., 1992; Holtzapple et al., 1990). There is an ongoing debate regarding the type of
inhibition exhibited by cellulases. Although extensive research has shown that cellulases are competitively inhibited, there is convincing evidence that some cellulases are noncompetitively inhibited (Breuil et al., 1992; Holtzapple et al., 1990).

1.9.3.2.2 Hydrolysis of solids in the water soluble stream

Another method for increasing the concentration of sugars available for fermentation is to include the water soluble stream in the enzymatic hydrolysis step. It has been shown that during hydrolysis of pretreated spruce (Picea abies), when the water soluble fraction was combined with the water insoluble stream during the saccharification process, cellulose conversion was reduced by up to 36% (Tengborg et al., 2001). In order to overcome this problem, washing the pretreated softwood or hardwood solids with cold or hot water prior to enzymatic hydrolysis became a common laboratory procedure (Nagle et al., 2002; Robinson et al., 2003). In addition, according to Tengborg et al., (2001), to increase conversion of cellulose to glucose, one can ferment the water soluble fraction prior to hydrolysis. Based on this study, it was suggested that the main inhibition of hydrolysis by various compounds in the water soluble stream was due to aliphatic acids, furan derivatives and phenolic compounds rather than monomeric sugars and pretreatment by-products, such as furfurals and 5-hydroxymethylfurfurals (5-HMFs) (Larsson, 1999; Tengborg et al., 2001). On the other hand, Robinson’s work suggested that the end-product inhibition was the main problem during enzymatic hydrolysis of combined water soluble and insoluble fractions of pretreated Douglas-fir (Robinson, 2003).

1.9.3.2.3 Concentration of the water soluble stream

Other methods for increasing the concentration of sugars in the water soluble stream prior to fermentation are based on removing water from the process stream, such as freeze
drying, evaporation, spray-drying and ultrafiltration. Although sugar concentration can be increased dramatically by using these methods, the amplified concentration of inhibitors negatively affecting fermentation along with the high capital cost and energy demands, makes this option technically and economically unattractive (Robinson et al., 2003).

1.9.3.2.4 Mixing of water soluble fraction with the cellulose hydrolysate

A more promising technique for increasing the concentration of sugars available for further fermentation is the supplementation of the water soluble fraction with a cellulose hydrolysate. Although a dramatic increase (56%) in the sugar concentration compared to the water soluble stream alone was observed with pretreated Douglas-fir (Robinson, 2003), in order to meet the target concentration of 100 g L\(^{-1}\) of hexoses in the mixture, the cellulose hydrolysis needed to be performed at approximately 18\% (w/v). However, by using a high concentration of solids during hydrolysis, significant end-product inhibition by glucose and cellobiose was observed (Robinson, 2003).

1.9.4 Fermentation

It is well known that more severe conditions during steam pretreatment will cause greater degradation of hemicellulose-derived sugars (Boussaid et al., 1999; Heitz et al., 1991). However, a high degree of severity is required to promote enzymatic hydrolysis of the cellulose fibres, especially in softwood (Nguyen et al., 2000). Therefore, during steam pretreatment of softwoods, the pentoses and hexoses liberated from the hydrolysed hemicellulose and cellulose may be further degraded to furfural, 5-hydroxymethylfurfural (5-HMF), levullinic acid, acetic and formic acids. Two major groups of potential inhibitors have been found in the liquid fraction after pretreatment of softwood residues: process-derived inhibitors created during pretreatment (e.g., lignin and sugar degradation products),
and naturally-occurring inhibitors from the feedstock (e.g., sterols, acetic and uronic acids and resin/fatty acids) (Olsson and Hahn-Hägerdal, 1996; Palmqvist, 1998), all of which may cause adverse effects during fermentation (Tengborg et al., 2001). It is apparent, based on the chemical composition of corn fibre, that primarily process derived inhibitors will be present in the water soluble stream after pretreatment, which can be minimized by careful tailoring of the severity of steam explosion. Therefore, it should be possible to combine the water soluble and insoluble streams during consecutive hydrolysis and fermentation processes.

As mentioned above, cellulase activity is inhibited by cellobiose and glucose, and thus several methods have been developed to reduce this end-product inhibition, including supplementation with excess β-glucosidase during hydrolysis and the use of a high concentration of enzymes and removal of sugars during hydrolysis by simultaneous saccharification and fermentation (SSF). Keeping in mind that a reduction in the cost of ethanol production can be achieved either by reducing the cost of raw materials or the cellulase enzymes, the supplementation of β-glucosidase during hydrolysis and the use of high enzyme concentrations are not economically feasible options. Therefore, one of the remaining options is the use of SSF process, which has several advantages over separate hydolysis and fermentation (SHF).

1.9.4.1 Comparison of SSF with SHF

Simultaneous saccharification and fermentation (SSF) is an alternative to separate hydrolysis and fermentation (SHF). The SSF procedure is a one-stage process involving the enzymatic saccharification of cellulose and simultaneous fermentation of fermentable sugars by yeast or bacteria in a single reactor.
SSF is advantageous over SHF due to the process integration obtained when hydrolysis and fermentation are performed in one reactor, which reduces capital cost and decreases saccharification and fermentation time (Stenberg et al., 2000b). In addition, the SSF approach eliminates the need for a solid-liquid separation step.

More importantly, SSF addresses one of the most serious shortcomings of current cellulolytic enzymes, i.e., sensitivity to end-product inhibition. The cellulase complex produced by commercial and laboratory mutants is strongly inhibited by low concentrations of glucose and cellobiose (Grohmann, 1993). Results from different studies (Fujii et al., 1991; Ooshima et al., 1985) showed that the Trichoderma cellulase enzyme system is strongly inhibited by glucose and cellobiose at concentrations much lower than 1% (w/v). On the other hand, efficient ethanol recovery seems to require a fermentable sugars concentration higher than 8% (w/v). By increasing β-glucosidase and cellulase loadings several times, this requirement can be fulfilled and severe end-product inhibition overcome, at a proportionately increased enzyme cost (Nguyen, 1993a). Therefore, by employing SSF one can achieve higher productivity, as end-product inhibition by glucose accumulation is reduced, and concurrently, lower enzyme loadings are required.

Another often-claimed advantage is reduced sensitivity to infection in SSF. The maintenance of low sugars concentration throughout SSFs and accumulation of relatively high concentrations of ethanol also helps to alleviate any problems with microbial contamination (Grohmann, 1993). However, other studies employing SSF of steam-pretreated spruce showed that this was not the case (Stenberg et al., 2000b).

The main disadvantage of the SSF approach is the loss of freedom in the adjustment of important parameters for hydrolysis and fermentation. The enzymes and microorganisms
have to be matched with respect to temperature, absence of antibiotics in enzyme preparations and the restriction of proteolytic or other enzyme-inactivating activities which may be secreted by fermenting microorganisms (Grohmann, 1993). Most of the organisms used in the fermentation of lignocellulosic hydrolysates e.g., *Saccharomyces cerevisiae* (Olsson and Hahn-Hägerdal, 1996), *Escherichia coli* (Dien et al., 1999) or *Zymomonas mobilis* (Olsson and Hahn-Hägerdal, 1993) limit the temperature to below 40°C, whereas the optimal temperature for enzyme hydrolysis has been suggested to be 45°C (Bura et al., 2002). This has a negative impact on productivity because hydrolysis is the rate-limiting step in SSF (Stenberg *et al.*, 2000b).

The other drawback of SSF is the difficulty in separating yeast from solids residue after SSF, which makes it more difficult to recover and recycle yeast cells. Therefore, it may be necessary to prepare a new yeast inoculum for each SSF run (Stenberg *et al.*, 2000a). For SHF, yeast recovery from a clean beer (containing little wood fibres and lignin) is much simpler (Nguyen, 1993a).

The final disadvantage of using SSF process over SHF is the inhibition of cellulase activity by ethanol. Wu and Lee (1997) found that at an ethanol concentration of 9, 35 and 60 g L$^{-1}$, cellulase activity was reduced by 9%, 36% and 64% compared to the original, during a SSF process at 38°C. The highest ethanol concentration ever reported for an SSF process was about 57 g L$^{-1}$, using 20.2% straw concentration (Mohagheghi *et al.*, 1992). At this substrate and ethanol level, a 70% sugar to ethanol conversion was achieved.

### 1.9.4.2 Microorganisms in corn fibre to ethanol fermentation

Mixed sugars derived from corn fibre are potential substrates for fermentation to a variety of valuable products, including ethanol, vitamins, amino acids and many others. The
microorganism most widely used in the fermentation to ethanol process is *Saccharomyces cerevisiae*. However, this yeast is unable to ferment pentose sugars, which can comprise an appreciable fraction of corn fibre hydrolysates. There are essentially no commercially suitable wild type or naturally occurring bacteria or yeast for fermenting xylose and arabinose to ethanol. For example, naturally occurring yeast that do ferment xylose require aeration for growth, have low productivity, are very sensitive to inhibitors, especially acetate, and have a low ethanol tolerance (Bothast *et al.*, 1999; Dien *et al.*, 2002).

It is important that microorganisms selected for the conversion of hemicellulose hydrolysates have the ability to ferment the sugars rapidly (ethanol productivity should be higher than 1 g L\(^{-1}\) h\(^{-1}\)), at high yield (higher than 90% of theoretical), and should have a high ethanol tolerance (higher than 40 g L\(^{-1}\)) in order to maximize conversion performance (Dien *et al.*, 2003). In addition, the selected microorganisms should be robust growers, which would require an inexpensive medium formulation, and very resistant to inhibitors (generated in the pretreatment step) (Dien *et al.*, 2003).

Both yeast (such as *Saccharomyces* and *Pichia* species) and bacteria (such as *Escherichia coli*, *Klebsiella*, and *Zymomonas*) have been genetically engineered to ferment glucose and xylose (Bothast *et al.*, 1999; Dien *et al.*, 2003; McMillan, 1996). Xylose-fermenting yeast such as *P. stipitis* has shown a great potential for achieving high conversion yields on detoxified hydrolysates. However, aeration is required and there is a significant performance tradeoff between yield and productivity (often 0.3-0.4 g L\(^{-1}\) h\(^{-1}\)) (McMillan, 1996; Slininger *et al.*, 1985). In contrast, recombinant bacteria achieved high yield (up to 0.3-0.5 g (g corn fibre sugars\(^{-1}\)) and high productivity (1.0-1.6 g L\(^{-1}\) h\(^{-1}\)) under anaerobic conditions (Bothast *et al.*, 1999; Hahn-Hägerdal *et al.*, 1994b). Moreover, *E. coli,*
K. oxytoca and Z. mobilis can ferment arabinose, which makes them superior microorganisms to ferment pretreated and hydrolysed lignocellulosic biomass, compared to Pichia stipitis (Dien et al., 2003). Nevertheless, inhibitor tolerance remains a concern for all of these strains. For example, Z. mobilis is extremely sensitive to acetic acid (Dien et al., 2003). Therefore, future development for these Gram-negative bacteria should emphasize increasing inhibitor tolerance, reducing growth factors and improving ethanol productivity.

Representative strains of recombinant E. coli, K. oxytoca, and Z. mobilis strains are currently being considered for commercial scale-up, however, the commercial ethanol industry has shown a bias for relying on Saccharomyces strains. In the future, the willingness of ethanol producers to consider using bacterial strains instead of Saccharomyces will depend on demonstrating that bacterial stains are capable of producing ethanol reliably in large bioreactors, that fermentations need not be fully aseptic to avoid contamination, and that strains can be developed that have qualitative advantages compared to yeast, such as a reduced need for saccharification enzymes (eg., cellulases) (Dien et al., 2003; Lynd et al., 2002; McMillan, 1996).

1.9.5 Techno-economic modeling

As shown previously in Figure 6, and as will be discussed in the Results section, when comparing a number of subprocesses during the bioconversion of softwood to ethanol with corn fibre to ethanol, the latter process option contains only two major steps: pretreatment and simultaneous saccharification and fermentation (SSF), compared to five stages used during bioconversion of softwoods (pretreatment, fractionation, delignification, hydrolysis and fermentation). What are the economic impacts of reducing the number of process steps involved in the bioconversion of lignocellulosics to ethanol? What are the
economic benefits of different process configurations during corn fibre to ethanol processing: SHF versus SSF?

Techno-economic models have been used in the past to provide assessments of both process and subprocess maturity and the production cost of a product (Gregg et al., 1998; Nguyen and Saddler, 1991; von Sivers and Zacchi, 1995; Wingren et al., 2003). It is generally recognized that, due to the immature state of the technology and the cost of pilot- and demonstration-scale facilities, models are one of the most efficient ways of assessing the technical and economic feasibility of lignocellulosic to ethanol processes. These models have generally been based on information obtained from both lab and pilot studies, and from the operation of similar processes in other industries or in commercial-scale acid hydrolysis plants (Gregg et al., 1998; Nguyen and Saddler, 1991). Most of the models have been used to assess not only the current techno-economic status of a particular process (both process step and overall process), but also the future potential of the various bioconversion processes.

According to Galbe and Zacchi (2002a), the estimated cost of producing ethanol from wood residues varies widely between different investigations, and ranges from US$0.32 to 1.00 per litre of ethanol (Lynd, 1996; von Sivers and Zacchi, 1995; Wyman, 1999). In addition, most cost estimations are based on lab-scale and, to some extent, pilot-scale data for individual process steps and thus should not be used to obtain an absolute production cost. However, the cost estimations are useful not only for identification of bottlenecks (by analysis of relative percentage contributions of each subprocess steps to the total process ethanol selling price), but also for comparison of the relative costs of different
process strategies, for example comparison of SHF with SSF on an equal footing (Galbe and Zacchi, 2002a).

1.10 Objectives and overview of the thesis

The goal of this Ph.D. project was to determine if corn fibre, due to its unique chemical composition (high carbohydrate content and low lignin concentration) and wide availability in most ethanol producing plants at low cost, would be a lignocellulosic substrate that could be used to demonstrate the technical and economical feasibility of a bioconversion to ethanol process. In this work I have assessed whether it is possible to use SO₂-catalysed steam explosion to recover most of the sugars in a hydrolysable and fermentable form. We also hypothesized that it would be possible to perform all the processes after pretreatment in one reactor. The proposed process would include pretreatment of the corn fibre, followed by pH adjustment of the whole slurry (water soluble and insoluble fractions) and completion of the hydrolysis and fermentation steps in a single reactor. The specific objectives of this project were:

- Assessment of SO₂-catalysed steam explosion of corn fibre for ethanol production;
- Evaluation of the hydrolysability of high cellulose concentrations created using high consistency solids and combining the water soluble and insoluble streams during the saccharification process;
- Optimisation and comparison of different process options: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF);
- Estimation of the economic impacts of the different process alternatives.

To evaluate the technical feasibility of achieving good carbohydrate recovery and ethanol conversion after SO₂-catalysed steam explosion of corn fibre, the optimal
pretreatment conditions were first established. The main challenge during designing the optimal pretreatment conditions for lignocellulosic biomass is balancing the trade-offs. For example, by using low pretreatment severity, one can obtain maximal yields of hemicellulose, however, this is not achieved with good cellulose hydrolysis. Conversely, by using severe pretreatment conditions, enzymatic digestibility of solids and lignin extraction can be improved, but a significant loss of hemicellulose sugars and the formation of inhibitory degradation products occurs. As shown previously, due to the chemical and structural nature of softwoods, it was possible to optimise the SO₂-catalysed steam explosion in terms of hemicellulose recovery, but to fully convert the cellulose in the solid fraction, a delignification process was required (Boussaid et al., 2000; Robinson, 2003; Yang et al., 2002). Therefore, in the work reported in this thesis, a compromised condition had to be defined during the pretreatment of corn fibre to recover the maximum amount of sugars in a hydrolysable and fermentable form, and concurrently to produce a minimum amount of sugar degradation products.

Although many corn fibre pretreatment methods have been proposed (enzymatic, acid hydrolysis, hot water and AFEX), none has been proven ideal. In an attempt to establish and optimise the bioconversion of corn fibre to ethanol, an alternate pretreatment method, SO₂-catalysed steam explosion, was evaluated. We hypothesized that SO₂-catalysed steam explosion can effectively pretreat corn fibre; and, by using relatively mild pretreatment conditions, achieve high overall sugar recovery in an enzymatically digestible and fermentable form.

Surface response modeling was employed to establish the optimum pretreatment conditions for corn fibre in terms of maximum hemicellulose and cellulose recovery in
hydrolysable and fermentable form. By using various severities controlled by altering temperature, residence time and SO₂ concentration, 13 sets of experimental conditions were tested for maximum sugar recovery. This statistical analysis involved fitting an empirical model to the experimental data and identifying the optimal residence time, temperature and SO₂-impregnation level in the pretreatment stage. During optimisation of the pretreatment method, all saccharification experiments were initially performed at a low solids consistency. Bearing in mind that economic studies have shown substrate concentration to have a major impact on process costs (Nguyen and Saddler, 1991), the hydrolysability of sugars at higher substrate concentrations was next evaluated.

We hypothesized that after mild SO₂-catalysed steam explosion, we should obtain good hemicellulose recovery and effective solids hydrolysis at high consistency without the need for prior lignin removal. Two methods were used to increase the concentration of sugars available for consequent fermentation. These included the hydrolysis of high concentration solids (up to 12%) and supplementation of the hemicellulose fraction during the saccharification process. The latter approach allowed us to concurrently hydrolyse the oligomer-rich hemicellulose fraction, along with the solids fraction.

It has been shown that during the hydrolysis of sugar-rich streams, end-product inhibition was the main factor influencing incomplete cellulose conversion (Gregg and Saddler, 1996; Robinson, 2003). Therefore, increased hydrolysis time, enzyme loadings, and pretreatment of the water soluble stream prior to the saccharification step were assessed in terms of improvement in cellulose conversion.

In order to minimize end-product inhibition, and to simplify the overall bioconversion process of corn fibre to ethanol, simultaneous saccharification and
fermentation (SSF) was next evaluated. It has been hypothesized that due to the unique nature of the substrate, it should be possible to combine several subprocesses (fractionation, hydrolysis and fermentation) during the bioconversion of corn fibre to ethanol.

The last objective was to evaluate the economic impact of process modifications (SHF vs. SSF) and this was simulated by using an existing techno-economic model. This is possible due to the unique characteristics of corn fibre, which allows us to pretreat the material, adjust the pH of the whole slurry and, without additional fractionation, delignification, or water-wash, to perform the saccharification and fermentation in a single reactor, using low enzyme loadings, and yeast concentration. This work was carried out not to determine the absolute cost of ethanol production, but was rather aimed at comparing the production cost of the two processes on an equal footing, and studying the effects of different parameters on the ethanol production cost.

In summary, the work described in this thesis investigated the technical and economic feasibility of achieving good carbohydrate recovery and ethanol conversion after steam explosion of corn fibre. It evaluated the technical and economic potential of completion of the hydrolysis and fermentation steps in one reactor after pretreatment of the corn fibre, followed by pH adjustment of the whole slurry (water soluble and insoluble fractions).
2.0 MATERIALS AND METHODS

2.1 Steam explosion of corn fibre

Corn fibre (60.0 % moisture content) was obtained from the National Center for Agricultural Utilisation Research (Peoria, IL, U.S.) and stored at -20°C until use. Prior to SO₂-catalysed steam explosion, corn fibre samples were thawed and left in a sealed plastic container to ensure uniform moisture content. Samples of 300g oven-dried weight (ODW) were impregnated overnight with anhydrous SO₂ in plastic bags. The uptake of SO₂, expressed as a percentage of the corn fibre (ODW), was measured by weighing the corn fibre before and after SO₂ addition. The samples were then loaded, in 50g batches, into a preheated 2L Stake Tech II steam gun (Stake Technologies, Norvall, ON, CAN) and exploded at different severities (temperatures ranging from 150-230°C; time ranging from 1.5-5 minutes; SO₂ concentration ranging from 0-6% weight/oven-dried weight of fibre) (Table 2). The severity of the steam explosion pretreatment was represented by a severity factor Log R₀ as defined by Overend and Chornet (1987) and shown in Equation 1.1 (section 1.9.1.1.1). The recovered slurries were separated by centrifugation (10 min at 7000 × g) and frozen at -20°C until used.
Table 2 Conditions for SO₂-catalysed steam pretreatment of corn fibre based on surface respond design.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Severity of pretreatment (Log ( R_0 ))</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>SO₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.06</td>
<td>170</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2.17</td>
<td>150</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2.76</td>
<td>170</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2.76</td>
<td>170</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>3.02</td>
<td>170</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
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<td>3</td>
</tr>
<tr>
<td>9</td>
<td>3.58</td>
<td>210</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>3.58</td>
<td>210</td>
<td>2.2</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>4.13</td>
<td>210</td>
<td>7.8</td>
<td>1</td>
</tr>
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<td>5</td>
</tr>
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<td>4.53</td>
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<td>3</td>
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<tr>
<td>14</td>
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<td>190</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
2.2 Enzymatic hydrolysis

Enzymatic hydrolysis experiments generally followed a similar scheme. However, specific conditions such as enzyme loadings, solid consistency, type of buffer, etc. varied in different experiments and are outlined specifically in the following sections.

Water-washed pretreated solids (or unwashed solids) obtained during steam explosion were hydrolysed at various consistencies ranging from 2 to 12.5% (w/v) solid concentration. During hydrolysis, one of the following solutions was used:

- 50 mM sodium acetate buffer (pH 4.8),
- unbuffered water soluble fraction (pH adjusted to 4.8 with 0.5M NaOH),
- synthetic carbohydrate solutions, composed of sodium acetate buffer supplemented with anhydrous glucose, xylose and arabinose,
- “cocktail” of synthetic sugars at the concentrations mimicking the concentration of monomers in the water soluble stream (arabinose, galactose, glucose, xylose, and mannose) in the acetate buffer.

The hydrolysis took place at 45°C with continuous agitation (200 rpm) for a period of up to 100 hours (average duration up to 48 hours). During hydrolysis, a complete cellulase preparation (Celluclast 1.5L) obtained from the fungi, *Trichoderma reesei*, supplied commercially by Novozymes North America Incorporated (Franklinton, NC, U.S.) was used. Each 125 mL Erlenmeyer flask containing 50 mL of total liquid was also supplemented with additional β-glucosidase enzyme (Novozym-188®) originating from *Aspergillus niger*. The hydrolytic reaction mixtures were inoculated with enzymes based on the amount of filter paper units (FPU) g cellulose⁻¹ of cellulase. Novozym-188® was loaded to achieve an IU (international units) to FPU ratio of 2:1. The cellulase and β-glucosidase
preparations were assayed for total cellulase activity and β-glucosidase activity as described in section 2.5.3.1 and 2.5.3.2, respectively, with results presented in Table 3.

During hydrolysis, two antibiotics: cyclohexamide (Sigma Chemical Co., St. Louis, MO, U.S.) and tetracycline (Sigma) were added to prevent microbial contamination at concentrations of 30 μg mL⁻¹ and 40 μg mL⁻¹, respectively. There was no antibiotic supplementation in the experiments where the hydrolysate was further used in the fermentation process such as during the comparison study between simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF).

Aliquots of 0.50 mL were aseptically removed at different reaction intervals, boiled for 5 minutes to inactivate the enzymes, and then centrifuged for 5 min at 15000 × g and 4°C. The supernatant was filtered using a syringe filter 4mm (Chromatographic Specialties Inc., Brockville, ON, CAN) and then stored at -20°C until further analysis by HPLC. All hydrolysis experiments were performed at least in duplicate. In addition, during each experiment, controls were run in parallel (enzymes plus buffer, corn fibre plus buffer).

The extent or yield of hydrolysis was expressed at the percentage of the theoretical glucose content in the feedstock at the start of hydrolysis that was recovered as monomeric glucose (i.e., the glucose yield). The determination of the theoretical glucose content of the feedstock was based on Klason analysis of corn fibre solids, and assumed all available glucose was present as cellulose (section 2.5.1.1). A conversion factor was applied in the calculation of the carbohydrate content to account for the hydration of the cellulose during cleavage (Allen et al., 2001).
Table 3 Cellulase and β-glucosidase activities in the enzyme preparations used during hydrolysis experiments.

<table>
<thead>
<tr>
<th></th>
<th>Total Protein Content (mg mL⁻¹)</th>
<th>FPU Activity (FPU mL⁻¹)</th>
<th>β-glucosidase Activity (IU mL⁻¹)</th>
<th>Xylanase Activity (IU mL⁻¹)</th>
<th>Mannanase Activity (IU mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celluclast 1.5L</td>
<td>47</td>
<td>61</td>
<td>38</td>
<td>201</td>
<td>120</td>
</tr>
<tr>
<td>Novozym-188</td>
<td>45</td>
<td>4</td>
<td>397</td>
<td>87</td>
<td>86</td>
</tr>
</tbody>
</table>
2.2.1 Optimisation of hydrolysis of corn fibre (temperature, enzyme loading and pH)

This procedure was performed to determine the optimal hydrolysis conditions for the unwashed, pretreated corn fibre solids at 5 % (w/v) consistency, supplemented with the water soluble fraction. During hydrolysis, three different temperatures (37, 45 and 53°C) and 20 FPU g cellulose"1 loadings were used. Additionally, the influence of pH (pH 4.8 and 5.3) on the hydrolysis yield was tested. The pH of the mixture of water soluble and insoluble fractions was adjusted by using 0.5M NaOH.

2.2.2 High solids consistency hydrolysis

Throughout the prior work on optimisation of SO2-catalysed steam explosion of corn fibre, when the enzymatic hydrolysis of solids obtained from the 13 pretreatment conditions was tested, the corn fibre solids were water-washed with tap water (20 x volume), and a 2% (w/v) solid consistency was employed (Chapter 3.1). These hydrolysis experiments were performed in an acetate buffer at an enzyme concentration of 20 FPU g cellulose"1 for 48 hours.

Various concentrations of pretreated, water-washed corn fibre solids were used (2.5, 5, 7.5, 10, and 12 % (w/v)) to assess the influence of solid consistency on the hydrolysis profile. Additionally, the influence of hydrolysis time was also investigated. These experiments were performed in an acetate buffer at an enzyme concentration of 10 FPU g cellulose"1 and an IU: FPU ratio of 2:1, for up to 100 hours (section 3.2.2.1).

2.2.3 Hydrolysis of the pretreated corn fibre solids in the water soluble fraction

An alternative method for increasing the concentration of sugars during hydrolysis was investigated by supplementation of the water insoluble fraction with the water soluble
stream. Unwashed or water-washed corn fibre solids at 5 % (w/v) consistency were hydrolysed at 20 and 40 FPU g cellulose$^{-1}$ for up to 144 hours.

2.2.4 Hydrolysis of the pretreated corn fibre solids in the synthetic carbohydrate buffer

To analyse the effect of high sugar concentrations in the water soluble fraction during the saccharification of the combined hemicellulose and cellulose streams, the hydrolysability of the pretreated, water-washed corn fibre solids was tested. This was carried out using single synthetic sugars: glucose at 30, 40, 50 g L$^{-1}$ concentrations, xylose at 10, 30, and 40 g L$^{-1}$ concentrations, or arabinose at 10, 30, and 40 g L$^{-1}$ concentrations in the acetate buffer. In addition, a “cocktail” of synthetic sugars (arabinose, galactose, glucose, xylose and mannose) was used to achieve a final concentration of approximately 9, 3, 30, 11, and 1 g L$^{-1}$ respectively, prior to hydrolysis, to analyse the influence of non-carbohydrate inhibitors on hydrolysis yield. Control hydrolyses were performed in an acetate buffer and in the water soluble stream generated from steam explosion.

2.2.5 Hydrolysis of the water soluble fraction

Hydrolysis of the hemicellulose fraction by cellulases and xylanases was performed prior to saccharification of the cellulosic stream (both water insoluble and soluble streams). Enzymes were added in excess during hydrolysis at pH 4.8, and temperature 45°C. After 6 hours, the flasks were placed in a boiling water bath for 10 min to denature the enzymes. The mixture was then centrifuged for 10 min at 7000 $\times$ g, and the supernatant was used as liquid media for subsequent hydrolysis experiments.

2.2.6 Separate hydrolysis and fermentation (SHF), the hydrolysis stage

The effectiveness of two processes, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF), was analysed and compared in terms
of sugar to ethanol conversions. Both processes were run concurrently, at similar solid consistency and enzyme loadings. However, the time, temperature and pH differed for the two processes. The methodological procedure for SSF is described in section 2.4. The SHF required a separate saccharification, which is outlined here. Unwashed or water-washed, pretreated corn fibre solids at 5, 7.5 and 10 % (w/v) consistency were hydrolysed at 10, 20, and 40 FPU g cellulose$^{-1}$ of cellulase enzyme loadings for 48 hours in the water soluble fraction. During hydrolysis there was no antibiotic supplementation. After 48 hours, the flasks were placed in a boiling water bath for 10 min to denature the enzymes; the mixture was then centrifuged for 10 min at 7000 × g, and the supernatant was used as a liquid media for subsequent fermentation experiments.

2.3 Fermentation

2.3.1 Fermentation microorganisms

A spent sulphite liquor (SSL)-adapted strain of *Saccharomyces cerevisiae* was generously provided by Tembec Ltd. (Témiscaming, QC, CAN) and used for all fermentations. As mentioned previously, *S. cerevisiae* was only able to ferment six carbon sugars. The yeast was maintained at 4°C on solid GMYP medium containing 1% glucose, 0.5% malt extract (Difco Laboratories, Detroit, MI, U.S.), 0.3% yeast extract (Difco), 0.5% peptone (Difco) and 1.5% agarose (Difco). For each fermentation, *S. cerevisiae* was pre-grown in 500mL YP media (1% glucose, 1% yeast extract and 1% peptone) at 30°C for 3 days with constant shaking (250 rpm), then harvested after 24 hours, and resuspended in fresh YP media. After extensive washing (3 times with distilled, sterile water), the inoculum cell concentration was adjusted with sterile, distilled water to provide a final cell concentration of 2g L$^{-1}$ (oven-dried cell weight). The adjustment of the cell concentration
was done based on a standard curve correlating the inoculum's oven-dried weight (ODW) and optical density (OD, determined spectrophotometrically at 600 nm) (Figure 7). The final dry weight of the inoculum was confirmed by overnight drying of a culture sample at 105°C.

During the SSF experiments, to establish the optimum conditions for simultaneous saccharification and fermentation, *S. cerevisiae* was pre-grown in 500mL YP media at 37°C and 40°C for 3 days. A new standard curve correlating the cells' optical density and oven-dried weight (ODW) was established for 37°C and 40°C (data not shown). This was the only difference with regard to growth and harvesting the yeast for the SSF process.
Figure 7 Correlation of dry weight of yeast versus optical density at 600 nm for standard curve for *S. cerevisiae* incubated at 30°C ($r^2=0.99$).
2.3.2 Fermentation of corn fibre hydrolysate

During the optimisation of the SO₂-catalysed steam explosion of corn fibre, the water soluble fractions obtained from each of the pretreatment, post-hydrolysis and enzymatic hydrolysis steps were assessed for their efficiency during fermentation to ethanol, without employing any detoxification steps.

Fermentation of the liquid sugar fractions (water soluble, post-hydrolysed water soluble and liquid fraction obtained during enzymatic hydrolysis) was conducted in 125 mL serum bottles containing 50 mL medium pre-adjusted to pH 6.0 with 0.5 M sodium hydroxide. The serum bottles were vented using a syringe needle fit with a 0.22 μm filter. Control fermentations were run in parallel using glucose-based media. The fermentation vessels were maintained at 30°C with continuous agitation (200 rpm). Samples (0.5 mL) were withdrawn aseptically by syringe, centrifuged for 5 min at 15000 × g and 4°C and the supernatant was filtered by using a 4mm syringe filter (Chromatographic Specialties Inc., Brockville, ON, CAN) and then stored at -20°C until analysis. Sugars, ethanol, 5-HMF and furfurals were determined periodically from the aliquot culture samples during the course of the fermentation. The relative ethanol yield, \( Y_{\text{EtOH}} (Y_{\text{refEtOH}})^{-1} \) was defined as the ratio of the ethanol yield of the filtrate and the theoretical fermentation. The theoretical yield for ethanol production from glucose is 0.51 g ethanol g⁻¹ glucose (Olsson and Hahn-Hägerdal, 1996). Each experiment was run in duplicate and the range value reported.

2.3.3 Optimisation of fermentation for SSF process

As mentioned in the Introduction (section 1.9.4.1), the main disadvantage of the SSF approach is that compromise conditions have to be used with regard to the temperature and pH for the integrated hydrolysis and fermentation step. The enzymes and microorganisms
must be matched with respect to temperature, absence of the antibiotics in enzyme preparations and the restriction of proteolytic or other enzyme-inactivating activities which may be secreted by fermenting microorganisms (Grohmann, 1993). To test the feasibility of doing the saccharification at a different temperature and pH, the optimisation of hydrolysis was performed (section 2.2.1). In addition, the influence of temperature and pH on the fermentation profiles had to be tested. Thus, as part of the preliminary work, the fermentability of the water soluble fraction obtained after steam explosion of corn fibre at optimal pretreatment conditions (190°C, 5min and 3% SO2) was tested at different temperatures (30, 37 and 40°C) and pH (5, 6) during a 48 hour time course. To simulate the concentration of sugars present during the SSF, anhydrous glucose was added to achieve a final concentration of 60 g L\(^{-1}\).

2.3.4 Separate hydrolysis and fermentation (SHF), the fermentation stage

The hydrolysate obtained from 48 hours fermentation was used during the SHF process. The pH of the hydrolysate was adjusted to pH 6.0 with 0.5 M sodium hydroxide. The fermentation was run in duplicate up to \(t=48\) hours, and the range values reported. An identical fermentation procedure was conducted, as previously described (section 2.3.2).

2.4 Simultaneous saccharification and fermentation (SSF)

The SSF experiments were performed under nonsterile conditions in 125mL serum bottles, with \(S.\ cerevisiae\) as the sugar fermenting microorganism. The water insoluble fraction at 5, 7.5 and 10% (w/v) concentrations was supplemented with the water soluble streams during SSF experiments. Most of the experiments were performed with water-washed solids. However, to test the feasibility of combining the whole slurry after pretreatment, adjusting the pH to pH 5.0 and performing the SSF process, unwashed solids
were used. The fermentation vessels were maintained at 37°C with continuous agitation (200 rpm). The serum bottles were vented using a syringe needle fitted with a 0.22 μm filter. The SSF experiments were performed at enzyme concentrations of 10, 20 and 40 FPU g cellulose\(^{-1}\) and an IU: FPU ratio of 2:1, for 48 hours.

There was neither nutrient, nor antibiotic supplementation during most of the SSF experiments. The reaction vessel contained only \(S.\ \textit{cerevisiae}\) at a cell concentration of 2 g L\(^{-1}\), the enzymes, and the pretreated slurry. However, additional SSF experiments with the addition of the antibiotics streptomycin (20 mg L\(^{-1}\)) (Sigma) and penicillin (20 000 U mL\(^{-1}\)) (Sigma) were performed in triplicate and compared to the SSF profiles without antibiotic additions.

Samples (0.5 mL) were withdrawn aseptically by syringe, kept on ice until centrifugation (5 min at 15000 \(\times\) g and 4°C) and the supernatant filtered through a syringe filter 4mm (Chromatographic Specialties Inc., Brockville, ON, CAN) and then stored at -20°C until further analysis. In addition, during each experiment, controls were run in parallel (enzymes in buffer with yeast, water insoluble stream with yeast). Each experiment was run in triplicate or duplicate and the range value reported.

2.5 Analytic procedures

2.5.1 Compositional analysis of corn fibre

2.5.1.1 Analysis of solids

The chemical composition of the original starting material and the steam-explored solids were determined using a modified Klason lignin method derived from the TAPPI Standard method T222 om-88 (TAPPI, 1998b). Briefly, 0.2 g of sample (ground to pass through a 40-mesh screen) was incubated at 20°C with 3 mL of 72% \(\text{H}_2\text{SO}_4\) for 2 hours with
mixing every 10 minutes. The reaction was then diluted with 112 mL of deionized water
(final acid concentration 4% H₂SO₄) and then transferred to a serum bottle. The solution
was then subject to autoclaving at 121°C for 1 hour and, when cold, filtered through a
medium coarseness sintered glass filter for the gravimetric determination of the acid
insoluble lignin content. Klason lignin (acid insoluble lignin) was determined
gravimetrically after rinsing the solids in the crucibles with 200 mL nanopure water, and
overnight drying at 105°C. The concentration of sugars in the filtrate was measured by
HPLC and the acid soluble lignin was quantified by measuring the absorbance at 205 nm
according to the TAPPI Useful Method UM250 (TAPPI, 1998c). Each experiment was run
in triplicate.

2.5.1.2 Post-hydrolysis

Post-hydrolysis experiments were performed according to (Shevchenko et al., 2000)
Duplicate samples containing 27 mL of the water soluble fraction were post-hydrolysed
after adding concentrated sulphuric acid to achieve a final concentration of 3% acid. The
post-hydrolysis was performed by heating the solution at 121°C for 1 hour in an autoclave.
A batch of sugar standards was also autoclaved under the same conditions, to estimate any
hydrolysis loss. The sugar concentrations were quantified by HPLC.

2.5.1.3 Starch content

The analysis of starch content was performed according to Grohmann and Bothast
(1997). Twenty five milliliters of distilled water and 10mL of 2N NaOH were added to 0.5
g of corn fibre sample (ground to pass through a 40-mesh screen) and amylopectin standards
(Sigma) in serum bottles. The bottles were incubated at 90°C for 20 minutes with constant
mixing. Then 10 mL of 2N HCl were added, and the reaction mixture was cooled to 50°C.
After the addition of 10 mL of acetate buffer (pH 4.2) and 5 mL of amyloglucosidase (Sigma) to the mixture, the serum bottles were incubated for 1 hour at 40°C. Finally, 5 mL of 25% (w/v) trichloroacetic acid (TCA) was added and each hydrolysate was transferred into a 100 mL volumetric flask. The concentration of sugars in the filtrate was measured by HPLC. In parallel, blank solutions (pure enzymes-amyloglucosidase, and corn fibre without enzymes) were run. Each experiment was run in triplicate.

2.5.1.4 Ash content

The ash content of corn fibre was determined by ignition at 575°C, according to TAPPI standard T-211 (TAPPI, 1998a).

2.5.2 Analysis of the water soluble fraction

2.5.2.1 Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) was determined by HPLC analysis. The HPLC system (Dionex DX-500, Dionex Corp., Sunnyvale, CA, U.S.) was equipped with an ion exchange Carbopac PA-1 column (4 × 250 mm) equilibrated with 0.25 M NaOH and eluted with nanopure water at a flow rate of 1 mL min⁻¹ (Dionex Corp.), an ED40 electrochemical detector (gold electrode), AD20 absorbance detector and autosampler (Dionex Corp., Sunnyvale, CA, U.S.). Sodium hydroxide (0.2 M) was added post-column (for detection) at a flow rate of 0.6 mL min⁻¹. Prior to injection, samples were filtered through 0.45 mm HV filters (Millipore, MA, U.S.) and a volume of 20 μL was loaded. Analytical-grade standards: L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose (Sigma) were used to quantify the concentration of sugars. In addition, L-fucose (Sigma) was used as an internal standard.
2.5.2.2 Furfurals and HMFs

The concentration of sugar degradation products such as 5-hydroxymethylfurfural (5-HMF) and furfural were determined by HPLC by measuring the absorbance at 280 nm. For the product separation an Aminex HPX-87H column (7.8 × 300 mm) was used, and 5mM H₂SO₄-the mobile phase was run at a flow rate of 0.6 mL min⁻¹ (Bio-Rad). Standards were prepared from analytical-grade HMF (Sigma) and furfural (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.).

2.5.2.3 Ethanol

Ethanol concentrations were determined using a Hewlett-Packard 5890 gas chromatograph equipped with a 6890 autoinjector, splitless injection and flame ionization detector (Agilent Technologies, Palo Alto, CA, U.S.). Components were separated using 30m Stabilwax-DA column (inner diameter 0.53mm) supplemented with a 5m deactivated guard column (Restek Corp., Bellefonte, PA, U.S.). During analysis, the detector temperature was 250°C, injector temperature was 90°C, and helium was used as the carrier gas (flow rate 1 mL min⁻¹). The ethanol standards were prepared by using HPLC-grade absolute ethanol (Riedel-de Haën AG, Seelze, Germany). The internal standard was 1-Butanol (Fisher).

2.5.3 Enzyme assays

2.5.3.1 Cellulase activity

The measurement of total cellulase activity of the Celluclast preparation was determined by the filter paper assay as reported by Ghose (1987). The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of assay mixtures, blanks and controls and glucose standards. The substrate was a 0.05 g
Whatman No.1 filter paper strip (1.0 × 6.0 cm). Initially 0.5 mL of enzyme diluted in an acetate buffer (0.05 M, pH 4.8) was added to the test tubes containing strips of filter paper in 1mL of sodium acetate buffer and incubated for 1 hour at 50°C with continuous shaking at 150 rpm. After 1 hour, 3 mL of dinitrosalicylic acid reagent (DNS) was added to stop the reaction. To be able to measure the progress of the reaction spectrophotometrically, the test tubes were incubated in a boiling water bath for 5 min. Cellulase activity was determined spectrophotometrically at 540 nm, and corrected for enzyme and reagent blanks. The final activity was expressed in filter paper units (FPU), where 1 FPU = enzyme concentration required to liberate 2.0 mg glucose equivalents in 60 min. All the tests were performed in triplicate and range values were calculated.

2.5.3.2 β-glucosidase activity

The measurement of activity of the Novozym-188 preparation was determined colourimetrically by using p-nitrophenyl-β-D-glucoside, as described by Wood and Bhat (1988). A 200 μL aliquot of diluted enzyme was added to the mixture of 1mL of 5 mM p-nitrophenyl-β-D-glucoside (Sigma), prepared in sodium acetate buffer (50 mM, pH 4.8), and 1.8 mL of sodium acetate buffer (50 mM, pH 4.8). The mixture was vortexed vigorously and incubated at 50°C for 30 min. To stop the reaction, glycine buffer (4 mL, 0.4 M, pH 10.8) was added. The enzyme activity was monitored spectrophotometrically at 430 nm and expressed in international units (IU), where 1 IU = 1μmol p-nitrophenol liberated per minute.
2.5.3.3 Xylanase activity

The measurement of xylanase activity was determined as described by Bailey et al., (1992). A volume of 200 µL of diluted enzyme was added to 1.8 mL of 1% (w/v) birch wood xylan (Sigma). The mixture was vortexed vigorously and incubated at 50°C for 5 min, then 3.0 mL DNS was added to the test tube and mixed. After placing the reaction tube was put in a boiling water bath for 5 min, and the absorbance was measured at 540 nm. D-xylose (Sigma) was used as a standard solution for this reaction. Xylanase activity was expressed in international units, IU, where 1 IU = 1 µmol reducing sugar (xylose) liberated per minute.

2.6 Modeling

2.6.1 Response surface methodology

A response surface methodology (RSM) was used to study the effects of temperature, time and SO2 concentration on hemicellulose recovery, enzymatic digestability, and fermentability of corn fibre after steam explosion (Roquemore, 1976). A design consisted of 15 sets of experimental conditions, including the centre-point experiment, which was chosen based on the results of our previous findings (Bura et al., 2002), and was performed in triplicate (Table 2, section 2.1). In addition, to test the influence of SO2 impregnation during pretreatment, two additional steam explosion conditions were included in the study. Statistical analysis was performed by fitting Equation 2.1 to experimental response data \( Y \) by multiple linear regression, using Microsoft Excel:

\[
Y = \text{Intercept} + C_1T + C_2q + C_3S + C_4Tq + C_5TS + C_6qS + C_7T^2 + C_8q^2 + C_9S^2
\]

(Eq.2.1)
where $T=$ time (min), $q=$ temperature ($^\circ$C), $S=$ SO$_2$ (%w/w).

As a result, an empirical model for each dependant variable, in terms of residence time, temperature, and amount of SO$_2$ added, was obtained. The model contained no higher terms than quadratic to avoid modeling experimental errors. Terms which were statistically insignificant (at the 95% confidence level) were eliminated from equation (2.1) to give an empirical model for each dependant variable in terms of time, temperature and SO$_2$ concentration. The coefficient of determination, and $r^2$ are reported in Table 4 to indicate the adequacy of fit for each empirical model. Means and variances were calculated for three repetitions of the centre-point conditions.

Ideally, with unlimited resources one would use the full factorial design to find the optimum pretreatment conditions for corn fibre during SO$_2$-catalysed steam explosion. This method would provide a very good insight on the pretreatment optimization. However, with three different factors (time, temperature and SO$_2$ concentration) and five different levels there would be a very large number of experiments to perform (243 different steam explosion experiments). Therefore, based on quadratic response surface, only 15 different experimental conditions were used during optimization of SO$_2$-catalysed steam explosion of corn fibre (Roquemore, 1976). The similar number of experiments have been used by other researchers during optimization of SO$_2$-catalysed steam explosion of softwoods (Boussaid et al., 2000; Stenberg et al., 1998) and hardwoods (Clark et al., 1989; Clark and Mackie, 1987).
Table 4 Fitted coefficients in Equation 2.1 for empirical models and the corresponding r² values.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Monomer sugar recovery in water soluble fraction</th>
<th>Sugar hydrolysability</th>
<th>Water soluble fraction fermentability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>26.47</td>
<td>80.47</td>
<td>94.33</td>
</tr>
<tr>
<td>C₁</td>
<td>2.88</td>
<td>1.59</td>
<td>0</td>
</tr>
<tr>
<td>C₂</td>
<td>5.06</td>
<td>1.65</td>
<td>-2.38</td>
</tr>
<tr>
<td>C₃</td>
<td>2.51</td>
<td>2.91</td>
<td>0</td>
</tr>
<tr>
<td>C₄</td>
<td>-1.55</td>
<td>-3.61</td>
<td>0</td>
</tr>
<tr>
<td>C₅</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>C₆</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₇</td>
<td>0</td>
<td>0</td>
<td>-2.56</td>
</tr>
<tr>
<td>C₈</td>
<td>-3.03</td>
<td>-6.79</td>
<td>-5.83</td>
</tr>
<tr>
<td>C₉</td>
<td>0</td>
<td>-1.41</td>
<td>-2.94</td>
</tr>
<tr>
<td>r²</td>
<td>0.99</td>
<td>0.99</td>
<td>0.97</td>
</tr>
</tbody>
</table>
2.6.2 Techno-economic modeling

A flexible “STEAM” techno-economic model has been developed that can effectively model a “generic” hardwood/softwood-to-ethanol process. A detailed description of the “STEAM” techno-economic model has been published previously (Gregg, 1996; Gregg et al., 1998; Gregg and Saddler, 1995). General description of the model is presented in Appendix 4.

To assess the cost of implementing different process options during corn fibre to ethanol conversion (SHF versus SSF), and compare it with softwood to ethanol conversion, three model runs were undertaken. The more important process and economic assumptions for these cases are shown in Table 15—Appendix 1). Operating and capital cost have not been updated in the model and consequently the model results have been determined as the relative percentage contribution of each subprocess to the total process ethanol selling price.

For the softwood model, the cellulose hydrolysis was carried out in fed-batch reactor, at 45°C, and pH 4.8 at 2% (w/v) solids consistency at 20 FPU g cellulose$^{-1}$. In the case of bioconversion of corn fibre to ethanol, the hydrolysis was performed in the water soluble fraction, at 8% consistency and 10 FPU g cellulose$^{-1}$. For SSF, the combined SSF time of 28 hours and final glucose to ethanol conversion are shown. The detailed experimental data used in the corn to ethanol model are based on results from section 3.3.
3.0 RESULTS AND DISCUSSION

3.1 Optimisation of \(SO_2\)-catalysed steam explosion

3.1.1 Background

Pretreatment of lignocellulosic biomass is crucial if effective enzymatic hydrolysis is to be achieved. Ideally, pretreatment of biomass residues should lead to optimal recovery of the hemicellulose-derived sugars in a monomeric form and the production of an easily-hydrolysable water insoluble cellulosic residue. While several pretreatment options for corn fibre have been evaluated for their efficacy and high yield in fractionating, solubilising, hydrolysing and separating the major compositional constituents (Allen et al., 2001; Grohmann and Bothast, 1997; Moniruzzaman et al., 1997), as yet, no single process has been proven to be superior. Therefore, in an attempt to establish and optimise the bioconversion of corn fibre to ethanol, an alternate pretreatment method, \(SO_2\)-catalysed steam explosion, was evaluated in the first phase of this thesis.

In order to assess the technical feasibility of achieving good carbohydrate recovery and ethanol conversion after steam explosion of corn fibre, the optimum pretreatment conditions were initially established. The main challenge during the design of optimal pretreatment conditions for a particular feedstock is balancing the trade-offs. For example, it is clear that very severe conditions can result in good enzymatic hydrolysis of the pretreated substrate (Brownell, 1987; Brownell and Saddler, 1984). However, severe pretreatment conditions also promote hemicellulose and cellulose degradation and the formation of decomposition products that can be toxic, and thus inhibit fermentation. In contrast, mild conditions produce a cellulose fraction that is relatively resistant to enzymatic hydrolysis. Therefore, compromise conditions must be defined in the pretreatment of corn fibre.
fibre to recover the maximum amount of sugars in a digestible and fermentable form, while concurrently producing a minimum amount of soluble sugar degradation products.

Previous work has shown that SO₂-catalysed steam explosion can successfully pretreat softwood (Boussaid et al., 2000; Clark et al., 1989; Clark and Mackie, 1987); and hardwood residues (Mackie et al., 1985; Schwald, 1988) during the bioconversion process. However, due to their chemical characteristics (high guaiacyl lignin content), softwood residues have proven to be more recalcitrant toward enzymatic hydrolysis when using the optimum pretreatment conditions, allowing for maximum hemicellulose and cellulose recovery in a fermentable form (Boussaid et al., 2000; Robinson, 2003; Yang et al., 2002). Therefore, an additional delignification process was required in the bioconversion of softwood to ethanol prior to enzymatic hydrolysis, and consequently increasing the overall cost of the process.

3.1.1.2 Introduction to results

We have hypothesized that SO₂-catalysed steam explosion can effectively pretreat corn fibre (high carbohydrate and low lignin content) and, by using relatively mild pretreatment conditions, achieve very good overall sugar recovery and effective solids hydrolysis without prior lignin removal. To optimise SO₂-catalysed steam explosion of corn fibre, a response surface methodology (RSM) was employed to study the effects of time, temperature and SO₂ concentration on hemicellulose recovery, enzymatic digestibility, and the fermentability of the corn fibre hydrolysate after pretreatment. A design, consisting of 15 sets of experimental conditions, including the centre-point experiment, was chosen (Table 2, section 2.1). The conditions for the centre-point experiment were designed based
on a preliminary study, the results of which are presented in Appendix 2 (Figure 33) (Bura et al., 2002).

Following impregnation with gaseous SO₂, corn fibre was steam exploded in a batch reactor at 13 different conditions, with temperatures ranging from 150-230°C, time varying from 1-9 minutes and SO₂-concentration changing from 0-6% (Table 2, section 2.1). As previously noted, one of the requirements for an ideal pretreatment condition is maximum recovery of the hemicellulose stream in monomeric form, while producing a minimum amount of inhibitors. Therefore, to optimise the SO₂-catalysed steam explosion operation, first the concentration of water soluble monomeric, oligomeric sugars and inhibitors were measured at all tested conditions (Figure 8). Once the conditions for allowing maximum recovery of hemicellulose sugars (while minimizing sugar decomposition products) were established, it was assessed whether the resulting water insoluble cellulosic fraction was readily hydrolysed. The water-washed insoluble fractions were enzymatically hydrolysed to determine their enzymatic digestibility with the use of cellulolytic enzymes at low enzyme loadings and substrate concentrations (2% (w/v)).

As previously mentioned, one of the problems associated with other types of pretreatments tested during the bioconversion of corn fibre to ethanol was the production of inhibitors affecting sugar fermentation. Consequently, the water soluble fractions obtained from each of the pretreatment, post-treatment and enzymatic hydrolysis steps were assessed for their efficiency in fermentation to ethanol by yeast (Saccharomyces cerevisiae) (Figure 8).

A statistical analysis of data based on a second-order surface-response design was employed to test the influence of pretreatment parameters on hemicellulose and cellulose
recovery, digestability and fermentability. For each parameter tested (shot yield, monomeric, oligomeric sugar recovery in the water soluble stream, hydrolysability of solids and the fermentability of water soluble, post-treated water soluble and saccharified water soluble streams) the statistical significance of the pretreatment conditions (temperature, time and SO₂ concentrations) were analysed. Terms that were statistically insignificant (at the 95% confidence level) were eliminated from Equation 2.1 (section 2.6.1). Only the values of total monomer and oligomer sugar yield, percentage conversion of cellulose to glucose during enzymatic hydrolysis and sugar to ethanol conversion were fit to a linear model using multiple linear regression. The compromise, the optimum pretreatment conditions with regard to hemicellulose recovery and enzymatic digestibility and sugar fermentability were chosen, based on the surface response model.

The final section of this chapter includes the mass-balance analysis of the "compromised" pretreatment conditions in terms of carbohydrate recovery and the main contributors to carbohydrate loss throughout the pretreatment, fractionation, water-wash, hydrolysis and fermentation are described. The predictability of the severity factor with regard to hemicellulose recovery and fermentability, solids digestability, and production of inhibitors is also discussed.
Figure 8 Experimental flow diagram describing the SO$_2$-catalysed steam explosion of corn fibre and consequent post-treatment, fermentation, and enzymatic hydrolysis.
3.1.2 Results and discussion

3.1.2.1 Feedstock composition

The chemical composition of the original untreated corn fibre was initially determined. For the purpose of this study, carbohydrates (from starch, hemicellulose and cellulose), Klason lignin and ash components were measured. The total polysaccharide content proved to be very high (~76%), as was previously observed by other investigators (Grohmann and Bothast, 1997). This high carbohydrate content should make this agricultural residue an attractive material for saccharification and fermentation processes (Table 5). The remaining components of the original corn fibre feedstock which were not quantified during these initial analyses include protein (~11% weight) and crude fat (2.5% weight) (Grohmann and Bothast, 1997).

Glucose (~46%), followed by xylose (~17%) and arabinose (10%) were the most abundant components of the corn fibre, as determined by secondary acid hydrolysis of constituent polysaccharides (Table 5). The compositional analysis of corn fibre can vary considerably, according to the source of the material and analytic methods used. In particular, adherent starch levels fluctuate by production facility and on a day-to-day basis. In this study, the concentration of glucose was reported as total monomeric concentration obtained from cellulose (25.6%) and starch (20%) (Table 5) and is comparable with previous analyses (Grohmann and Bothast, 1997; Leathers, 2003).

It has been shown that arabinoglucuronoxylan is the major hemicellulose found in corn fibre (corn fibre gum) with branches containing xylose, arabinose, galactose, and glucuronic acid units in descending order of abundance (Montgomery and Smith, 1957; Whistler and BeMiller, 1956). Agricultural residues generally contain a considerably lower
Table 5 Composition of corn fibre (carbohydrates, lignin and inorganic) (% weight).

<table>
<thead>
<tr>
<th>Ara</th>
<th>Gal</th>
<th>Glu (total)*</th>
<th>Xyl</th>
<th>Man</th>
<th>Klason lignin</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.3</td>
<td>2.2</td>
<td>45.6</td>
<td>17.1</td>
<td>0.7</td>
<td>8.7</td>
<td>0.7</td>
</tr>
<tr>
<td>(0.3)</td>
<td>(0.2)</td>
<td>(1.1)</td>
<td>(0.6)</td>
<td>(0)</td>
<td>(0.2)</td>
<td>(0.0)</td>
</tr>
</tbody>
</table>

* total glucose (from cellulose 25.6% and starch 20%)

Values in parentheses are standard deviation, where n=9
lignin content compared to softwood and hardwood residues. In this study, the lignin (Klason lignin) content was determined to be ~9%, which concurs with previous findings using similar quantification techniques (Grohmann and Bothast, 1997).

3.1.2.2 Solids recovery (shot yield)

As mentioned previously, it is important that pretreatment conditions are defined which ensure good hydrolysis of the water insoluble cellulose stream, but are not so severe that the overall yield of recovered carbohydrate is unacceptably low. When a range of conditions for pretreating corn fibre was assessed it was apparent that the severity had a substantial effect on total recovery of solids after steam explosion, with the shot yield ranging from 74 to 97% (Figure 9). Clearly, increased pretreatment severity reduced the solids recovery of the original corn fibre. The best recovery of solids was obtained when the corn fibre was treated at lower temperatures (150-170°C) with Log R_o=2.06-3.02, while lowest shot yield was obtained at 235°C (Log R_o=4.53). At the same temperatures (170°C and 210°C), longer reaction times led to lower shot yields (Table 6). It was apparent that more severe steam explosion conditions significantly decreased the shot yield for the recovery of solid material. It is probable that the reduction in the amount of fibrous material with increasing severity could be attributed to the solubilisation of the hemicellulose, as similar results have been reported during comparison of liquid hot water and steam pretreatments of sugar cane bagasse (Laser et al., 2002), SO₂-catalysed steam explosion of Pinus radiata (Clark and Mackie, 1987) and mixtures of Picea abies and Pinus silvestris (Stenberg et al., 1998). In addition, at the most severe conditions (Log R_o 4.13 and 4.53) some of the cellulose was also solubilised. The insoluble corn fibre content for the centre-point experiment (190°C, 5 min, 3% SO₂) varied between 91 and 86% with an average value
of 88%. A very low standard deviation (less than 3%), indicated good reproducibility of the steam explosion experiments (Table 6). It was apparent that there were no statistical correlations between time, temperature, and SO$_2$-concentration and shot yield values (most of the coefficient tested had p-values higher than 0.05).
Figure 9 Shot yields expressed as a percentage of the original oven dried corn fibre after steam explosion as function of severity (Log $R_o$).
Table 6 Conditions for SO$_2$-catalysed steam pretreatment of corn fibre and shot yield expressed as a percentage of the original oven dried corn fibre after steam explosion.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Severity of pretreatment (Log R$_0$)</th>
<th>Temperature ($^\circ$C)</th>
<th>Time (min)</th>
<th>SO$_2$ (%)</th>
<th>Shot yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.06</td>
<td>170</td>
<td>1</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>2.17</td>
<td>150</td>
<td>5</td>
<td>3</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td>2.76</td>
<td>170</td>
<td>5</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>4</td>
<td>2.76</td>
<td>170</td>
<td>5</td>
<td>6</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>3.02</td>
<td>170</td>
<td>9</td>
<td>3</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>3</td>
<td>87*</td>
</tr>
<tr>
<td>7</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>3</td>
<td>91*</td>
</tr>
<tr>
<td>8</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>3</td>
<td>86*</td>
</tr>
<tr>
<td>9</td>
<td>3.58</td>
<td>210</td>
<td>2.2</td>
<td>1</td>
<td>89</td>
</tr>
<tr>
<td>10</td>
<td>3.58</td>
<td>210</td>
<td>2.2</td>
<td>5</td>
<td>86</td>
</tr>
<tr>
<td>11</td>
<td>4.13</td>
<td>210</td>
<td>7.8</td>
<td>1</td>
<td>79</td>
</tr>
<tr>
<td>12</td>
<td>4.13</td>
<td>210</td>
<td>7.8</td>
<td>5</td>
<td>81</td>
</tr>
<tr>
<td>13</td>
<td>4.53</td>
<td>230</td>
<td>5</td>
<td>3</td>
<td>74</td>
</tr>
<tr>
<td>14</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>15</td>
<td>3.35</td>
<td>190</td>
<td>5</td>
<td>6</td>
<td>86</td>
</tr>
</tbody>
</table>

* average shot yield-88 %, standard deviation-3%
3.1.2.3 Monomer sugar yield

Once the influence of pretreatment severity on solids recovery was tested, next the nature and concentration of sugars in the water soluble, hemicellulose-rich fraction has been assessed. As shown in Figure 8, after pretreatment, the hydrolysate was fractionated by centrifugation into solid and liquid fractions. When analysing Table 7, it was apparent that following steam explosion, the highest percentage of all monomeric hemicellulose-derived sugars was recovered at temperatures ranging from 170-210°C.

As the pretreatment severity was increased, the concentration of monomers in the water soluble fraction increased up to Log $R_0$ equal to 3.35 (Table 7). At each of the pretreatment temperatures used (170, 190, and 210°C), increased pretreatment times and SO$_2$ concentrations yielded higher recovery of monomeric hemicellulose-derived sugars. According to the surface response modeling, temperature, time and SO$_2$-concentration had an effect on the hemicellulose monomeric sugar recovery. According to the model, maximum recoveries of the hemicellulose-derived monomers (34%) were obtained at temperatures between 190°C and 220°C and pretreatment times of 6-7 min (Figure 10 A).
Table 7 Yield of hemicellulose derived sugars (excluding glucose) in the water soluble fraction after steam explosion (monomers) and steam explosion and post-hydrolysis (oligomers and total sugars), expressed as g per 100g of hemicellulose in the original corn fibre.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of pretreatment (Log $R_0$)</th>
<th>Monomers (%)</th>
<th>Oligomers (%)</th>
<th>Total sugars (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>170°C, 1 min, 3%</td>
<td>2.06</td>
<td>6.1</td>
<td>10.4</td>
<td>16.5</td>
</tr>
<tr>
<td>150°C, 5 min, 3%</td>
<td>2.17</td>
<td>5.7</td>
<td>7.1</td>
<td>12.8</td>
</tr>
<tr>
<td>170°C, 5 min, 0%</td>
<td>2.76</td>
<td>10.9</td>
<td>18.6</td>
<td>29.5</td>
</tr>
<tr>
<td>170°C, 5 min, 6%</td>
<td>2.76</td>
<td>18.5</td>
<td>24.5</td>
<td>43.0</td>
</tr>
<tr>
<td>170°C, 9 min, 3%</td>
<td>3.02</td>
<td>23.8</td>
<td>31.0</td>
<td>54.8</td>
</tr>
<tr>
<td>190°C, 5 min, 3%</td>
<td>3.35</td>
<td>26.5 (1.0)</td>
<td>23.8 (1.6)</td>
<td>50.3 (0.8)</td>
</tr>
<tr>
<td>210°C, 2.2 min, 1%</td>
<td>3.58</td>
<td>21.5</td>
<td>19.2</td>
<td>40.8</td>
</tr>
<tr>
<td>210°C, 2.2 min, 5%</td>
<td>3.58</td>
<td>27.6</td>
<td>6.3</td>
<td>33.9</td>
</tr>
<tr>
<td>210°C, 7.8 min, 1%</td>
<td>4.13</td>
<td>22.5</td>
<td>12.5</td>
<td>35.0</td>
</tr>
<tr>
<td>210°C, 7.8 min, 5%</td>
<td>4.13</td>
<td>24.1</td>
<td>5.9</td>
<td>30.0</td>
</tr>
<tr>
<td>230°C, 5 min, 3%</td>
<td>4.53</td>
<td>23.0</td>
<td>4.1</td>
<td>27.1</td>
</tr>
<tr>
<td>190°C, 5 min, 0%</td>
<td>3.35</td>
<td>15.4</td>
<td>30.6</td>
<td>46.0</td>
</tr>
<tr>
<td>190°C, 5 min, 6%</td>
<td>3.35</td>
<td>27.8</td>
<td>19.2</td>
<td>46.9</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviation, where $n=9$. 
Figure 10 Response surface graph for monomeric hemicellulose recovery in the water soluble fraction as a function of (A) temperature (°C) and time (min), and (B) temperature (°C) and SO₂ concentration (% w/w).
Although temperature and time appeared to be the main factors influencing recovery, the interaction between temperature and SO2 concentration also appeared to influence the hemicellulose recovery. Maximum recovery of the hemicellulose-derived monomers (36%) was obtained at temperatures between 190 and 220°C and at SO2 concentrations between 4 and 5% (Figure 10 B). In addition, increasing the SO2 concentration also increased monomer sugar yield, however it reached a plateau at roughly 3% (Table 7). The impregnation of additional SO2 prior to pretreatment did not seem to significantly improve monomer sugar recovery, although it did result in a decrease in the concentration of total sugars. Similar findings were previously reported by Clark and Mackie (1987), where the effect of SO2 loading on total sugar recovery of steam exploded Pinus radiata was significant up to approximately 3% (w/w) dry matter. In related work, during the comparison of different pretreatment method of sugarcane bagasse, Martin et al., (2002) observed that the addition of SO2 increased the yield of hemicellulose compared with the steam pretreatment without impregnation. These findings imply that the observed decrease in water soluble oligomer recovery with increased SO2 concentration results from the lower pH, which facilitates the complete depolymerization of oligomeric hemicellulose.

3.1.2.4 Oligomer and total sugar yield

It was apparent that post-hydrolysis of the recovered water soluble fractions using 3% sulphuric acid (120°C, 1 hour) effectively depolymerized the solubilised oligomeric hemicellulose-derived sugars. The percentage of oligomers present in the water soluble fraction ranged from 4 to 31% of the total soluble sugars (Table 7). The influence of time and SO2 concentration had different effects on oligomer and total sugar recovery, depending on the pretreatment temperature. At the lower temperatures (170°C), longer times and
higher SO2 concentrations led to better oligomer and total sugar recoveries. Conversely, at higher temperatures (210°C), longer times and higher SO2 concentrations led to decreased oligomer and total sugar recoveries. Similar effects have been reported by San Martin et al., (1995) where the water soluble fraction obtained from steam-exploded Pinus radiata at 220°C and 30 sec contained 50% oligomers, while samples steam exploded at the same temperature for 2 min contained only 15% oligomers. Although the ratio of monomers to oligomers depends on the severity of pretreatment, there was no statistically significant correlation between time, temperature and SO2 concentration with regard to oligomer recovery. However, there was an interaction between temperature and time in terms of total sugar recoveries, which was illustrated in the surface response contour (Figure 11).

Maximum recoveries of total-hemicellulose sugars (64%) were obtained at temperatures ranging from 160 to 190°C and times from 6-7 min. Previous research has indicated that hemicellulose-derived sugars are less amenable to inhibitory byproduct generation at temperatures lower than or near 200°C for softwood and hardwood residues (Clark and Mackie, 1987; Excoffier et al., 1991). Low concentrations of monomeric hemicellulose-derived sugars and high concentrations of oligomers have also been observed during the pretreatment of corn fibre by hot water and steam fractionation (Allen et al., 2001) or by ammonia fibre explosion system (Hespell et al., 1997; Moniruzzaman et al., 1997). It is likely that the production of oligosaccharides and consequently the incomplete hydrolysis of xylans during pretreatment are partially due to their structure. As observed by Hespell et al., (1997), during enzymatic hydrolysis of AFEX-treated corn fibre, starch and cellulose were converted solely to glucose. However, oligosaccharides represented 30-40% of the xylan degradation products. In the case of hot water and steam fractionation, the
**Figure 11** Response surface graph for total hemicellulose recovery in the water soluble fraction as a function of temperature (°C) and time (min).
majority of the solubilised pentosans liberated after both pretreatments were oligomers (Allen et al., 2001). Corn fibre xylan is poorly degraded and structural analyses suggest that over 70% of the xylose backbone residues have one or more arabinose, 4-O-methylglucuronic acid, or other sidechains. As a result, there are few unsubstituted regions in corn fibre xylan (Figure 4) (Montgomery and Smith, 1957; Saulnier et al., 1995).

Apart from substitution of the xylose backbone with arabinose, the presence of diferulic bridges and structural wall proteins cross-link the sugars, creating a network in which cellulosic microfibrils are embedded, thus forming an insoluble and hard-to-hydrolyse network (Saulnier and Thibault, 1999).

3.1.2.5 Sugar degradation products

One of the characteristics of a good pretreatment method is to ensure the minimum formation of sugar degradation products and in so doing, to recover a maximum amount of sugars in a fermentable form. Therefore, as can be seen from Figure 8, the water soluble fraction was analysed for its concentration of 5-hydroxymethyl furfural (5-HMF) and furfural at all pretreatment conditions employed, and reported as a percentage of the original sugar. It is not surprising that as the severity of the pretreatment increased, the concentration of 5-HMF and furfural increased concurrently (Figure 12). However, the values are generally low, and ranges were observed from 0.01 to 1.73%, and 0.02 to 1.93%, for 5-HMF and furfural respectively (Figure 12). A comparable amount of sugar degradation products was reported by Allen et al., (2001) during evaluation of hot liquid water pretreatment and steam fractionations of corn fibre (0.7-1.5% of furfurals and 0.1-0.5% HMFs).
**Figure 12** Concentration of 5-HMF and furfural in water soluble fraction (original sugar %) as a function of pretreatment severity.
The relative concentration of furfural was typically higher compared to 5-HMF (Figure 12). This can be explained by the chemical structure of corn fibre and the origin of the inhibitors. During pretreatment, six-carbon sugars decompose under first-order reactions to yield furan-type compounds (e.g., 5-hydroxymethylfurfurals, 5-methylfurfurals), which can be further degraded to acids (e.g., formic and levulinic acids), while five-carbon sugars predominantly yield 2-furfurals and condensation products (Clark and Mackie, 1984; Ulbricht et al., 1984). Corn fibre hemicellulose, which is largely composed of xylose and arabinose (pentoses), is more susceptible to hydrolysis when more severe pretreatment conditions are applied (increased pretreatment temperature and time). Since hemicellulose derived sugars, such as xylose and arabinose, are formed at an early stage, they are thus exposed to the dehydrating agents for a longer time, and as a result, furfurals (pentose sugar degradation products) are formed in larger quantities compared to 5-HMF.

As previously mentioned, sulphur dioxide catalysis caused a higher conversion of hemicellulose during pretreatment conditions of 190°C, 5 min, and 3% SO₂ compared with the treatment when corn fibre was not impregnated with SO₂ (190°C, 5 min, and 0% SO₂) (Table 7). In addition, the lower concentration of sugar degradation products at this condition (0.24 and 1.17% for 5-HMF and furfural, respectively) compared with pretreatment where SO₂ was not used (0.26 and 1.25% for 5-HMF and furfural, respectively), might suggest that the presence of catalyst reduced the amount of inhibitors (Table 8). Furthermore, it might imply that the pretreatment severity (190°C, 5 min, and 3% SO₂) is optimal in terms of time and temperature, and the pH final adjustment may enhance overall sugar recovery.
On the other hand, when Martin et al. (2002) evaluated methods for the pretreatment of sugarcane bagasse, the concentrations of furfural and HMF in the water soluble stream for non-impregnated and SO₂-impregnated biomass were the same: 1.6% and 0.7% respectively, which might suggest that conditions for this pretreatment were too severe in terms of other steam explosion parameters (temperature and time). In the same study, when H₂SO₄ was used as the impregnating agent, the concentration of sugar degradation products increased threefold. It has been previously shown that the catalysing effect of sulphur dioxide was weaker than that of sulphuric acid on the release of monomeric sugars (Söderström et al., 2002; Söderström et al., 2003). For example when sulphuric acid was used, the amount of sugar degradation products was greater during dilute acid hydrolysis, which may explain why during dilute acid hydrolysis of corn fibre pretreated at 140-160°C for 10-30 minutes, significant sugar degradation products formation occurred, which inhibited the fermentation process (Grohmann and Bothast, 1997).
Table 8 Concentration of 5-HMF and furfural in water soluble fraction (original sugar %).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of pretreatment (Log $R_0$)</th>
<th>5-HMF (%)</th>
<th>Furfural (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>170°C, 1 min, 3%</td>
<td>2.06</td>
<td>0.01</td>
<td>0.57</td>
</tr>
<tr>
<td>150 °C, 5 min, 3%</td>
<td>2.17</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>170°C, 5 min, 0%</td>
<td>2.76</td>
<td>0.03</td>
<td>0.34</td>
</tr>
<tr>
<td>170°C, 5 min, 6%</td>
<td>2.76</td>
<td>0.07</td>
<td>0.87</td>
</tr>
<tr>
<td>170°C, 9 min, 3%</td>
<td>3.02</td>
<td>0.12</td>
<td>1.10</td>
</tr>
<tr>
<td>190°C, 5 min, 3%</td>
<td>3.35</td>
<td>0.24 (0.01)</td>
<td>1.17 (0.07)</td>
</tr>
<tr>
<td>210°C, 2.2 min, 1%</td>
<td>3.58</td>
<td>0.29</td>
<td>0.97</td>
</tr>
<tr>
<td>210°C, 2.2 min, 5%</td>
<td>3.58</td>
<td>0.45</td>
<td>1.03</td>
</tr>
<tr>
<td>210°C, 7.8 min, 1%</td>
<td>4.13</td>
<td>1.07</td>
<td>1.05</td>
</tr>
<tr>
<td>210°C, 7.8 min, 5%</td>
<td>4.13</td>
<td>1.47</td>
<td>1.38</td>
</tr>
<tr>
<td>230°C, 5 min, 3%</td>
<td>4.53</td>
<td>1.73</td>
<td>1.93</td>
</tr>
<tr>
<td>190°C, 5 min, 0%</td>
<td>3.35</td>
<td>0.26</td>
<td>1.25</td>
</tr>
<tr>
<td>190°C, 5 min, 6%</td>
<td>3.35</td>
<td>0.26</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviation, where $n=9$
3.1.2.6 **Enzymatic hydrolysis**

Once we established the conditions which would allow maximal recovery of the hemicellulose sugars, while minimizing sugar decomposition products, we next had to assess whether the resulting water insoluble cellulosic fraction was readily hydrolysable. The recovered pretreated water-washed corn fibre solids (2% consistency) were subjected to enzymatic hydrolysis for 72 hours with cellulases supplemented with an excess of β-glucosidase (Figure 8). The results indicated that the hydrolysability of the solids improved as pretreatment conditions became more severe, reaching a maximum at severity of 3.35 (190°C, 5 min and 3% SO₂) (Figure 13).

Evidently, pretreatment at lower severities (Log \( R_0 = 2.06-3.35 \)) increased the hydrolysability of the corn fibre insoluble fraction (Figure 13). On the other hand, at higher severities (Log \( R_0 = 3.58-4.53 \)) a considerable decrease in total sugar yield was observed (Figure 13) due to the loss of hemicellulose sugars and partial solubilisation of cellulose. As mentioned earlier, the success of enzymatic hydrolysis depends on the accessibility of cellulose to enzymes. It is hypothesized that more severe pretreatments result in material that is more accessible to enzymes, however, if the biomass is treated under very severe conditions, much of the cellulose will be released during pretreatment, and concurrently, formation of sugar degradation products will be observed. In this study, the medium severity set of conditions (190°C, 5 min and 3% SO₂) appeared to be a good compromise between extremes, because it resulted in a considerable improvement in the hydrolysability of solids without significant carbohydrate degradation, and thus the highest overall monomeric sugar yield. Similar trends have been previously observed with softwood residues, where optimal enzymatic hydrolysis of the cellulosic component occurred at higher
Figure 13 The percentage of monomeric sugars in the water soluble fraction of steam exploded corn fibre and water insoluble stream following enzymatic hydrolysis, as a function of pretreatment severity.
pretreatment severities (Boussaid et al., 2000; Robinson, 2003; Stenberg et al., 1998).

According to the model, the optimum in yield of fermentable sugars after pretreatment and enzymatic hydrolysis was obtained at 170-200°C and 4-7 min (Figure 14 A). It was also apparent that SO$_2$ concentrations influenced the recovery of monomeric sugars after pretreatment and hydrolysis. Consequently, maximum recovery of monomeric sugars (85%) was obtained at temperatures between 185 and 205°C and SO$_2$ concentrations between 3 and 5% (Figure 14 B).

Steam explosion pretreatment, followed by enzymatic hydrolysis of corn fibre showed an 81% conversion of all original polysaccharides to monomeric sugars in the current study, which is comparable to the work of Grohmann and Bothast (1997), who successfully converted 85% of the total available carbohydrate using dilute acid treatment (H$_2$SO$_4$) in combination with enzyme hydrolysis. The lower yield in the current study is due to higher concentrations of oligomers (xylose) present in the water soluble fraction after steam explosion. One of the advantages of using H$_2$SO$_4$ as a catalyst during pretreatment is a greater hydrolysability of the biomass. However, the significantly higher concentration of inhibitors may make this option less attractive.

In addition, the influence of SO$_2$ impregnation on monomer recovery at a given temperature was investigated at 190°C, 5 min and SO$_2$ concentrations of 0, 3 and 6%. Total sugar recoveries of 77, 81 and 74% were observed, respectively (Table 9). Similar to previous findings by Clark et al., (1989) using softwood, the enzymatic digestibility of the water insoluble corn fibre and monomer sugar yield were substantially improved over the situation where no SO$_2$ impregnation was employed. However, above 3% SO$_2$ concentration, there were no further observable improvements. Although the value of using
SO₂ in the steam explosion pretreatment of corn fibre was evident, the mode of action of SO₂ is less clear. It is probable that it may act simply as an acid catalyst, lowering the pH of the treatment and so preventing degradation of carbohydrates (Weil et al., 2002). Due to the lower pH, SO₂ also enhances the hydrolysis and solubilisation of the hemicellulose and cellulose fractions, thus the balance between carbohydrate hydrolysis and degradation is pushed in favor of the hydrolytic reaction.
Figure 14 Response surface graph for monomeric sugar recovery after pretreatment and enzymatic hydrolysis as a function of (A) temperature (°C) and time (min), and (B) temperature (°C) and SO₂ concentration (% w/w).
Table 9 Percentage of monomeric sugars in water soluble fraction of steam exploded corn fibre following different pretreatment conditions, and water insoluble stream following enzymatic hydrolysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of pretreatment (Log Ro)</th>
<th>Monomers-water soluble fraction (%)</th>
<th>Monomers after enzyme hydrolysis-water insoluble fraction (%)</th>
<th>Total monomers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>170°C, 1 min, 3%</td>
<td>2.06</td>
<td>2.3</td>
<td>57.4</td>
<td>59.7</td>
</tr>
<tr>
<td>150 °C, 5 min, 3%</td>
<td>2.17</td>
<td>2.1</td>
<td>46.8</td>
<td>48.9</td>
</tr>
<tr>
<td>170°C, 5 min, 0%</td>
<td>2.76</td>
<td>4.0</td>
<td>57.5</td>
<td>61.5</td>
</tr>
<tr>
<td>170°C, 5 min, 6%</td>
<td>2.76</td>
<td>7.0</td>
<td>65.5</td>
<td>72.5</td>
</tr>
<tr>
<td>170°C, 9 min, 3%</td>
<td>3.02</td>
<td>9.4</td>
<td>71.1</td>
<td>80.5</td>
</tr>
<tr>
<td>190°C, 5 min, 3%</td>
<td>3.35</td>
<td>11.4 (0.5)</td>
<td>69.1 (2.1)</td>
<td>80.5 (2.6)</td>
</tr>
<tr>
<td>210°C, 2.2 min, 1%</td>
<td>3.58</td>
<td>9.3</td>
<td>62.9</td>
<td>72.2</td>
</tr>
<tr>
<td>210°C, 2.2 min, 5%</td>
<td>3.58</td>
<td>13.6</td>
<td>61.2</td>
<td>74.8</td>
</tr>
<tr>
<td>210°C, 7.8 min, 1%</td>
<td>4.13</td>
<td>13.3</td>
<td>47.1</td>
<td>60.4</td>
</tr>
<tr>
<td>210°C, 7.8 min, 5%</td>
<td>4.13</td>
<td>22.2</td>
<td>53.0</td>
<td>75.2</td>
</tr>
<tr>
<td>230°C, 5 min, 3%</td>
<td>4.53</td>
<td>16.2</td>
<td>41.6</td>
<td>57.8</td>
</tr>
<tr>
<td>190°C, 5 min, 0%</td>
<td>3.35</td>
<td>5.9</td>
<td>70.7</td>
<td>76.6</td>
</tr>
<tr>
<td>190°C, 5 min, 6%</td>
<td>3.35</td>
<td>12.8</td>
<td>61.4</td>
<td>74.2</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviation, where n=9
3.1.2.7 Fermentation of corn fibre hydrolysate

As mentioned earlier, the overall ethanol yield depends not only on the sugar yield, but also on the fermentability of the liberated sugars, which reflects the concentration of various soluble substances released from corn fibre and formed in the pretreatment step. The concentration of inhibitors in the fermentation step depends on the configuration of the preceding steps. For example, one of the major problems associated with dilute acid hydrolysis of corn fibre and other types of lignocellulosic biomass is the poor fermentability of the hydrolysates produced (Grohmann and Bothast, 1997; Olsson and Hahn-Hägerdal, 1996; Tengborg et al., 1998). Therefore, to test the feasibility of using SO2-catalysed steam explosion as the pretreatment method for a technically and economically feasible corn fibre to ethanol process, the water soluble fractions obtained from each of the pretreatment, post-treatment and enzymatic hydrolysis steps were next assessed for their efficiency of fermentation to ethanol, without employing any detoxification steps (Figure 8). The ethanol yield in the fermentation of sugars present in the liquid fractions obtained after pretreatment, post-treatment and enzymatic hydrolysis was determined (Table 10). The relative ethanol yield, \( Y_{\text{EtOH}} (Y_{\text{ref EtOH}})^{-1} \) was defined as a ratio of the ethanol yield of the filtrate and the theoretical fermentation (0.51 g ethanol g\(^{-1}\) glucose—theoretical yield for ethanol production from glucose). This gives a measure of the fermentability of the filtrate, which can reflect the amount of inhibitors present.

There was a clear relationship between the fermentability of the water soluble fraction obtained after steam explosion and the severity factor used, up to \( \log R_o=3.35 \) (Table 10). As expected, after 6 hours, only the hexose sugars glucose and mannose, liberated from the corn fibre in the water soluble stream, were effectively consumed by \( S. \)
cerevisiae. Conversions to ethanol increased with increased severity of pretreatment, reaching a maximum at 190°C, 5 min and 3% SO₂, demonstrating a yield of 94% of theoretical (Table 10). However, beyond Log Ro=3.35, relative ethanol yield decreased, to as low as 69%.

The surface response model predicted a range of optimal pretreatment conditions with regard to time, temperature and SO₂ concentrations (Figure 15 A, B). According to the model, the optimum of relative ethanol yield (96%) from fermentable sugars after pretreatment (water soluble fraction) was obtained at 181-193°C, 4-5.5 min and SO₂ concentrations from 2.2 to 3.6 % (Figure 15 A, B). However, at severities higher than 3.35, a distinct decrease in fermentability was observed. It is well established that as steam explosion severity increases, so does the degradation of monomeric sugars, by dehydration and condensation reactions to furfural like compounds (Robinson, 2003; Söderström et al., 2002; Stenberg et al., 1998). Possible mechanisms of furfural toxicity include chemical reactivity with cellular components, damage to the cellular membrane, and inhibition of metabolism (Zaldivar et al., 1999). As shown previously in Figure 12, the amount of 5-HMF and furfural present increased with increasing severity of pretreatment. However, the low concentration of inhibitors generated at low temperatures (lower than 190°C), and the poor fermentability of the water soluble stream obtained at these conditions suggests that other by-products may be acting as inhibitors. Its has been shown that sugar degradation products such as 5-HMFs and furfurals are less inhibitory than lignin-derived compounds such as vanillin or syringylaldehyde on ethanol fermentation by S. cerevisiae (Clark and Mackie, 1984; Martín et al., 2002; Weil et al., 2002).
**Table 10** Relative ethanol yield for the liquid fraction obtained after: pretreatment (water soluble fraction) (6 hours), enzymatic hydrolysis (2 hours) and post-treatment (24 hours) $Y_{\text{EtOH}} (Y_{\text{ref EtOH}})^{-1}$ (%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of pretreatment (Log $R_0$)</th>
<th>Pretreatment $Y_{\text{EtOH}} (Y_{\text{ref EtOH}})^{-1}$ (%)</th>
<th>Enzymatic hydrolysis $Y_{\text{EtOH}} (Y_{\text{ref EtOH}})^{-1}$ (%)</th>
<th>Post-treatment $Y_{\text{EtOH}} (Y_{\text{ref EtOH}})^{-1}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>170°C, 1 min, 3%</td>
<td>2.06</td>
<td>79</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>150 °C, 5 min, 3%</td>
<td>2.17</td>
<td>85</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>170°C, 5 min, 0%</td>
<td>2.76</td>
<td>79</td>
<td>83</td>
<td>81</td>
</tr>
<tr>
<td>170°C, 5 min, 6%</td>
<td>2.76</td>
<td>82</td>
<td>81</td>
<td>89</td>
</tr>
<tr>
<td>170°C, 9 min, 3%</td>
<td>3.02</td>
<td>85</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>190°C, 5 min, 3%</td>
<td>3.35</td>
<td>94 (1)</td>
<td>89 (1)</td>
<td>85 (4)</td>
</tr>
<tr>
<td>210°C, 2.2 min, 1%</td>
<td>3.58</td>
<td>79</td>
<td>81</td>
<td>86</td>
</tr>
<tr>
<td>210°C, 2.2 min, 5%</td>
<td>3.58</td>
<td>81</td>
<td>87</td>
<td>84</td>
</tr>
<tr>
<td>210°C, 7.8 min, 1%</td>
<td>4.13</td>
<td>66</td>
<td>92</td>
<td>84</td>
</tr>
<tr>
<td>210°C, 7.8 min, 5%</td>
<td>4.13</td>
<td>69</td>
<td>95</td>
<td>82</td>
</tr>
<tr>
<td>230°C, 5 min, 3%</td>
<td>4.53</td>
<td>69</td>
<td>96</td>
<td>81</td>
</tr>
<tr>
<td>190°C, 5 min, 0%</td>
<td>3.35</td>
<td>91</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>190°C, 5 min, 6%</td>
<td>3.35</td>
<td>95</td>
<td>89</td>
<td>89</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviation, where n=9
It was apparent that there was no clear relationship between the fermentability of the liquid fractions obtained after enzymatic hydrolysis and post-treatment and the pretreatment severity (Table 10). The relative ethanol yields were high, and ranged from 81% to 100%, and 80% to 89% for sugars obtained after enzymatic hydrolysis and post-hydrolysis, respectively. The relatively high ethanol yields of the liquid fraction collected after enzymatic hydrolysis were comparable to the results previously obtained by Allen et al., (2001). After simultaneous saccharification and fermentation (SSF) of corn fibre pretreated by hot water and steam explosion, 86% and 90% conversion of glucan to ethanol by \textit{S. cerevisiae} was observed, respectively. Post-treatment, using the sulphuric acid catalysed aqueous solution obtained from SO$_2$-catalysed steam explosion of corn fibre, depolymerized the soluble oligomeric hemicelluloses and released most of the available carbohydrates in a fermentable form. At optimal pretreatment conditions, post-treatment increased the concentration of sugars available for fermentation by 10%. These results were similar to previous work done by Schevchenko \textit{et al.}, (2000), who reported 100% conversion rates for hexoses present in the post hydrolysates of SO$_2$-catalysed steam exploded Douglas-fir.
Figure 15 Response surface graph for the relative ethanol yield from water soluble fraction obtained after pretreatment as influenced by (A) temperature (°C) and time (min), and (B) temperature (°C) and SO$_2$ concentration (% w/w).
3.1.2.8 Mass-balance

As was mentioned in the general introduction, the production cost for biomass-to-ethanol must be competitive with that of fossil fuels such as oil and gasoline. As previously shown, the highest costs in the bioconversion of biomass to ethanol are those of raw material and enzymes (Boussaid et al., 1999; Galbe and Zacchi, 2002a; Gregg et al., 1998). Consequently, it is important to ensure a high degree of utilisation of all carbohydrate components in the feedstock (Wu et al., 1999). In addition, overall yield has been found to be the most important parameter when evaluating the production cost of bioethanol (von Sivers and Zacchi, 1995).

It was also apparent that the pretreatment conditions used had a dramatic effect on all aspects of the process, from sugar recovery to hydrolysis yield, on all of the downstream process steps and that a defined, optimum condition will always be a compromise. As an example of a typical mass-balance calculation for all the carbohydrate components, the case of the optimum pretreatment conditions (190°C, 5 min, 3% SO2) was selected. As can be seen from Figure 16, even these pretreatment conditions still contributed to a significant loss in sugars (3.7%), followed by the fractionation and water-wash (3.4%). During pretreatment, 2.2%, 1.3% and 0.2% (Figure 16) of arabinose, xylose and glucose, respectively, were either converted to sugar degradation products, furfurals (1.17%) and 5-HMFs (0.25%), or not recovered from the receiving vessel after steam explosion. Not surprisingly, the greatest losses were of arabinose, followed by xylose, which concurs with previous findings (Grohmann and Bothast, 1997). It has been suggested that the high susceptibility of arabinosyl linkages to hydrolysis may be in part responsible for
fragmentation and solubilisation of cell wall components in the corn fibre, and thus formation of degradation products at elevated temperatures (BeMilller, 1967).

As can be seen in Figure 16, about 3.4% of the total carbohydrates were lost during fractionation and water-wash steps, which is comparable with the sugar recovery of steam exploded Douglas-fir (Yang et al., 2002). One of the problems associated with the fractionation and subsequent water-wash is a loss of carbohydrates either during centrifugation or in using copious amounts of water and generation of dilute sugar streams. Therefore, it might be possible to combine the water soluble and insoluble streams after pretreatment, thus simplifying the process, producing a more concentrated carbohydrate stream, and consequently improving sugar recovery.

When 2% consistency solids were used during hydrolysis in an acetate buffer, complete conversion of cellulose to glucose was observed, without any additional carbohydrate loss. Moreover, the hexose sugars (glucose and mannose) in the water soluble fraction and the hydrolysate after saccharification were completely converted to ethanol during fermentation by *S. cerevisiae* (Figure 16). The residual galactose, xylose and arabinose present in the media were reflective of the ability of yeast to selectively ferment glucose and mannose.

However, despite the various "losses", the overall recovery of sugars during SO₂-catalysed steam explosion at 190°C, 5 min, and 3% SO₂ was very high (~93%). It was higher than sugar recovery during SO₂-catalysed steam explosion of Douglas-fir (~88%) (Yang et al., 2002), and dilute acid hydrolysis of corn fibre at 160°C (~85%) (Grohmann and Bothast, 1997).
Figure 16 Mass-balance of all corn fibre carbohydrate components after pretreatment at 190°C, 5min and 3% SO₂, fractionation, water-wash, hydrolysis (2% consistency) and fermentation by *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>Loss</th>
<th>Glucose-0.2</th>
<th>Arabinose-2.2</th>
<th>Galactose-0</th>
<th>Xylose-1.3</th>
<th>Mannose-0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractionation and water-wash</td>
<td>Loss</td>
<td>Glucose-0</td>
<td>Arabinose-2</td>
<td>Galactose-0.2</td>
<td>Xylose-0.2</td>
<td>Mannose-0</td>
</tr>
<tr>
<td>Solid fraction</td>
<td>Glucose-31.7</td>
<td>Arabinose-0</td>
<td>Galactose-0</td>
<td>Xylose-0.6</td>
<td>Mannose-0</td>
<td></td>
</tr>
<tr>
<td>Liquid fraction</td>
<td>Glucose-13.7</td>
<td>Arabinose-6.1</td>
<td>Galactose-2.0</td>
<td>Xylose-14.0</td>
<td>Mannose-0.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrolysis</th>
<th>Fermentation</th>
<th>Residues</th>
<th>Fermentation</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2%, consistency) in acetate buffer</td>
<td>(six-carbon carbohydrates)</td>
<td>Glucose-0</td>
<td>Arabinose-0</td>
<td>Galactose-0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glucose-0</td>
<td>Arabinose-6.1</td>
</tr>
</tbody>
</table>
3.1.2.9 Severity factor

Previous work (Overend and Chornet, 1987) and the work reported here shows that the severity factor gives a good prediction of different proportions of monomers and oligomers in the water soluble fraction as well as sugar degradation products. As the pretreatment severity increased, the yield of monomers (Table 7) and sugar degradation products in the water soluble fraction increased (Figure 12). On the other hand, the concentration of oligomers in the water soluble stream decreased (Table 7). In this study, as severity was increased, the glucose yield increased up to Log $R_0 = 3.35$ but subsequently decreased dramatically (Table 9). Similar glucose yields were obtained at severity factor $Log R_0 = 2.76$ (170°C, 5 min and 6% SO$_2$) and 3.58 (210°C, 2.2 min and 1% SO$_2$) (~73%); and $Log R_0 = 3.02$ (170°C, 9 min and 3% SO$_2$) and 3.35 (190°C, 5 min and 3% SO$_2$) (~81%) (Table 9). Other workers (Heitz et al., 1991) have also shown that, after enzymatic hydrolysis, maximum glucose recovery (90% of theoretical) was obtained at severities greater than $Log R_0 = 4.0$. However, this is not the case in the current investigation; at $Log R_0 = 4.0$, less than 60% of total glucose was recovered after enzymatic hydrolysis. It was probable that this discrepancy was due to the nature of the feedstock as Heitz et al., (1991) used hardwood (*Populus tremuloides*), which requires harsher pretreatment conditions. It could also possibly be due to differing data analyses as the progression of enzymatic hydrolysis could be measured as a percentage of the amount of glucose present in the pretreated solids, not in the original feedstock, as was likely used by Heitz et al., (1991). However, this method does not reflect the amount of sugars lost due to the formation of degradation products at higher pretreatment severity. It has been shown that, as pretreatment severity is increased, the enzymatic digestibility of solids concurrently
increases. However, precise tailoring of conditions is required due to the formation of sugar degradation products beyond a certain Log \((R_0)\), thus deceasing overall carbohydrate yield (Boussaid \textit{et al.}, 2000; Robinson \textit{et al.}, 2003; Söderström \textit{et al.}, 2002; Söderström \textit{et al.}, 2003).

Schwald \textit{et al.}, (1988) also showed that steam treatment of aspen wood cannot be characterized by one severity factor over temperature ranges greater that 40°C. Analysis of hemicellulose-derived products demonstrated that the compositions of the water soluble fractions were different, while accessibility and digestibility of the cellulosic fractions appeared to be identical. Although the substrates were obtained after pretreatment of aspen wood with a different reaction coordinate \(R_0\), the cellulosic fraction for both substrates (190°C, for 25 min and 240°C for 80sec) was hydrolysed completely within 30 hours.

It is well established that, as steam explosion severity increases, so does the degradation of monomeric sugars, by dehydration and condensation reactions that occur at higher temperatures and longer cooking times (Schwald \textit{et al.}, 1989b). However, as can be seen from Table 10, the fermentability of the water soluble fraction cannot be predicted by the severity factor. As the severity increased, the ethanol yield increased up to Log \(R_0=3.35\), but subsequently decreased dramatically (Table 10). Moreover, similar relative ethanol yields were obtained at severity factors Log \(R_0=3.76\) and \(2.58 (-81\%)\). As also reported by Söderström \textit{et al.}, (2003), during a two-step steam pretreatment process with dilute \(\text{H}_2\text{SO}_4\) impregnation, concepts of severity factors and the combined severity are unreliable methods for the evaluation of simultaneous saccharification and fermentation (SSF) of pretreated \textit{Picea abies}. Söderström \textit{et al.}, (2003) concluded that ethanol yield in SSF was mainly affected by the temperature and concentration of \(\text{H}_2\text{SO}_4\).
As mentioned previously, one of the main problems associated with optimising pretreatment conditions is finding compromise conditions, in which cellulose hydrolysis in the water insoluble stream occurs, with conversion of oligomeric sugars to monomers in the water soluble fraction, and concurrent minimal formation of sugar degradation products. This is even further complicated by the fact that optimal treatment severities for maximum recovery of hemicellulose, lignin and cellulose are not the same (Heitz et al., 1991). Cellulose and lignin require higher severity treatments to be recovered in high yields, however, these are obtained at the expense of increasing hemicellulose destruction, which can be recalcitrant towards further fermentation processing. Thus, a two-stage treatment cycle, with removal of the bulk of the hemicellulose at low severity, in consequence generating the minimum amount of inhibitors, and a post-treatment (delignification) to obtain the cellulose in high purity, was used during SO$_2$-catalysed steam explosion of Douglas-fir (Robinson, 2003; Yang et al., 2002). In this study, it was shown that for corn fibre, one could define the optimum pretreatment condition in terms of total sugar recovery in hydrolysable and fermentable form, without an additional delignification process. Moreover, as expected, to obtain maximum sugar recovery for corn fibre, the optimum severity of pretreatment (Log $R_0$=3.35) was lower compared with softwoods (Log $R_0$=3.45), and hardwoods (Log $R_0$=3.40), due to the chemical characteristics of the feedstocks (Boussaid et al., 2000; Ramos et al., 1992; Robinson, 2003). Thus, tailoring of pretreatment conditions for each type of biomass is required.
3.1.3 Conclusions

Each of the parameters investigated during steam pretreatment by response-surface modeling (temperature, time and SO$_2$ concentration) had an effect on the hemicellulose monomer recovery, its subsequent fermentability, and the enzymatic hydrolysis of the cellulose stream. Although the wide range of pretreatment conditions investigated precluded an exact determination of optimal conditions, the predicted “compromise-optimal” pretreatment conditions (190°C, 5 min, 3% SO$_2$) were in agreement with the optimum conditions observed for maximum sugar yield obtained after pretreatment and enzymatic hydrolysis, and fermentability of the monomeric sugars. The results show that SO$_2$ impregnation of corn fibre prior to steam explosion is beneficial.

A two-stage treatment for corn fibre processing, comprised of SO$_2$-catalysed steam explosion and hydrolysis by a mixture of cellulolytic enzymes, has proven to be a very effective method for converting the available polysaccharides in the residue to monomeric sugars, as evidenced by the 81% conversion observed without the need for a detoxification step. Maximum sugar yields (soluble and following enzymatic hydrolysis) were recovered from corn fibre pretreated at 190°C for 5 min with 3% SO$_2$. Post-treatment of the aqueous solution from SO$_2$-catalysed steam explosion effectively depolymerised the soluble oligomeric hemicellulose and increased the concentration of carbohydrates in fermentable form by 10% at the optimum pretreatment conditions.

The results also demonstrate that, probably due to the low concentration of sugar degradation products formed during the “compromise” pretreatment condition (190°C for 5 min with 3% SO$_2$), *Saccharomyces cerevisiae* was able to convert the resulting corn fibre hydrolysates, hemicellulose stream and liquid fraction from post-hydrolysis to ethanol.
efficiently, yielding 89%, 94% and 85% of theoretical conversion, respectively, from the hexose sugars.

According to this work, the severity factor provides a good prediction of the different proportions of monomers and oligomers in the water soluble fraction, and sugar degradation products. However, the severity factor is not an accurate measurement in the evaluation of hydrolysability and fermentability of pretreated corn fibre.

It is possible to find the optimum pretreatment condition for corn fibre in terms of total sugar recovery in hydrolysable and fermentable form, without an additional delignification process, thus simplifying the bioconversion to ethanol. As expected, to obtain maximal sugar recovery for corn fibre, the optimum severity of the pretreatment (Log R₀ = 3.35) was lower compared to both softwoods and hardwoods, due to the differing chemical characteristics of the feedstocks.

Finally, according to mass-balance analysis, the overall recovery of sugars during SO₂-catalysed steam explosion at 190°C, 5 min, 3% SO₂ was very high (~93%), with the pretreatment, fractionation and water-wash steps, being the main contributors to the overall carbohydrate loss.


3.2 Hydrolysability of high concentration sugars

3.2.1 Background

Based on the surface response modeling described in the previous chapter, the optimum pretreatment conditions for maximal hemicellulose and cellulose recovery in a hydrolysable and fermentable form were established (190°C, 5 minutes and 3% SO₂). However, all the saccharification experiments were performed at a low solid consistency (2% w/v) in an acetate buffer. Moreover, during optimisation of SO₂-catalysed steam explosion of corn fibre, multiple-step processing was employed: i.e., pretreatment, fractionation, water-wash of solid fraction, enzymatic hydrolysis and fermentation.

As stated previously, most economic studies have shown that the sugar concentration used for subsequent fermentation steps has a major impact on the overall process costs (Nguyen, 1993b; Nguyen and Saddler, 1991). The sugar concentration that can be established in hydrolysis reactors can affect production costs in several ways. For example, by using a concentrated sugar stream (100-300 g L⁻¹), smaller-volume ethanol fermenters are required, thus lowering capital costs. Concurrently, concentrated sugar streams yield a higher concentration of ethanol, which can lower the steam requirement in the ethanol stripping tower (Nguyen, 1993b). As reported by Zacchi and Axelsson (1989), doubling the ethanol concentration from 2.5 to 5% reduces the energy required to distill a fermentation broth to high-purity ethanol (93.5%) using conventional distillation techniques.

There are several ways to increase the concentration of sugars available for saccharification and further fermentation. For example, one can, a) use a higher concentration of solids during the hydrolysis, b) combine the water soluble and insoluble streams during hydrolysis, c) physically concentrate the water soluble fraction or d)
supplement the water soluble fraction with glucose derived from separate enzymatic hydrolysis. Due primarily to the high cost associated with physical concentration of the water soluble fraction and the contemporaneous potential for concentrating inhibitors, the first two approaches for increasing the concentration of sugars available for fermentation were investigated (Figure 17).

By using the first approach, it should be possible to generate a concentrated sugar stream after saccharification, however, it has been shown at the Plant Development Unit (PDU) at the National Renewable Energy Laboratory (NREL) to be difficult to handle pretreated solids at consistencies higher than 25% (w/v) (Tucker, 2003). Therefore, a more attractive option would be to combine the water soluble and insoluble streams during hydrolysis.

In most laboratory-scale investigations, as well as in the optimisation of steam explosion of corn fibre experiments described in the previous chapter, the solids are separated from the liquid in the fractionation step after pretreatment, and water-washed prior to enzymatic hydrolysis (Gregg and Saddler, 1996; Robinson, 2003; Stenberg et al., 1998; Tengborg et al., 1998). For the bioconversion of Douglas-fir to ethanol, the need for extensive water-washing arises from the requirement to remove delignification chemicals and lignin prior to enzymatic hydrolysis (Boussaid et al., 2000; Robinson, 2003; Yang et al., 2002). From an economic point of view, it is preferable to include the water soluble fraction in the hydrolysis of solids because it reduces the need for fresh water, and simplifies the process overall by precluding the fractionation and water-wash steps. Moreover, by combining the streams one can overcome the dilute sugar stream problem, which occurs when there is the need for complete recovery of the hemicellulose fraction after fractionation.
and water-washing. Previously Zacchi and Axelsson (1989), investigated the influence of ethanol concentration in the feed on energy consumption in a distillation unit. It was found that, when washed solid material (5% dry matter) was used in enzymatic hydrolysis, the energy demand for the concentration of the dilute ethanol stream was twice as high as when feed obtained using the whole slurry was employed.

3.2.1.1 Introduction to results

We hypothesised that, after mild SO₂-catalysed steam explosion, we can obtain good hemicellulose recovery and effective solids hydrolysis at high consistency without prior lignin removal. Two methods were used in attempts to increase the concentration of sugars available for consequent fermentation. The first was to carry out hydrolysis at high concentration solids, up to 12% (w/v) in acetate buffer. The second involved supplementation of the hemicellulose fraction during the saccharification process (Figure 17). The latter approach allowed us to hydrolyse the oligomer-rich hemicellulose fraction, which comprises up to 50% of the total sugars recovered in the water soluble fraction, along with the cellulose-rich water insoluble fraction. Moreover, it simplifies the bioconversion of corn fibre to ethanol by removing the fractionation and water-wash steps, and therefore creates a high carbohydrate stream which will lead to a higher ethanol concentration and subsequently reduces distillation costs. In addition, as shown in the previous chapter, fractionation and water-wash are two of the main contributors to carbohydrate loss. Thus, by combining the water soluble and insoluble streams during hydrolysis, it should be possible to increase the overall carbohydrate recovery.

Initially, the influence of high consistency solids on the hydrolysis profiles of pretreated, water-washed corn fibre solids in acetate buffer was assessed (Figure 17). To be
able to evaluate the hydrolysability of mixed fractions (water soluble and insoluble), we first optimised the hydrolysis conditions (temperature and pH) used (Figure 17). Once the saccharification conditions were established, the influence of enzyme loading, hydrolysis time, mode of enzyme addition and additional pretreatment of the water soluble stream (treatment with protein) were tested during hydrolysis of the water soluble and insoluble fractions at 5% (w/v) consistencies (Figure 17). We then investigated the role of the carbohydrate and non-carbohydrate derived inhibitors on the hydrolysis profiles of combined streams, and the supplementation of the solid fraction with synthetic sugar streams equivalent in concentration to the original water soluble fraction. The need for extensive water-wash of solids prior to enzymatic hydrolysis of combined streams was also examined, to assess the possible role of non carbohydrate inhibitors on the saccharification process.
Figure 17 Experimental procedure for hydrolysis at high sugar concentrations.

Corn fibre

Pretreatment
190°C, 5 min, 3% SO₂

Fractionation (Centrifugation)

Sugar analysis

Water soluble fraction

Sugar analysis

Water insoluble fraction

Sugar analysis

Water insoluble+soluble fractions

Preliminary experiments
Hydrolysis in water soluble fraction, 5% (w/v)
- Temperature (37, 45 and 53°C)
- pH (4.8, 5.3)
- Enzyme loading 20 FPU g cellulose⁻¹

Sugar analysis

Hydrolysis in water soluble fraction, 5% (w/v), 45°C, pH 4.8, 20 FPU g cellulose⁻¹
- Enzyme loading
- Hydrolysis time
- Treatment of water soluble fraction

Sugar analysis

Hydrolysis in acetate buffer 2.5, 5, 7.5, 10, 12% (w/v)

Sugar analysis
3.2.2 Results and discussion

3.2.2.1 Hydrolysis of high consistency solids

It was previously shown that pretreated, water-washed corn fibre solids, although containing 29% “pseudolignin” (Table 16—Appendix 3), can be completely hydrolysed at low consistency (2% w/v) without requiring a prior delignification step. However, economic studies have shown that substrate concentrations have a major impact on process costs (Nguyen and Saddler, 1991). Thus, to make the biomass to ethanol process economically feasible, i.e., increasing the sugar concentration available for further fermentation process, higher solids concentrations were used during the saccharification process. The pretreated, water-washed corn fibre solids were subject to enzymatic hydrolysis for 96 hours with a combination of cellulases and an excess of β-glucosidase in the hydrolysis buffer (50mM, pH 4.8). The hydrolysability of the pretreated (190°C, 5min and 3% SO₂), water-washed corn fibre solids with varying consistencies (of 2.5-12% w/v) at 10FPU g cellulose⁻¹ loadings and IU:FPU ratio of 2:1 are shown in Figure 18.

When the glucose yields were compared against increasing concentrations of steam exploded corn fibre, hydrolysed by the same enzyme loading, it was apparent that after 24 hours, the yield at higher substrate concentrations decreased significantly. Glucose yields of 68 and 98% were obtained within the first 24 hours for 12% and 2% (w/v) solids concentrations respectively, and a further 72 hours incubation was required to achieve almost complete hydrolysis (95%) for 5, 7.5 and 10% (w/v) of corn fibre solids. For 12% (w/v) solids concentration, a 90% glucose yield was achieved after 72 hours of hydrolysis, which corresponded to a 55 g L⁻¹ glucose content.
The hydrolysis yields were affected by the concentration of corn fibre. However, keeping in mind that all experiments were performed in regular shake flasks, without prior delignification of solids, it would be feasible to achieve almost complete hydrolysis (90%) at 12% solids concentration within 72 hours, thus generating concentrated sugar streams available for further fermentation (55 g L\(^{-1}\) glucose concentration). As observed by Schwald et al., (1989a) and Gregg and Saddler (1996), both the batch enzymatic hydrolysis yield, and the initial hydrolysis rate were influenced by the concentration of the substrate. There is an inverse relationship between the concentration of the substrate and the enzymatic hydrolysis yield, with the highest rate of hydrolysis and subsequent yield obtained over the first 24 hours. Gregg and Saddler (1996) reported that glucose yields of 50 and 80% were obtained within the first 24 hours of hydrolysis of peroxide-treated steam-exploded aspen at 10% and 2% substrate concentrations, respectively. A further 72 hours’ incubation was required to obtain final yields of 80% and 95%, respectively (Gregg and Saddler, 1996).
Figure 18 The hydrolysability of pretreated, water-washed corn fibre solids with varying consistency (2.5%, 5%, 7.5%, 10% and 12 %-w/v) at 10 FPU g cellulose$^1$ loadings and IU:FPU ratio 2:1. Error bars represent the range of duplicate hydrolysis.
3.2.2.2 Hydrolysis of solids in the water soluble fraction

Although using high consistency solids of up to 12% (w/v) during enzymatic hydrolysis may be a feasible option, the increased hydrolysis time to achieve 90% cellulose conversion makes this option less favourable. Moreover, it became apparent that mixing of the fibrous material at concentrations higher than 10% (w/v) was a very difficult task when using the regular shake flasks. As stated previously, another method by which it is possible to increase sugar concentrations available for further fermentation is to include the hemicellulose-rich fraction, i.e., the liquid obtained after the pretreatment step, in the enzymatic hydrolysis of the cellulose-rich, water insoluble stream. In addition, mixing the hemicellulose and cellulose fractions during the saccharification process may reduce the need for acidic post-treatment to depolymerize oligomers, shown previously to comprise up to 50% of the recovered hemicellulose stream (Bura et al., 2003).

Based on the compositional differences of the hydrolysis media (acetate buffer and water soluble fraction) and the presence of inhibitors in the hemicellulose stream, one might predict different behaviour in the two mixtures during enzymatic hydrolysis. For example, Robinson (2003) combined the water soluble and delignified water insoluble streams of SO$_2$-catalysed steam exploded Douglas-fir at medium severity and obtained 76% hydrolysis yield after 72 hours of saccharification.

One potential drawback is that the additional sugar content or other potential inhibitors in the water soluble stream could result in increased end-product inhibition of the *Trichoderma* complex enzymes. As discussed previously, it is a common laboratory procedure to fractionate solids and liquids, thus removing inhibitors present in the hemicellulose fraction from the solids, and further water-wash the solids fraction prior to
enzymatic hydrolysis (Mes-Hartree and Saddler, 1983; Sinitsyn et al., 1982). However, keeping in mind that the SO₂-catalysed steam explosion was optimised for maximum recovery of hemicellulose, with minimum formation of sugar degradation products by using medium pretreatment severity, it may be possible to eliminate the fractionation and water-wash steps prior to hydrolysis. Thus, it should be possible for the whole slurry to be used during the saccharification process.

Due to the difference in the hydrolytic buffer (i.e., change from acetate buffer to the hemicellulose-rich fraction), preliminary experiments were performed in order to optimise hydrolysis conditions with regard to temperature and pH (Figure 19). The hydrolysis yields of the pretreated corn fibre solids at 190°C, 5 minutes and 3% SO₂, performed in the hemicellulose-rich fraction at 5% (w/v) consistency with varying pH and temperatures, at 20 FPU g cellulose⁻¹ and 2:1 loading of β-glucosidase activity (IU) to filter paper activity (FPU) are presented in Figure 19.

As predicted, the optimum temperature and pH for hydrolysis of mixed fractions was equivalent to saccharification in the acetate buffer (50mM, pH 4.8). Based on Figure 19, the highest hydrolysis yield (85%) was achieved at 45°C, after 100 hours of hydrolysis, and the lowest (73%) at 37°C. At all three different temperatures tested, pH 4.8 produced a better environment for the hydrolysis of the water soluble and insoluble fractions by the cellulolytic enzymes. Therefore, in the following experiments, the optimal hydrolysis conditions, pH 4.8 and temperature 45°C were used for hydrolysis of the pretreated corn fibre slurry.
Figure 19 The hydrolysis yield of the pretreated corn fibre solids at 5% (w/v) consistency with varying pH (4.8 and 5.3), temperature (37, 45 and 53°C) at 20 FPU g cellulose⁻¹ and IU:FPU ratio 2:1. Error bars represent the range of duplicate hydrolysis.
3.2.2.2.1 Enzyme loading, hydrolysis time and mode of enzyme supplementation

For an industrial process to be more attractive it is preferable to hydrolyse high dry mass content without separation of the filtrate from the solids, and in doing so to obtain a high ethanol concentration, thus reducing the cost of subsequent distillation. However, from the preliminary results, it became apparent that the hydrolysis yield of combined streams was 15% lower compared to saccharification in an acetate buffer (Figure 19), probably due to the end-product inhibition of the enzymes. Therefore, the influence of enzyme loadings on hydrolysis yield of combined streams obtained during SO$_2$-catalysed steam explosion of corn fibre was next assessed. The hydrolysability of the pretreated corn fibre solids, performed in the hemicellulose-rich fraction at 5% (w/v) consistency with varying enzyme loadings (10, 20 and 30 FPU g cellulose$^{-1}$) is shown in Figure 20.

It was apparent that the inclusion of the water soluble fraction during hydrolysis of pretreated corn fibre solids inhibited the saccharification process (Figure 20). However, as the amount of enzymes added was increased, the cellulose conversion increased compared to the control hydrolysis where sodium acetate buffer was used. Not surprisingly, the highest cellulose conversion (92%) was obtained after 96 hours, when 30 FPU g cellulose$^{-1}$ was used, and the lowest (80%) was achieved for 10 FPU g cellulose$^{-1}$, (Figure 20). For the control hydrolysis solids in the acetate buffer, completion of saccharification occurred after 48 hours for both enzyme loadings of 10 and 30 FPU g cellulose$^{-1}$. As shown previously by Tengborg et al., (2001), when the hemicellulose-rich fraction was mixed with pretreated spruce (Picea abies) without prior delignification during enzymatic hydrolysis, 19.5% cellulose conversion was achieved, which is comparable with work by Robinson (2003) who showed a 17.3% hydrolysis with Douglas-fir. Thus by increasing the dosage of cellulases in
the process it is possible, to a certain extent, to enhance the yield and rate of hydrolysis. Unfortunately, this might significantly increase the cost of the overall process (Gregg and Saddler, 1996). However, in the case of enzymatic hydrolysis of pretreated corn fibre solids, it is possible to use as little as 10 FPU g cellulose$^{-1}$ loadings to achieve high cellulose conversion (80%), increasing the overall sugar concentration available for further fermentation processes to 65 g L$^{-1}$. 
Figure 20 The influence of different enzyme loadings (10, 20 and 30 FPU g cellulose\(^{-1}\)) on the enzymatic hydrolysis of pretreated corn fibre solids at 5% (w/v) consistency at an IU:FPU ratio of 2:1 in the water soluble fraction and acetate buffer. Error bars represent the range of duplicate hydrolysis.
The effect of hydrolysis time on cellulose conversion was also investigated. The pretreated corn fibre, water-washed solids mixed with hemicellulose-rich fraction, was subjected to enzymatic hydrolysis at 20 and 40 FPU g cellulose\(^{-1}\) for 144 hours and compared to a control hydrolysis in an acetate buffer (Figure 21). One of the factors affecting enzymatic hydrolysis of lignocellulosic biomass, apart from enzyme inactivation due to denaturation, end-product inhibition or unproductive binding of active enzyme to the substrate, is substrate transformation (Eriksson et al., 2002). Increased substrate recalcitrance has been proposed to explain the declining rates of hydrolysis (Eriksson et al., 2002). Easily hydrolysed parts of the cellulose substrate might be hydrolysed in the early stages, leaving recalcitrant parts for later stages. Therefore, in this set of experiments, the influence of fresh enzyme addition was also evaluated, by supplementing the media first with 20 FPU g cellulose\(^{-1}\) at 0 hours and sequentially adding 20 FPU g cellulose\(^{-1}\) after 20 hours or 40 hours of hydrolysis and comparing the hydrolysis profile with that of 40FPU g cellulose\(^{-1}\) added at the beginning of reaction.

The results indicated that the hydrolysability of the solids in the water soluble fraction improved as hydrolysis time increased, reaching almost complete cellulose conversion (96%) for 40 FPU g cellulose\(^{-1}\) loading at 144 hours. The extended hydrolysis time did not improve hydrolysability of the slurry when 20 FPU g cellulose\(^{-1}\) was used. With the supplementation of the saccharification media by fresh enzyme (20 FPU g-cellulose\(^{-1}\)) after 20 and 49 hours, hydrolysis yield increased by 8% and 12% respectively, compared to the control (20 FPU g cellulose\(^{-1}\) added at the beginning of hydrolysis). In the case of hydrolysis of pretreated solids in the acetate buffer, the addition of fresh enzyme at
Figure 21 The influence of hydrolysis time and enzyme supplementation on the extent of cellulose conversion in a mixture of the water soluble fraction and the pretreated corn fibre solids at 20 and 40 FPU g cellulose\(^{-1}\) at IU:FPU ratio 2:1 in the water soluble fraction and acetate buffer at 5% (w/v) consistency. Error bars represent the range of duplicate hydrolysis.
20 hours changed neither the hydrolysis yield nor the rate compared to the control, which might be explained by the already high conversion yield after 24 hours (92%).

As can be seen from the data presented in Figure 21, only the sequential supplementation with fresh enzyme after 48 hours of hydrolysis of combined streams (when hydrolysis at 20 FPU g cellulose\(^{-1}\) almost reached a plateau) improved the conversion yield, compared to the case when 40 FPU g cellulose\(^{-1}\) was added at the beginning of the reaction. This seems to indicate that enzyme deactivation or unproductive binding plays a more important role than increased substrate recalcitrance for the decrease in hydrolysis yield. These results might also suggest that supplementation with a fresh enzyme in a sequential manner (0 hours, 48 hours) is a possible option for increasing hydrolysis yield. However, the lengthened saccharification time (more that 48 hours), for only 4% improvement in the hydrolysis yield, does not make this option particularly attractive.

3.2.2.2 Potential inhibitors during hydrolysis of combined streams

Increased hydrolysis time, enzyme loading and sequencing enzyme addition improved the sugar yield during hydrolysis of solids in the hemicellulose stream. However, most of the options do not appear to be feasible at the commercial level. What are the reasons for the impairment of hydrolytic abilities of cellulolytic enzymes when the water soluble and insoluble streams are combined during the saccharification process? The following sets of experiments evaluated the influence of different types of inhibitors (carbohydrate and non-carbohydrate) on the progress of enzymatic hydrolysis of combined fractions. First of all, as mentioned previously, the water soluble fraction obtained after pretreatment of corn fibre contains various chemical compounds such as organic acids, phenolics and sugar degradation products, which can potentially inhibit the cellulolytic
action of enzymes employed during the saccharification process (Grohmann and Bothast, 1997; Tengborg et al., 2001; Weil et al., 2002). Apart from phenolic compounds, aliphatic acids and furan derivatives, the sugars present in the water soluble streams all could potentially increase inhibition of the cellulase and β-glucosidase enzymes. As discussed, the solids are separated from liquids in the fractionation step after pretreatment, and water-washing occurs prior to enzymatic hydrolysis (Gregg and Saddler, 1996; Robinson, 2003; Stenberg et al., 1998; Tengborg et al., 1998). As reported previously (Mes-Hartree and Saddler, 1983; Nagle et al., 2002; Sinitsyn et al., 1982), extensive water-washing of the solids fraction after fractionation improved hydrolysis yield. In addition, it was also shown that by washing the pretreated solids at higher temperatures (>135°C), a solids substrate with lower lignin content was produced, and higher amounts of solubilised lignin in the wash liquor were present (Nagle et al., 2002). Therefore, by simplifying the overall bioconversion of corn fibre to ethanol process through elimination of the fractionation and water-wash steps, i.e., by hydrolysing combined streams, one might anticipate that the inhibitors present might possibly inhibit the saccharification process.

3.2.2.2.1 Sugar inhibitors

To evaluate the role of individual sugars present in the water soluble fraction on the hydrolysis profile of combined streams, the acetate buffer was supplemented with either synthetic glucose, xylose or arabinose at different concentrations during saccharification of a 5% consistency pretreated corn fibre solids. Glucose, xylose and arabinose are the main monomeric sugars present in the water soluble stream obtained from pretreated corn fibre, at concentrations of ~30, 11 and 9 g L⁻¹, respectively. Although it was shown that mannose and galactose have some impact on hydrolysis yield (Robinson, 2003), due to the very low
concentration of these sugars in the water soluble fraction (less than 0.4 and 2 g L\(^{-1}\) respectively) only synthetic glucose, xylose and arabionse were used in the following experiments.

The hydrolysability of pretreated corn fibre solids at 5% (w/v) consistency in the presence of: synthetic sugars (glucose, xylose and arabinose) at various concentrations is shown in Figures 22 A, B, and C, respectively.

Not surprisingly, supplementation of the water insoluble stream in the acetate buffer with synthetic glucose at all concentrations (30, 40 and 50 g L\(^{-1}\)) reduced the hydrolysis yield by 11, 13 and 14%, respectively at 48 hours, compared to the corresponding control (Figure 22 A). However, the influence of increased glucose concentrations on the hydrolysis profile was only apparent at 12 hours of saccharification, when the hydrolysis rates were calculated [dividing the concentration of sugars (g L\(^{-1}\)) over 12 hours]. As the concentration of synthetic glucose was increased from 30 to 50 g L\(^{-1}\), the corresponding hydrolysis rates at 12 hours decreased from 1.02 g L\(^{-1}\) hour\(^{-1}\) to 0.87 g L\(^{-1}\) hour\(^{-1}\) and 0.71 g L\(^{-1}\) hour\(^{-1}\), respectively. Xylose was the next most inhibitory sugar, however, supplementation of the acetate buffer and water insoluble fraction with synthetic xylose did not have the same inhibitory effect as did glucose. Only a 6% decrease in the cellulose to glucose conversion was observed for all of the concentrations tested (Figure 22 B). As can be seen from Figure 22 C, arabinose caused the smallest reduction in hydrolysis yield. Therefore, even at the high concentration of 40 g L\(^{-1}\) (more than 4 times higher than the concentration in the water soluble fraction), it was still possible to achieve very high hydrolysis yields (close to 97% cellulose to glucose conversion) (Figure 22 C). Keeping in mind that the concentration of
Figure 22 The hydrolysability of pretreated corn fibre solids at 5% (w/v) consistency in the presence of synthetic: A) glucose, B) xylose, and C) arabinose, water soluble fraction and acetate buffer at 20 FPU g cellulose\(^{-1}\), IU:FPU ratio 2:1.
glucose, xylose and arabinose in the water soluble fraction are 30, 11 and 9 g L\(^{-1}\), respectively, it appears that it is primarily the glucose present in the hemicellulose fraction that is the most potent inhibitor during saccharification of the combined water soluble and insoluble streams.

Glucose is well recognized for its inhibition of cellulose hydrolysis (Holtzapple et al., 1990). The buildup of sugars during enzymatic hydrolysis is known to slow the hydrolysis of cellulose to glucose. The end-product inhibition caused by the liberation of cellobiose and glucose from the cellulose can result in substantial inhibition of the major (the endo- and exo-acting) cellulases and \(\beta\)-glucosidase components of *Trichoderma* (Holtzapple et al., 1990). The type of inhibition of the cellulases is the subject of considerable discussion, and it has been modeled as competitive, non-competitive, and uncompetitive in nature by various researchers (Holtzapple et al., 1990; Mandels and Reese, 1963). Although, cellobiose is generally reported to be a stronger inhibitor of the cellulases than glucose (Holtzapple et al., 1990), Marsden et al., (1983) have shown a 50% reduction in activity using a 4% glucose medium with a *Trichoderma reesei* cellulase preparation. Xylose (Dekker, 1988) and xylans (Mes-Hartree and Saddler, 1983) have also been reported to inhibit the enzymatic hydrolysis of lignocellulosic substrate, but one should keep in mind that the influence of a particular sugar will largely depend on its concentration. Therefore, the concentration of the sugars in the water soluble fraction must first be established and then accordingly, the influence of that sugar's inhibition on the hydrolysis yield can be properly tested.

However, if only monomeric sugars affected the enzymatic hydrolysis, similar conversion yields would be seen for the hydrolysis of the combined fraction (water soluble
and insoluble) and that of synthetic glucose in acetate buffer and the water insoluble stream. By analysing the conversion yields (Figure 22 A), it can be seen that this was not the case. When the water insoluble fraction was supplemented with the water soluble stream, after 48 hours, 80% cellulose to glucose conversion was observed. Even at the highest concentration of glucose tested ($50 \text{ g L}^{-1}$), an 86% hydrolysis yield was observed. It was apparent that non-carbohydrate inhibitors were also responsible for some of the observed decrease in hydrolysis yields.

3.2.2.2.2 Non-sugar inhibitors

The hemicellulose stream obtained after pretreatment of corn fibre contains various chemical compounds such as organic acids, phenolics and sugar degradation products which could potentially inhibit the cellulosytic action of enzymes employed during the saccharification process (Grohmann and Bothast, 1997; Tengborg et al., 2001; Weil et al., 2002). In previous research it was shown that a decrease in enzyme activity can be caused by phenolic compounds derived from lignin, extractives and carbohydrate decomposition products (Mandels and Reese, 1963), through reversible and irreversible binding, precipitation and deactivation of the enzymes (Jung and Fahey, 1983). Corn fibre contains a minimal amount of lignin (~7%) in the form of phenolic acids (~5%), ferulic acid, and p-coumaric acid (Saulnier and Thibault, 1999). The pretreatment conditions were optimised for minimum formation of sugar degradation products; it was hypothesized that non-carbohydrate inhibitors from water soluble streams additionally influenced the incomplete hydrolysis of combined streams.

Pretreated unwashed and washed corn fibre solids at a 5% consistency were hydrolysed in the presence of 50% diluted water soluble fraction, undiluted water soluble
fraction, and synthetic sugars that mimicked the concentration of monomers in the hemicellulose stream at 20 FPUg cellulose\(^{-1}\) for 72 hours (Figure 23). Not surprisingly, the highest conversion after 72 hours (100%) was obtained when the washed pretreated corn fibre solids were hydrolysed in acetate buffer (Figure 23). The hydrolysis yield decreased by 5% when unwashed corn fibre solids were tested in acetate buffer during saccharification. Once the whole slurry was analysed after pretreatment, the lowest conversion (80%) was obtained. As the amount of water soluble fraction increased from 50 to 100%, the cellulose conversion decreased by 14 and 20%, respectively. However, additional washing of solids when supplemented with the 100% hemicellulose stream did not improve the conversion yield (Figure 23). When a sugar solution with the same concentration of monomeric sugars as in the case of the 100% water soluble stream, was added to the unwashed solids fraction, a conversion of 88% was achieved. It was apparent that the presence of non-carbohydrate compounds in the water soluble fraction in a saccharification mixture was responsible for at least partial inhibition of the cellulolytic enzymes.

Nevertheless, the carbohydrate and non-carbohydrate inhibitors from the water soluble stream of pretreated corn fibre were not as detrimental to cellulolytic enzymes (80% conversion) as reported for pretreated softwood residues, where 19.5% and 17.3% conversion occurred when combined streams were saccharified (Robinson, 2003; Tengborg \textit{et al.}, 2001).
Figure 23 The hydrolysability of unwashed and washed pretreated corn fibre solids at 5% (w/v) consistency in the presence of: synthetic sugars, water soluble fraction, dilute water soluble stream and acetate buffer at 20 FPU g cellulose⁻¹, IU:FPU ratio 2:1. Error bars represent the range of duplicate hydrolysis.
3.2.2.2.3 Reduction of non-sugar inhibitors by treatment of hemicellulose fraction

It was anticipated that by removal of the non-carbohydrate inhibitors through treatment of the water soluble fraction prior to hydrolysis the saccharification yield would be increased. Previously, it has been shown that during the hydrolysis of water soluble and insoluble streams of pretreated spruce, the inhibition created by the hemicellulose fraction could be overcome by fermentation prior to the saccharification process (Tengborg et al., 2001). Sewalt et al., (1997) also reported that the negative impact of lignin on the enzymatic hydrolysis of cellulose could be countered by the addition of nitrogen-containing compounds, such as gelatin and ovalbumin.

In this study, the influence of enzymes (xylanases and cellulases) and albumin treatment of the water soluble fraction prior to hydrolysis on saccharification yield were tested (Figure 24). In separate flasks, excesses of xylanase, cellulase and bovine albumin were added to the water soluble streams and incubated for 6 hours at 45°C. The proteins were then removed from the hemicellulose fractions by sequential boiling and centrifugation and the “treated” water soluble streams were used in consecutive saccharifications of combined fractions (Figure 24).

The “treatment” of the water soluble fraction by cellulases and xylanases improved the cellulose conversion by 10% after 72 hours (Figure 24). However, the same effect was also observed when the hemicellulose fraction was treated with bovine albumin prior to the saccharification process (Figure 24). Therefore, the removal of inhibitory compounds by lignin-protein interaction, rather than depolymerization of oligomeric sugars, was likely the mechanism influencing improved cellulose conversion. It was previously shown that the inhibition of enzymatic hydrolysis of a model substrate (filter paper with the addition of
Figure 24 The influence of pretreatment of the water soluble fraction prior to saccharification with: xylanase, cellulase and albumin on the conversion of cellulose during enzymatic hydrolysis at 20 FPU g cellulose$^{-1}$, IU:FPU ratio 2:1, of pretreated corn fibre solids at 5% consistency, compared to control (pretreated solids in the water soluble fraction). Error bars represent the range of duplicate hydrolysis.
solid lignin) was almost completely overcome by pretreatment with ammonia (Tengborg et al., 2001). In addition, it was proposed that polyphenolics form physical complexes with cellulose, which can be broken by the addition of another protein (Sewalt et al., 1997). Ooshima et al., (1990) also reported the decrease of nonspecific bindings of the cellulase enzyme to the lignin fractions could be overcome by the addition of protein. Although treatment of the water soluble fraction with proteins prior to hydrolysis improved digestibility of the substrate, it would not eliminate end-product inhibition due to carbohydrate presence. This study has shown that, from a strictly technical point of view, “protein treatment” of the water soluble stream prior to the saccharification step could be a possible solution to the inhibition issue. However, the main drawback of the process configuration is the fact that it is an additional step, which could add significantly to the costs of the overall process.

3.2.3 Conclusions

In our efforts to increase the sugar concentration during corn fibre-to-ethanol processing, two methods were tested: use of a higher concentration of solids during hydrolysis, and combining the water soluble and insoluble streams during saccharification. It was established that it is possible to hydrolyse high consistency pretreated corn fibre solids without prior delignification, for up to a 12% solids concentration in acetate buffer when using regular shake flasks; we achieved 90% cellulose conversion. After steam explosion of corn fibre at the optimum pretreatment conditions (190°C, 5min and 3% SO2), it was possible to combine the hemicellulose-rich fraction with the cellulose-rich water insoluble stream during hydrolysis, thus simplifying the process by removal of the fractionation and water-wash steps. However, during hydrolysis of high-sugar combined
streams (water soluble and insoluble fractions), it became apparent that end-product inhibition was the main factor influencing incomplete cellulose conversion (having achieved 80% cellulose conversion at 10 FPU g cellulose\(^{-1}\) and 5% w/v solids consistency).

Conversely, increased hydrolysis time (up to 144 hours) and enzyme loading (40 FPU g cellulose\(^{-1}\)) resulted in near complete (96%) cellulose to glucose conversion. During hydrolysis of combined streams, a sequential enzyme addition enhanced cellulose to glucose conversion. The possible removal of inhibitory compounds by lignin-protein interaction also improved cellulose conversion by 10%.

Although increased hydrolysis time, enzyme loadings, and addition of protein prior to the saccharification step improved cellulose conversion, most of the options proposed do not appear to be feasible at the commercial level. Additionally, it was clearly shown that the presence of non-carbohydrate inhibitors from the water soluble stream in the saccharification mixture was responsible for at least partial inhibition of cellulosolytic enzymes, but did not appear to be as detrimental as reported for pretreated softwood residues.
3.3 Simultaneous saccharification and fermentation versus separate hydrolysis and fermentation

3.3.1 Background

Based on the ease of hydrolysis of high-cellulose concentrations, which was described in the previous chapter, it was established that it is possible to hydrolyse high consistency solids, without prior delignification at up to a 12% solids concentration. Although ninety percent cellulose conversion was achieved during hydrolysis of the combined cellulose and hemicellulose streams (water soluble and insoluble fractions), it became apparent that end-product inhibition was one of the main factors influencing incomplete cellulose hydrolysis (80% conversion). However, by increasing the hydrolysis time, enzyme loading, and the addition of protein prior to the saccharification step, cellulose conversion was improved. Unfortunately, it is likely that all of these options would add to the already-high cost of converting corn fibre to ethanol. Therefore, in an attempt to increase the hydrolysis yield while minimizing end-product inhibition and simplify the overall process, the use of a simultaneous saccharification and fermentation (SSF) process was investigated and compared to the separate hydrolysis and fermentation (SHF) process.

It was hypothesized that after pretreating corn fibre at compromise conditions for obtaining maximum sugar recovery and minimum formation of sugar degradation products, it would be possible to adjust the pH of the hydrolysate (water soluble and insoluble fractions) and subsequently enzymatically digest and ferment the mixture in a single reactor (SSF), without the need for fractionation, delignification, water-wash and detoxification steps.
The SSF process has several advantages over the SHF process, such as increased hydrolysis rates by conversion of sugars that inhibit cellulase activity, lower enzyme requirements, higher product yields, shorter processing times and lower reaction volumes required since a single reactor is employed. However, the disadvantages of SSF include incompatible temperatures required for hydrolysis and fermentation, limited ethanol tolerance of microbes, sensitivity to bacterial infections and difficulties in yeast recycling (Stenberg et al., 2000b).

Furthermore, in choosing a SSF process, one should also consider the appropriate fermentation microorganism. As mentioned earlier, although Saccharomyces cerevisiae and Zymomonas mobilis can ferment glucose to ethanol rapidly and efficiently, they cannot ferment other sugars, such as xylose and arabinose. Both yeast (Saccharomyces and Pichia species) and bacteria (as Escherichia coli, Klebsiella, and Zymomonas) have been genetically engineered to ferment glucose and xylose to ethanol (Bothast et al., 1999; Dien et al., 2003; McMillan, 1996). However, commercial exploitations of these yeast and bacteria species are still somewhat restricted, mainly due to their low ethanol tolerance, slow rates of fermentation, difficulty in maintaining an optimal oxygen supply, and sensitivity to inhibitors generated during pretreatment of lignocellulosic substrates (Hahn-Hägerdal et al., 1994a; Saha, 2003). Therefore, during this comparison, a wild-type Saccharomyces cerevisiae was employed.

3.3.1.1 Introduction to results

As part of the initial work, the “compromise” conditions for SSF were first established. Then, in order to produce an ethanol stream of higher concentration, the effects of solids concentration and enzyme loading were compared when the SSF and SHF
processes were used (Figure 25). As previously mentioned, one of the drawbacks associated with SSF is the sensitivity to bacterial infection (Stenberg et al., 2000b), however this was overcome by supplementation with antibiotics during SHF and SSF processes (Figure 25).

Finally, the influences of constant pH and mode of substrate supplementation (batch or fed-batch system) were compared during combined hydrolysis and fermentation experiments (Figure 25).
Figure 25 Experimental procedure for comparison of SSF with SHF.

- **Corn fibre**
  - Pretreatment: 190°C, 5 min, 3% SO₂
  - Water soluble + insoluble streams
    - Separate Hydrolysis and Fermentation (SHF)
      - Solid consistency
      - Enzyme loading
      - Addition of antibiotics
    - Simultaneous Saccharification and Fermentation (SSF)
      - Constant pH
      - Batch/fed-batch system
  - Analysis of sugars, 5-HMF, furfurals, ethanol
3.3.2 Results and discussion

3.3.2.1 High substrate concentration SSF

The fermentability of the water soluble fraction obtained after steam explosion of corn fibre at the optimum pretreatment conditions (190°C, 5 min and 3% SO₂) was initially assessed at different temperatures (30, 37 and 40°C) and pH (5, 6) (data not shown). To simulate SSF conditions in terms of glucose concentration, the water soluble fraction was supplemented with synthetic glucose to obtain a final concentration of ~60 g L⁻¹. Conversions to ethanol with relative yields approaching 90% were attained at all temperatures and pH levels after 24 hours with the relative ethanol yield-\(Y_{\text{EtOH}}\) defined at the ratio of the ethanol yield of the filtrate and the reference fermentation. However, the fastest glucose consumption and ethanol production was observed at 30°C, followed by 37°C and 40°C. The pH did not influence the glucose to ethanol conversion at the temperatures evaluated.

It appeared that the water soluble fraction was readily fermented at 37°C. The hydrolysability of the pretreated corn fibre solids in the water insoluble fraction at 37°C and 45°C, and 5% consistency, was tested previously and demonstrated a 10% decrease in glucose yield at the lower temperature (37°C), after 100 hours of saccharification reactions (Figure 19, section 3.2.2.2). It was apparent that compromise conditions of pH 5 and a temperature of 37°C provided the best conditions for effective SSF.

The concentration of cellulose or cellulosic substrates used during SSF and SHF processes is an important considerations. Substrate loading affects production costs in several ways such as, higher substrate loading results in smaller-volume hydrolysis and fermentation reactors, thus lowering capital cost. It also yields higher ethanol
concentrations in the beer, and concurrently lowers the steam requirement in the ethanol stripping tower. Cellulosic substrates can be highly fibrous or have bulky irregular particles, and at high concentrations they are difficult to stir (Mohagheghi et al., 1991).

Distillation of the fermentation broth is energy demanding and for ethanol concentrations below 2% (w/w) the energy demand rises steeply (Olsson and Hahn-Hägerdal, 1996). Thus, high substrate concentrations, i.e., high concentrations of fermentable carbohydrates and thus high final ethanol concentration, are preferred (Olsson and Hahn-Hägerdal, 1996). Nevertheless, microorganisms have a limited tolerance to either the substrate or ethanol, or both, and sensitivity differs between genera. For example, *S. cerevisiae* has high ethanol tolerance, being able to grow at ethanol concentrations up to 120 g L\(^{-1}\) and fermenting over 300 g L\(^{-1}\) ethanol (Olsson and Hahn-Hägerdal, 1996). Similarly, Mohagheghi et al., (1991) showed that pretreated wheat straw can be efficiently fermented into ethanol at up to 15% cellulose concentration, but above this level, the yeast cells lose their viability due to ethanol inhibition (higher than 57 g L\(^{-1}\)). Therefore, during the initial comparison of SSF and SHF, the influence of solids concentration on the hydrolysis and fermentation profiles was first examined. Due to the fact that all experiments were performed in regular shake flasks, it was possible to use up to a 10% solids consistency during hydrolysis and fermentation of combined fractions (water soluble and insoluble streams).

The effects of solids consistency of pretreated corn fibre solids on the performance of SSF and SHF in the water soluble fraction are shown in Figures 26 A and B. First, a SSF of the mixture of water soluble and insoluble pretreated corn fibre (at 190°C, 5min and 3%SO\(_2\)) was performed, based on the preliminary results at compromise conditions (37°C
and pH 5) for 24 hours (Figure 26 A). For the SHF, the hydrolysis portion of the process was performed at 45°C and pH 4.8, for 48 hours (previously established optimum conditions for hydrolysis-Figure 19). After 48 hours, the enzymes were deactivated (by boiling) and the solid and liquid streams were separated by centrifugation. Then the pH of the liquid fraction was adjusted to pH 6 and the fermentation proceeded at 30°C for 24 hours (Figure 26 B). Therefore, the X-axis in Figure 26 B starts at t 48 hours, or the time when hydrolysis ended and fermentation started. Thus, the total time for hydrolysis and fermentation was 72 hours.

As is shown in Figure 26 A, the initial hexose concentration was between 23-27 g L⁻¹, but the final ethanol concentration was 22-32 g L⁻¹, which initially does not appear to follow the theoretical ethanol yield from glucose. However, it should be noted that, in the SSF process, hydrolysis and fermentation subprocesses take place simultaneously and, as soon as the glucose molecules are released during the hydrolysis process, S. cerevisiae converts them to ethanol. The initial hexose concentration, presented in Figure 26 A, only represents the concentration of sugars in the water soluble fraction, not the combined fractions, as the water insoluble cellulose fractions were still in their polymeric forms. Therefore, the starting sugar concentration was low. In the case of the SHF conditions, the concentration of hexose sugars at the beginning of the fermentation reflects the combined concentrations of monomers from the water soluble and insoluble streams, as the cellulose stream was 80% hydrolysed during the prior saccharification step.
Figure 26 Hexose consumption and ethanol production during A) SSF and B) SHF (with 10 FPU g cellulose$^{-1}$, an IU:FPU ratio of 2:1) of combined water soluble and insoluble fractions at 5, 7.5, and 10% consistency solids, pH 5 and no antibiotic supplementation. Error bars represent the range of duplicate experiments. The numbers in the legend present the relative ethanol yield (%). For SHF the X-axis shows the combined time of hydrolysis and fermentation and it starts at t=48 hours when the hydrolysis was completed.
As the consistency of pretreated corn fibre solids increased from 5 to 10%, conversion to ethanol increased, with yields of 81-87% of theoretical, obtained with the SSF experiments (Figure 26 A). Although the concentration of lactic acid was not measured, the final pH of the hydrolysates was measured at the end of each fermentation, and the cells were analysed under a microscope for the presence of \textit{Lactobacillus}. Although no contamination was apparent when 7.5 and 10% (w/v) substrate concentrations were used, when 5% (w/v) solids were mixed with the water soluble fraction, the pH at the end of the reaction increased to pH 6.5. Microscopic analysis of the sample indicated that it was likely contaminated by \textit{Lactobacillus} rods.

Substrate concentrations of 7.5% and 10% were found to be optimal for the SSF process. This was also observed in earlier studies (Mohagheghi \textit{et al.}, 1991; 2000a; Stenberg \textit{et al.}, 2000b). However, it does not explain why a lower conversion yield was observed when 5% consistency solids were used. It has been previously shown that low dry weights in the SSF process caused an increase in lactic acid production (Stenberg \textit{et al.}, 2000a; Stenberg \textit{et al.}, 2000b). The explanation for this behaviour may be that one or more components in the liquid from the pretreatment stage (which is present at higher concentrations when the dry matter content is higher) suppresses the growth of lactic acid bacteria (Stenberg \textit{et al.}, 2000a; 2000b). Another possible explanation is that the yeast releases substances such as amino acids (thiamine and guanine), necessary for growth of lactic acid bacteria (Challinor and Rose, 1954; de Olivia-Neto and Yokoya, 1997). Stenberg \textit{et al.}, (2000b) suggested that the release of these necessary substances takes place when the glucose level is low, probably because the yeast are stressed or dying. In this study, the volume of the water soluble fraction at all consistencies was constant because: a) all the
experiments were performed in the hemicellulose fraction, b) the solids were unwashed (the presence of water soluble fraction), and c) the final reaction volumes were the same (50mL). Moreover, the concentration of sugar degradation products (furfural and 5-HMF) was similar for all consistencies tested (Figure 29). Therefore, the lower concentration of glucose available during 5% SSF, compared to 7.5 and 10%, was probably due to the growth of lactic acid bacteria and thus lower ethanol yield, rather than the presence of different amounts of inhibitors in the water soluble streams.

It was apparent that the increased solids consistency had a greater negative effect on ethanol yield when using the SHF process, i.e., 6-17% lower, than when using SSF (Figure 26 B). Although the hexose to ethanol conversion was complete in all cases the cellulose to glucose conversion was the limiting step in this series of experiments (Figure 26 B). As expected, incomplete hydrolysis, especially at higher solids consistency, affected the overall ethanol yield. Moreover, it took 24 hours to complete all the SSF experiments (81-87% ethanol conversion), whereas 72 hours were needed to obtain 70-75% ethanol conversion during SHF.

3.3.2.2 Enzyme loading

Enzyme loading is one of the most important variables in SSF and saccharification, because it determines the yield, rate, and to a large extent, the cost of the process. During this series of experiments, an 8% (w/v) solids concentration was used for the reason that it seems to be the optimum consistency of solids for the SSF experiments (previous results), and it also mimicked the concentration of solids in the pretreated slurry after steam explosion. An increase in cellulase concentration from 10 to 20 and 40 FPU g cellulose\(^{-1}\) during 8% consistency SSF resulted in an increase in ethanol yield from 86 to 93 and 97%,
respectively, during the fermentation period investigated (Figure 27). However, the small differences in ethanol productivity at 20 and 40 FPU g cellulose$^{-1}$ (1.95 and 2.06 g L$^{-1}$h$^{-1}$, respectively) and ethanol yield suggested there was little benefit in increasing the cellulase loading past 20 FPU. Considering the economic pressures of the overall bioconversion of corn fibre to ethanol process, it may be possible to use as little as 10 FPU g cellulose$^{-1}$ to achieve 86% hexose conversion after 28 hours with 1.8 g L$^{-1}$h$^{-1}$ ethanol productivity. Spindler et al., (1991; 1989) performed several investigations using different levels of cellulase activity (7 to 26 FPU g cellulose$^{-1}$) and $\beta$-glucosidase:cellulase activity (0:1 to 8:1) on various herbaceous crops, and showed an increase in ethanol yield with increasing cellulase loading, although saturation seem to occur above 20 FPU g cellulose$^{-1}$, especially at higher ratios of $\beta$-glucosidase:cellulase. An increase in cellulase concentration from 10 to 20 and 40 FPU g cellulose$^{-1}$ during 8% (w/v) solids consistency SHF did not considerably influence ethanol yield during the saccharification and fermentation periods investigated (Figure 27). Similar ethanol yields (76-82%) were likely due to the incomplete hydrolysis of water soluble and insoluble streams after 48 hours of saccharification. Thus, the starting hexose concentration was comparable, producing similar ethanol concentrations (~21 g L$^{-1}$). In comparing SHF to SSF, a fivefold decrease in ethanol productivity (0.35 g L$^{-1}$h$^{-1}$) was observed due to SHF’s requiring sixty hours of combined time for hydrolysis and fermentation, versus SSF’s needing only twenty-eight hours.
Figure 27 Hexose consumption and ethanol production during SSF and SHF at 37°C with 10, 20 and 40 FPU g cellulose⁻¹ of the combined water soluble and insoluble fractions at 8% (w/v) consistency solids at an IU:FPU ratio of 2:1. Error bars represent the range of duplicate experiments. The numbers in the legend show the relative ethanol yield (%) and ethanol productivity (g L⁻¹ h⁻¹). The ethanol productivity was calculated as ethanol produced (g L⁻¹) within first 12 hours for SSF and 12+48 hours for SHF.
3.3.2.3 Antibiotics and nutrients supplementation

Once the effects of solids concentration and enzyme loading on the SSF profile had been established, the influence of antibiotics and nutrient supplementations were next tested. It has been often claimed that SSF is much less sensitive to contamination than SHF (Wyman et al., 1992). However, other studies have shown that this was not the case and, in fact, it was very difficult to avoid infection using an SSF approach (Stenberg et al., 2000a; Stenberg et al., 2000b). The effect of antibiotic supplementation (penicillin and streptomycin) during SSF and SHF was studied at different substrate consistencies in the water soluble fraction (Figure 28). It should be noted that, during the SSF and SHF experiments, non-sterile conditions were used.

As can be seen from Figure 28 hexose consumption and ethanol production during SSF and SHF with/without antibiotic supplementation were almost identical. The high fermentation yield during SSF (87-88% of theoretical) achieved after 24 hours for 7.5 and 10% (w/v) solids consistency, without addition of antibiotics, was probably due to the positive effect of the liquid fraction from the pretreatment stage on the fermentation, and high concentration of sugar available for fermentation, which was also observed in previous studies when the whole slurry of steam exploded spruce (Picea abies) was used in SSF processes (Stenberg et al., 2000b; Stenberg et al., 1998). The water soluble fraction obtained after pretreatment may influence ethanol fermentation positively and lactic acid production negatively, due to differences in the sensitivity of yeast and bacteria to inhibitors in the liquid fraction (e.g., 5-hydroxymethylfurfurals, furfurals, acetic acid).
Figure 28 Hexose consumption and ethanol production during (A) SSF and (B) SHF at 10FPU g cellulose\(^{-1}\) enzyme loadings of the combined water soluble and insoluble fractions at 5, 7.5 and 10\% (w/v) consistency solids at an IU:FPU ratio of 2:1 with/without antibiotic supplementation. Error bars represent the range of duplicate experiments. The numbers in the legend show the relative ethanol yield (%).
On the other hand, too high a concentration could also negatively affect the yeast. During this research, the utilisation of furfurals during SSF and SHF processes by \textit{S. cerevisiae} was also evaluated (Figure 29). It was apparent that the concentration of inhibitors in the water soluble fraction [5-HMFs (~0.8 g L$^{-1}$), and furfurals (~1.1 g L$^{-1}$)] were not high enough to affect yeast fermentation at the range of consistencies tested (5-10%) during the SSF process (Figure 29 A).

As previously stated, pretreatment conditions can cause carbohydrates to be degraded to furfurals and 5-hydroxymethylfurfurals (5-HMFs). It was shown earlier during the optimisation of SO$_2$-catalysed steam explosion (Chapter 3.1) that the relative concentration of furfurals in the water soluble fraction was higher compared to 5-HMF, at all conditions tested (Figure 12). During comparison of utilisation of inhibitors during SHF and SSF (Figure 29), this was also the case. However, the starting concentrations of 5-HMFs and furfurals were higher during SSF (0.8 and 1.1 g L$^{-1}$, respectively) compared to SHF (0.6 and 1.0 g L$^{-1}$, respectively). The difference in the concentration of sugar degradation products might be due to the additional hydrolysis step during SHF. However, in both cases the low concentration of sugar degradation products meant that fermentation was not affected. Furfural toxicity has been extensively studied in relation to ethanol production. For example, \textit{Escherichia coli} strain KO11 has a maximum tolerance of 3 g L$^{-1}$. However, culture growth and ethanol production can be affected at concentrations as low as 1 g L$^{-1}$ (Beall \textit{et al}., 1991). In addition, strains such as xylose-fermenting \textit{Candida shehatae} and \textit{Pichia stipitis} have been observed to be almost completely inhibited by furfural concentrations of 2-4 g L$^{-1}$ due to chemical reactivity with cellular components, thus damaging the cellular membrane and inhibiting metabolism (Delgenes \textit{et al}., 1996).
Figure 29 Utilisation of 5-HMF and furfurals during A) SSF B) SHF at various solid consistencies by *Saccharomyces cerevisiae*. Error bars represent the range of duplicate experiments.
However, the pretreatment conditions for SO₂-catalysed steam explosion of corn fibre were carefully tailored, and consequently the production of sugar degradation products was limited and would not likely affect the metabolic activity of the microorganisms.

It was apparent that both 5-HMF and furfurals were utilized faster during SHF when compared with SSF (3 and 6 hours, respectively, Figures 29 A, B). The differences in fermentation conditions during SSF and SHF probably influenced the consumption of sugar degradation products. As mentioned previously, one of the disadvantages of SSF is a need to compromise the temperature and pH for both hydrolysis and fermentation. Thus, a temperature of 37°C and pH 5, which was not optimal for growth of *S. cerevisiae*, probably resulted in slower consumption rates of the inhibitors.

In this study, it was also shown that no additional nutrient supplementation (yeast extract, glucose, peptone) was required at low yeast concentration (2 g L⁻¹) to achieve very high hexose to ethanol conversion, where other studies have suggested that at low yeast concentration it was necessary to increase nutrient level (Stenberg *et al.*, 2000b) or adapt the cells to the water soluble fraction (Kadam and Newman, 1997). Similarly, Kadam and Newman (1997) have shown that, using hybrid poplar as a raw material, the need for nutrients was higher when the whole slurry was used as a substrate, as opposed to only washed material, unless the yeast was adapted to the solution during inoculation. In contrast, the present study has shown that there is no considerable difference in the SSF profiles of water-washed vs. unwashed solids in the water soluble fraction at 8% solid consistency (Figure 30).
Figure 30 SSF profiles of water-washed vs. unwashed solids in the water soluble fraction at 8% (w/v) solid consistency and 20 FPU g cellulose\textsuperscript{-1} enzyme loadings of the combined water soluble and insoluble fractions an IU:FPU ratio of 2:1. Error bars represent the range of six experiments.
The higher ethanol concentration obtained after 28 hours for SSF, where the combined fraction of water soluble and water-washed, insoluble streams was used (28 g L\(^{-1}\), compared with 27 g L\(^{-1}\)) was probably due to differences in the concentration of sugar degradation products (Figure 30). During this set of experiments, the small portion of sugars removed during water-washing was supplemented by synthetic carbohydrates to match the concentration of sugars at the beginning of the SSF processes. Therefore, a slightly higher concentration of ethanol was produced during SSF where water-washed insolubles were combined with the water soluble fraction, 28 g L\(^{-1}\), compared to 27 g L\(^{-1}\) where unwashed corn fibre solids were used. The minimal difference in sugar degradation products is probably due to the separation procedure of solid and liquid streams. During fractionation of the solid and liquid streams of pretreated corn fibre, the liquid fraction will have been effectively removed from the solid matrix by centrifugation processes (less the 2% of total sugars remaining in the corn fibre solids–data not shown). Thus, most of the non-fermentable substances and inhibitors were already present in the mixture in the water soluble fraction and additional water-wash of solids was not necessary for efficient sugar to ethanol conversion. The similar SSF profiles of the hydrolysates where the unwashed or water-washed solids were combined with the water soluble fraction suggests that it is technically feasible to pretreat corn fibre at 190°C, 5 min and 3% SO\(_2\), and obtain a slurry at 8% (w/v) solids consistency. Without any subsequent fractionation or water-wash of solids, we can adjust the pH of the mixture to pH 5, perform SSF at 37°C for 28 hours and achieve a 91% of theoretical hexose-to-ethanol conversion.
3.3.2.4 Small and larger scale SSF for batch and fed-batch systems

Usually most cost estimations are based on lab-scale and, to some extent, pilot-scale data for individual process steps (Galbe and Zacchi, 2002b; Nguyen and Saddler, 1991; Wingren et al., 2003). Throughout this work, SSF experiments were performed in shake flasks with a working volume of 50 mL, where agitation and temperature were controlled in the incubator-shaker. In order to test the effect of constant pH, larger scale fermentations were conducted in magnetically stirred 500mL covered beakers (Fleaker®, Corning Inc., Corning, NY) equipped with pH, temperature and agitation controls. During high consistency solids SSF experiments (Chapter 3.3.2.1), the pretreated corn fibre solids (5, 7.5 and 10%) were initially added to the water soluble stream, to simulate a batch system. One of the main disadvantages in using high solid concentrations during SSF is the difficulty in achieving effective mixing. To increase the mass transfer rate, one can either use a more powerful agitator, or run SSF in a fed-batch mode (Ballesteros et al., 2002). The advantage of a fed-batch as opposed to a simple batch mode is that the initial concentration of insoluble solid material can be kept at a low level and thus facilitate agitation. Thus, to test the effects of constant pH and different modes of solids addition, vis-à-vis batch versus fed-batch systems, 500mL Fleakers® were employed during SSF of 8% and 8+4% (w/v) consistency solids, and compared to the regular shake flask experiments where pH was adjusted once (at the beginning of the experiment) (Table 11). The fed-batch scenario was simulated by initial supplementation of the water soluble stream with 8% (w/v) corn fibre solids and sequential addition of 4% (w/v) solids after 12 hours of SSF. Stirring difficulties that were experienced at 12% (w/v) consistency during SSF experiments (larger scale) limited solid substrate loadings to 8% and 8+4% (w/v).
Table 11 Ethanol concentration (g L$^{-1}$) and percentage of theoretical yield of ethanol (%) (after 48 hours) for small and larger-scale SSF experiments (50 and 300 mL) at 10 FPU g cellulose$^{-1}$ enzyme loadings of the combined water soluble and insoluble fractions at an IU:FPU ratio of 2:1 for batch and fed-batch systems at different solids consistency (w/v). Range values of triplicate experiments are given in brackets.

<table>
<thead>
<tr>
<th>Substrate loading (%)</th>
<th>Ethanol (g L$^{-1}$)</th>
<th>Percentage of theoretical yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>25.6 (0.3)</td>
<td>91</td>
</tr>
<tr>
<td>Small scale 8+4 (fed-batch)</td>
<td>28.4 (0.4)</td>
<td>79</td>
</tr>
<tr>
<td>12</td>
<td>26.3 (0.1)</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>27.6 (0.2)</td>
<td>96</td>
</tr>
<tr>
<td>Larger scale 8+4 (fed-batch)</td>
<td>30.7 (0.4)</td>
<td>85</td>
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</tbody>
</table>
By maintaining a constant pH of 5 in the larger-scale Fleakers® at 8 and 8+4% (w/v) solids consistency, an increase in ethanol production by *S. cerevisiae* by \( \sim 2 \, \text{g L}^{-1} \) after 48 hours of SSF was observed compared to the control, where regular shake flasks were employed where pH was adjusted once at the beginning of the experiment (Table 11). The importance of controlling pH during fermentation has been described previously. For example, Dien *et al.*, (1999) reported that lowering the pH below the optimum 6.5 to pH 6 during fermentation of corn fibre hydrolyates (by recombinant *E. coli* FBR 3) decreased sugar consumption by half. Similarly, lactic acid-forming bacteria have been shown to be the main contaminants in commercial, potable alcohol plants, but contamination could be minimized by using pH values below 5 (Olsson and Hahn-Hägerdal, 1996).

As seen from the data in Table 11, when comparing results of both SSF procedures at 12% and 8+4% (w/v) substrate concentration at 10 FPU g cellulose\(^{-1}\) enzyme loadings (smaller scale), an increase in ethanol yield from 73 to 79% of theoretical was achieved. Fed-batch experiments resulted in a better SSF performance compared with experiments in which substrate was added all at once. This was because, after partial saccharification, the mixture became more fluid (after 12 hours) and more substrate could then be added. This procedure allowed us to achieve higher SSF yields at lower enzyme loadings, while simultaneously achieving higher ethanol concentrations. Similar results were obtained during SSF of recycled paper-derived material by employing a fed-batch procedure (Ballesteros *et al.*, 2002). Fed-batch SSF permitted the workers to increase ethanol yield from 56 to 80% of theoretical at low enzyme loadings (10 FPU g substrate\(^{-1}\)) at 10% and 5+3+2% (w/v) consistency respectively (Ballesteros *et al.*, 2002).
3.3.3 Conclusions

To increase cellulose conversion, minimize end-product inhibition and simplify the overall process, the use of a simultaneous saccharification and fermentation process after steam explosion was investigated and compared to a separate hydrolysis and fermentation approach. It was shown that a solids concentration of ~8% (w/v) in the water soluble fraction during SSF experiments was optimal with regard to ethanol yield and productivity. By using these high solids concentrations which correspond to the concentration of solids in the water soluble stream after steam explosion, while adding the minimum amount of cellulolytic enzymes (10 FPU g cellulose\(^{-1}\)), \textit{S. cerevisiae} was able to convert the hexoses very efficiently.

It is possible to steam explode corn fibre at the optimum pretreatment conditions (190°C, 5 min and 3% \(\text{SO}_2\)), without any solid/liquid separation nor washing of solids, adjust the pH of the slurry (pH 5) and perform SSF at 10 FPU g cellulose\(^{-1}\) enzyme loadings to achieve 86% hexose to ethanol conversion at 8% solids consistency within 28 hours. This is compared to 75% conversion to ethanol during 60 hours using SHF. The ethanol concentration during SSF could be increased by constant control of the pH at 5 during the reaction and by using a fed-batch rather than batch system during high consistency solids (12% w/v) SSF. The ethanol productivity was approximately five times greater for SSF (1.8 g L\(^{-1}\) h\(^{-1}\)), when compared to SHF (0.36 g L\(^{-1}\) h\(^{-1}\)).

The advantages gained in increased productivity by performing simultaneous saccharification and fermentation outweighed the drawbacks of different temperatures and pH optima for the enzymes and the yeast. Although a very small \textit{S. cerevisiae} inoculum was used (2 g L\(^{-1}\)) in the SHF and SSF experiments, there was no need for either additional
antibiotics or nutrient supplementation during fermentation. There appeared to be minimal amounts of sugar degradation or naturally occurring potentially inhibitory products obtained during steam explosion of corn fibre, *S. cerevisiae* was able to convert the existing hexose sugar to ethanol during SSF very efficiently (87% ethanol conversion compared to theoretical), without the use of any detoxification methods. In order to minimize bacterial contamination, the water soluble and insoluble fractions were combined at an 8% (w/v) solids consistency. This prevented sugar deficiency for *S. cerevisiae*, which could result in the release of nutrients from dead yeast cells, and thus potentially stimulating bacterial growth.

Due to the unique chemical properties of corn fibre, the process integration was shown to be technically feasible. This was realized by minimising hemicellulose loss and formation of sugar degradation products, and enhancing the enzymatic mediated hydrolysability of the water insoluble fraction after SO$_2$-catalysed steam explosion.

Corn fibre did seem to be an “ideal substrate” in that pretreatment conditions could be used that not only maximised the conversion of the cellulose and hemicellulose derived sugars to ethanol, but also allowed considerable process integration. As many process steps such as fractionation, water-wash and delignification were not required when corn fibre was used as a substrate it was of considerable interest to see what effect the high carbohydrate conversion to ethanol and the elimination of multiple process steps would have on the estimated cost of producing ethanol when it is compared to a more recalcitrant substrate such as softwood.
3.4 Techno-economic evaluation of different process alternatives

3.4.1 Background

As shown in the previous chapter, it was possible to obtain good yields of sugars from both the cellulose and hemicellulose components of corn fibre while using pretreatment conditions that allowed the hemicellulose recovery and cellulose hydrolysis steps to be combined with the fermentation step. This was accomplished without any solid/liquid separation or wash of solids, while the pH of the slurry was adjusted to pH 5. The subsequent SSF was performed at 10 FPU g cellulose\(^{-1}\) to achieve an 87% hexose to ethanol conversion at 8% solids consistency within 28 hours, compared to 75% conversion to ethanol during 60 hours of SHF. This obvious technical success begged the question, “what are the possible economic impacts of different process alternatives—SHF versus SSF?”

For example, in terms of the subprocesses required during bioconversion of softwood to ethanol versus corn fibre to ethanol, the latter process when using corn fibre involves only two major process steps: pretreatment and simultaneous saccharification and fermentation (SSF), compared to the five stages typically used during bioconversion of wood or other agricultural residues (pretreatment, fractionation, delignification, hydrolysis and fermentation). Thus, we next wanted to determine what the economic benefits of process integration might be during the bioconversion of biomass to ethanol, particularly in terms of increased hydrolysis and fermentation efficiency.

Techno-economic models have been used in the past to provide assessments of both process and subprocess maturity and the probable production cost of the product (Gregg et al., 1998; Nguyen and Saddler, 1991; von Sivers and Zacchi, 1995; Wingren et al., 2003). It has been found that with processes which are at an early stage of development, there are also
a greater number of potential technologies to be evaluated (Gregg, 1996). Proving the worth of these technologies at pilot- or demonstration-scale facilities is both expensive and time-consuming. Thus, mathematical models have been developed to assist in optimizing and evaluating the level of development of these processes.

For this study, "STEAM", a flexible techno-economic model that can effectively model a "generic" hardwood/softwood-to-ethanol process (Gregg, 1996) was used. The general information about the model is presented in Appendix 4. In the past, the model has been used to illustrate the differences and similarities of each of the process steps when different wood substrates (softwood and hardwood residues) were used as starting feedstocks (Gregg, 1996). The model has also been used to identify which changes in the various process steps may have the greatest impact on the final cost of producing ethanol from wood substrates (Gregg, 1996; Gregg et al., 1998). In this way, it provides a useful tool in directing where research and development efforts should be focused to reduce the cost of producing ethanol from biomass feedstocks. In this study, the economic impacts of subprocess integrations were analysed, by comparing the production costs of softwood to ethanol with corn fibre to ethanol.

Because most cost estimations are based on small lab-scale and, to a limited extent, pilot-scale data for individual process steps, it is difficult to use them to obtain an absolute production cost (Galbe and Zacchi, 2002a; Gregg, 1996; Wingren et al., 2003). Thus, this study is not aimed at determining the absolute cost of ethanol production, nor an economic optimisation of its production, but rather at comparing the production cost of the two processes—SSF versus SHF.
As mentioned in the Results section (3.1), corn fibre has been estimated to include up to 30% arabinoxylan. Although five carbon sugars fermentation was not tested during this study, a single-parameter sensitivity analysis was used to provide some insight into the effect of five carbon sugar utilisation on production costs during the fermentation process.

3.4.2 Results and discussion

3.4.2.1 Comparison of different process alternatives for Douglas-fir and corn fibre

Due to the recalcitrant nature of lignin in softwoods, and the need for a high degree of utilisation of all carbohydrate components in this feedstock, the overall process that is conceptualized for the production of ethanol is suggested to involve numerous steps, including pretreatment, fractionation, delignification, water-wash, hydrolysis and fermentation (Figure 31) (Gregg et al., 1998; Yang et al., 2002).

During the optimisation of SO₂-catalysed steam explosion conditions for softwood residues it was shown that there is always a compromise between maximum hemicellulose recovery in a fermentable form, and complete enzymatic digestibility of solids, as a function of severity. The Forest Products Biotechnology group has accomplished this through the use of a less severe, single-stage pretreatment step (195°C, 4.5 min and 4.5% SO₂) (Boussaid et al., 1999; Wu et al., 1999). However, as a consequence of using milder pretreatment conditions, to ensure an extensive enzymatic digestibility by cellulases, a delignification process has been shown to be necessary (Yang et al., 2002). Additionally, in order to the remove delignification products, a subsequent water-wash was proven to be essential, thus causing a supplementary loss of the hemicellulose fraction (Yang et al., 2002).
When substrates such as wood have been used in pre-treatment we and other groups have typically fractionated the solids and liquid streams prior to enzymatic hydrolysis, primarily to recover the hemicellulose fraction from the solids (Mes-Hartree and Saddler, 1983; Robinson, 2003; Sinitsyn et al., 1982). Additionally, with substrates such as steam pretreated Douglas-fir, it was shown that in order to achieve effective hydrolysis, a delignification step was required in addition to the water-wash steps if effective enzymatic hydrolysis of cellulose-rich, water insoluble fraction was to be achieved. This consequently increased the number of subprocesses that were involved in the bioconversion of the softwoods to ethanol, plus much of the residual hemicellulose associated with the water insoluble fraction was lost during the delignification step (Robinson, 2003). An additional concern regarding the bioconversion of softwoods to ethanol is the dilute nature of the water soluble fractions that are obtained. Complete recovery of the hemicellulose sugars following steam explosion can result in very dilute process streams (less than 50 g L\(^{-1}\)), which can create economic disadvantages due to the associated equipment and energy costs (Robinson, 2003). In contrast, the work described in this thesis has shown that it is technically possible to complete enzymatic hydrolysis and fermentation steps in one reactor after pretreatment of the corn fibre (190°C, 5 min and 3 % SO\(_2\) (Figure 31). A very high overall carbohydrate for fractionation, delignification and water-wash steps, and with minimal enzyme (10 FPU g cellulose\(^{-1}\)) and yeast supplementation (2 g L\(^{-1}\)). Thus, it substantially reduced the complexity of the overall process to two major process steps (pretreatment and simultaneous saccharification and fermentation). Additionally, the combination of the water soluble and recovery after pretreatment (95%), with minimal formation of sugar degradation products, allowed us to achieve a high sugar hexoses to
Figure 31 Schematic comparing the bioconversion of Douglas-fir and corn fibre to ethanol.
ethanol conversion (87%), without the need insoluble streams during hydrolysis and fermentation allowed for better recovery of the hemicellulose residual associated with the water insoluble, cellulose-rich stream.

The contributions of the various subprocess steps on the capital, operational and total cost during Douglas-fir and corn fibre to ethanol conversion are shown in Table 12. The operating and capital costs of corn fibre processing were all calculated as a percentage of Douglas-fir total cost. Thus, the corn fibre total cost was only 48% of the Douglas-fir total.

As discussed previously, the bioconversion of corn fibre to ethanol did not require water-wash, alkaline peroxide treatment or lignin recovery that are required for the effective conversion of wood substrates such as Douglas-fir. Additionally, the simultaneous saccharification and fermentation approach could be effectively applied to corn to ethanol conversion. As is apparent from its absence from Table 12, the fermentation of five carbon sugars was not included for in the assessment of either softwoods or corn fibre.

Not surprisingly, the operating cost of carrying out the alkaline peroxide delignification step was the biggest subprocess contributor to the total cost of Douglas-fir to ethanol, as it accounted from almost 52% of the total costs (Table 12). Due to the production of dilute sugar streams, and the use of low consistency solids during the 48-hour hydrolysis process (2% w/v), the fermentation and hydrolysis steps contributed significantly to the overall high cost of ethanol production, 12% and 9% respectively (Table 12).
Table 12 Subprocess contribution (in %) to the capital, operational and total cost of ethanol for Douglas-fir and corn fibre (using SSF option).

<table>
<thead>
<tr>
<th>Subprocess</th>
<th>Douglas-fir</th>
<th></th>
<th>Corn fibre</th>
<th></th>
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<td>Capital</td>
<td>Total</td>
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<tr>
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</tr>
<tr>
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<td>3</td>
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</tr>
<tr>
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<td>3</td>
<td>9</td>
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<td>13</td>
</tr>
<tr>
<td>C6 Fermentation</td>
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<td>4</td>
<td>12</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Ethanol Recovery</td>
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<td>8</td>
<td>1</td>
<td>9</td>
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</tbody>
</table>

Σ=100

Σ=48
Consequently, the production of a dilute ethanol stream added to the high cost of ethanol recovery, 8%. When the total cost of producing ethanol from softwoods was compared with the use of corn fibre as the feedstock the simplification of the overall bioconversion process (elimination of fractionation, delignification, and water-wash) reduced the overall cost of ethanol production by 52%. It was also apparent that during corn fibre-to-ethanol processing via SSF, enzymatic hydrolysis was the most expensive step (25% of the total cost), followed by pretreatment (20% of the total cost).

However, one would expect greater differences in the ethanol cost, when comparing the Douglas-fir to corn fibre processes. By using higher solids consistency (8% (w/v)) during hydrolysis of the combined water soluble and insoluble streams of pretreated corn fibre, decreasing the time for saccharification and fermentation (from 96 to 28 hours), and using lower enzyme loadings (10 FPU g cellulose$^{-1}$, instead of 20 FPU g cellulose$^{-1}$), a greater economic difference between two processes might have been anticipated (Table 15-Appendix 1). However, in order to explain the outcome of the “STEAM”—techno-economic model, the chemical composition and relative “recalcitrance” of Douglas-fir and corn fibre should be compared. Although the total carbohydrate content for Douglas-fir and corn fibre are fairly similar (69.5 % and 75.9 % respectively) the five carbon sugar content differs significantly (Robinson, 2003). Xylose (~17 %), followed by arabinose (~10 %) are the most abundant five carbon sugar components of corn fibre, whereas Douglas-fir contains only 4 % of xylose and 1.2 % of arabinose (Robinson, 2003). Given that the fermentation of five carbon sugars to ethanol was not included in the model, almost 30 % of the original carbohydrates were not utilized in the fermentation process, resulting in an increased final production cost of ethanol from corn fibre.
3.4.2.2 Comparison of different process options: SSF versus SHF for corn fibre

Having established that simplification of the bioconversion of biomass to ethanol process decreased the overall cost of bioconversion of corn fibre to ethanol compared to softwoods, we next compared the different process options, i.e., SSF versus SHF, in terms of the overall process costs.

As shown in section 3.1, by optimizing SO2-catalysed steam explosion at 190°C, 5 min and 3% SO2 (w/w), it was possible to recover 95% of the sugars after pretreatment in a hydrolysable and fermentable form. Earlier, in section 3.3 it was shown that by using the SSF process, it was possible to achieve 87% of theoretical ethanol yield by utilizing only six carbon sugars after 28 hours' saccharification and fermentation of combined streams at 8% (w/v) solids consistency, whereas by employing SHF only 75% of theoretical ethanol yield was obtained after 60 hours of processing (Figure 32).

As detailed in the previous chapter, the simultaneous saccharification and fermentation process of the combined streams was shown to be technically feasible. We next used the “STEAM” techno-economic model to test the economic merits of this process alternative. The economic impacts of different process alternatives during bioconversion of corn fibre to ethanol: SSF versus SHF are presented in Table 13. When we compared the total costs for the same feedstock, the operating and capital costs of corn fibre SSF processing were all calculated as a percentage of SHF total costs, and not as the percentage of softwood total, as in Table 12. Thus, the operating, capital and total cost contributions are different for corn fibre as outlined in Table 12.

During comparison of different process options (SSF versus SHF) in corn fibre-to-ethanol processing, the hydrolysis step was the biggest contributor to the total cost (25% and
Figure 32 Schematic representation of bioconversion of corn fibre to ethanol, separate hydrolysis and fermentation (SHF) versus simultaneous saccharification and fermentation (SSF) including sugar recovery after pretreatment, ethanol yield and combined reaction time.

Corn fibre $\xrightarrow{\text{Steam Explosion}}$ (solid + liquid fraction) $\xrightarrow{95\%}$ SSF (87%-6C)-28 hours

$\xleftarrow{\text{HSF (75%-6C)-60 hours}}$
26%, respectively), followed by pretreatment (20% and 21%, respectively), and ethanol recovery (18% and 19%, respectively). Similar trends were observed when an "NREL model" was employed to predict the economic impacts of agricultural residues as a potential feedstock on the overall cost of the process (Elander, 2003). These workers also found that the hydrolysis step was the most expensive subprocess during corn stover-to-ethanol conversion (Elander, 2003).

The economic benefits associated with combining the water soluble and insoluble streams during bioconversion of corn fibre to ethanol during the SSF process are clearly evident during hydrolysis and fermentation, where a ~4% decrease in hydrolysis and fermentation costs was observed (Table 13). It was apparent that the production of a more concentrated ethanol stream during SSF influenced the operating cost of enzyme production and decreased cost of ethanol recovery (Table 13). The higher ethanol yield and better ethanol productivity achieved during SSF resulted in a decrease in the total cost of all of the subprocesses by 21%, when compared to the SHF option. Similar trends were observed when SSF was compared with SHF during the bioconversion of spruce residues to ethanol (Wingren et al., 2003). The ethanol production cost for SSF was approximately 10% lower when compared to SHF during processing of 5% (w/w) pretreated solids (Wingren et al., 2003). According to Wingren et al., (2003), the main reason for this result was that capital costs were lower and the overall ethanol yield was higher, which also proved to be the case for this study.

As mentioned previously, 30% of the sugars present in corn fibre are five carbon sugars (xylose and arabinose). Although \textit{S. cerevisiae} was able to ferment only six carbon sugars to ethanol, it was important to test the economic outcome of incorporation of xylose
Table 13 Subprocess contribution (in %) to the capital, operational and total cost of ethanol for corn fibre: SSF versus SHF.

<table>
<thead>
<tr>
<th>Subprocess</th>
<th>Corn fibre SHF</th>
<th></th>
<th></th>
<th>Corn fibre SSF</th>
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<tr>
<td>Pretreatment</td>
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<td>20</td>
<td>10</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Enzyme Production</td>
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<td>6</td>
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<tr>
<td>C6 Hydrolysis</td>
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<tr>
<td>C6 Fermentation</td>
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</table>

Σ=100                   Σ=79
and arabinose fermentation in the process. The effects of concurrent five and six carbon sugar fermentation during the SSF process are shown in Table 14. During the modeling, it was assumed that xylose and arabinose were converted completely to ethanol with 100% of theoretical ethanol conversion. This was done to establish a “best case” while realizing a more likely conversion would probably be with the range of 80-90%. The operating and capital costs of corn fibre processing during SSF when five and six carbon sugars were utilized (5 and 6 SSF) were calculated as a percentage of SSF when only six carbon sugars were fermented to ethanol (6 SSF) (Table 14). As is described in more detail below, when 5 and 6 carbon sugars were utilized during SSF the total cost was approximately 84% of the cost established when only 6C were employed during SSF (Table 14).

When five and six carbon sugars were included in the SSF process, hydrolysis was still the main contributor to the overall cost of the process, but utilisation of all the sugars decreased the total cost of corn-fibre-to ethanol to 18% as opposed to 25% which was the scenario when only hexoses were utilized by *S. cerevisiae* (Table 14). The incorporation of pentose sugars in the process scheme decreased the cost of pretreatment, feedstock handling and ethanol recovery, by 4%, 3% and 3%, respectively (Table 14). Thus, the total production costs were reduced by as much as 16%, when five carbon sugars fermentation was incorporated into the corn fibre-to-ethanol process (Table 14).

The problems associated with five carbon sugar fermentation have been described in greater detail in other work. For example, Asghari *et al.*, (1996) showed that the pretreated corn fibre hydrolysate was converted to ethanol efficiently by using *Escherichia coli* strain KO11 (Asghari *et al.*, 1996). Ethanol yield was 86% of the maximum theoretical yield.
Table 14 Subprocess contribution (in %) to the capital, operational and total cost of ethanol for corn fibre during SSF with/without five carbon sugars fermentation.

<table>
<thead>
<tr>
<th>Subprocess</th>
<th>Corn fibre, 5C and 6C SSF</th>
<th>Corn fibre, 6C SSF</th>
</tr>
</thead>
<tbody>
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<td>Feedstock Handling</td>
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<td>Pretreatment</td>
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<td>4</td>
</tr>
<tr>
<td>Enzyme Production</td>
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<td>0</td>
</tr>
<tr>
<td>C6 Hydrolysis</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>C6 Fermentation</td>
<td>6</td>
<td>2</td>
</tr>
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<td>Utilities</td>
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</tbody>
</table>

Σ=84  Σ=100
(0.51g ethanol g sugar⁻¹) (Asghari et al., 1996). However, it should be noted that during this fermentation, additional nutrients were required (including yeast extract, tryptone); and that complete utilisation of xylose required 72 hours of fermentation, while further detoxification of the hydrolysate was also necessary (Asghari et al., 1996). Clearly, the utilisation of five carbon sugar during fermentation is an important economic factor and in any further analyses, the additional costs associated with xylose and arabinose fermentation (removal of inhibitors, almost tripled fermentation time and additional nutrient supplementation) should be incorporated into the model.

3.4.3. Conclusions

The aim of this chapter was to investigate the influence of the number of subprocesses on the total ethanol cost both for corn fibre itself and when it was compared with softwood feedstocks. Additionally, it was intended to be an evaluation of relative production cost of two processes—SSF versus SHF—during bioconversion of corn fibre to ethanol. In this way they could be assessed on an equal footing, as opposed to an estimate of the absolute cost of ethanol production, or an economic optimisation of its production.

The model was first used to establish the economic impact of a reduction in the number of subprocesses during the bioconversion of corn fibre to ethanol, as compared with softwood to ethanol processing. By comparing the operating and capital costs of ethanol from Douglas-fir with corn fibre, the model predicted that process integration of the bioconversion of corn fibre to ethanol process (elimination of fractionation, delignification, and water-wash) could reduce the overall cost of producing ethanol by more than 50%.

The model indicated that the contribution of each of the subprocesses to the total cost was feedstock dependent. Although the delignification process was the main contributor to
the total cost of the process during bioconversion of softwood to ethanol, the model indicated that the hydrolysis step was the main contributor to the total production cost for all the cases analysed during bioconversion of corn fibre to ethanol.

Other areas identified by the model showed that employing a SSF process instead of SHF provided a substantial (21%) decrease in the overall cost of the process, which was primarily achieved by the higher ethanol yield and better ethanol productivity obtained when using this process option. In a similar fashion, the model also indicated that the fermentation of five carbon sugars concurrently with six carbon sugars during a SSF process could reduce the final cost of ethanol by an additional 16%, if 100% of theoretical ethanol yield during fermentation of xylose and arabinose were assured. It was apparent that the “STEAM” model provided a way to assess the cost of individual and integrated steps and the effect of various feedstocks (Douglas-fir, corn fibre) and process options (SSF versus SHF) on the economics of the process. Finally, it was shown that technically feasible process integration during bioconversion of corn fibre to ethanol i.e., steam explosion of corn fibre at the optimum pretreatment conditions (190°C, 5 min and 3% SO₂), followed by simultaneous saccharification and fermentation of pretreated slurry at 8% (w/v) consistency solids, was proven to be economically attractive compared to bioconversion of softwoods to ethanol, or separate hydrolysis and fermentation option for corn fibre (SHF).
4.0 CONCLUSIONS AND FUTURE WORK

4.1 General conclusions

As we had hypothesized at the beginning of this work, corn fibre has been shown to be an ideal lignocellulosic substrate with which to investigate the technical and economic feasibility of the biomass-to-ethanol conversion process. Due to the chemical and biological nature of corn fibre (low lignin and high sugar content) we were able to establish the optimum, mild steam pretreatment conditions which facilitated the recovery of most of the hemicellulose and cellulose derived sugars in a hydrolysable and fermentable form. It was apparent that the mild pretreatment conditions employed had a dramatic effect on all aspects of the process, from sugar recovery to hydrolysis yield. Thus, due to good sugar recovery and minimal sugar degradation in the hemicellulose fraction and high digestibility of the cellulose stream, there was no need for fractionation, delignification or water-wash during corn fibre processing, which consequently reduced the number of subprocess steps to just pretreatment and SSF.

During optimisation of SO$_2$-catalysed steam explosion of corn fibre, response surface modeling indicated that maximum sugar yields of 93% (hemicellulose derived water soluble, and cellulose, following enzymatic hydrolysis) were recovered from corn fibre that was pretreated at 190°C for 5 minutes after exposure to 3% SO$_2$. Through tailoring of the optimum pretreatment conditions for corn fibre processing, the results showed that SO$_2$ impregnation of biomass prior to steam explosion was beneficial. The relatively good enzymatic digestibility of pretreated corn fibre solids was proven by achieving a conversion efficiency of 81% of the combined original hemicellulose and cellulose in the corn fibre to...
monomeric sugars, which was increased to 91% by additional post-treatment of the hemicellulose fraction. It was apparent that the water soluble and insoluble fractions were readily fermentable to ethanol, resulting in good yields that were 89% and 94% of theoretical conversion, respectively, from the available hexose sugars. These yields were likely a consequence of the low concentration of sugar degradation products formed during the “compromised” pretreatment conditions (190°C, 5min and 3% SO₂) and also the chemical and biological nature of corn fibre.

Although the pretreatment conditions used for SO₂-catalysed steam explosion were proven to be optimal for maximum hemicellulose and cellulose recovery, it was evident that when employing water-washing and fractionation, the ethanol concentrations after fermentation of separate hemicellulose and cellulose streams would be too dilute for an economically feasible process. In an effort to increase the low sugar concentration, a strategy of combining the water soluble and insoluble fractions was applied, thus concurrently reducing the number of subprocess steps to pretreatment, hydrolysis and fermentation. When hydrolysing the whole slurry after pretreatment (including the hemicellulose and cellulose-rich fractions in order to increase sugar concentration and resulting ethanol yield), there was an apparent correlation between high fibre content and low cellulose conversion (80%). This was likely due to end-product inhibition limiting enzymatic hydrolysis of the cellulose. By increasing hydrolysis times, enzyme loadings, and the addition of protein prior to the saccharification step we were able to improve cellulose conversion. However, most of the options proposed would add to the already high cost of bioconversion.
To increase cellulose conversion, minimize end-product inhibition, and simplify the overall process, the use of simultaneous saccharification and fermentation process after steam explosion was investigated. This approach allowed us to simplify the bioconversion to the two stages: of pretreatment and SSF. By using the SSF option during bioconversion of corn fibre to ethanol, the problem of end-product inhibition was resolved, as evidenced by very high (86%) hexose to ethanol conversion at 8% solids consistency with minimum enzyme loading (10 FPU g cellulose$^{-1}$). The overall efficiency of the process was also improved, as evidenced by the fact that ethanol productivity was approximately five times greater for SSF when compared to SHF.

As mentioned previously, the SO$_2$-catalysed steam pretreatment and hydrolysis of softwood residues has proven to be a complex, multi-component process that, despite having made significant progress, remains difficult to establish in an economically viable fashion. Due to the unique nature of the substrate, by minimizing hemicellulose loss and formation of sugar degradation products and enhancing hydrolysability of the water insoluble fraction, the process modification of the bioconversion of corn fibre to ethanol was shown to be technically feasible. The natural progression to possible industrial scale-up of the bioconversion of corn fibre to ethanol would typically involve an economic evaluation of the overall process and the various process steps. It was found that technically feasible process integration during bioconversion of corn fibre to ethanol, i.e., steam explosion of corn fibre at the optimum pretreatment conditions, followed by simultaneous saccharification and fermentation of pretreatment slurry, was an economically attractive option when compared to bioconversion of softwoods to ethanol.
The “STEAM” model indicated that the input of each of the subprocesses to the total cost was feedstock dependent. Even though the delignification process was the main contributor to the total cost of the process during bioconversion of softwood to ethanol, for corn fibres the hydrolysis step was the main contributor to the total production cost for all the cases assessed in this study.

The economic modeling also indicated the importance of being able to incorporate five carbon sugar fermentation in corn fibre to ethanol processing, as its lack of utilisation decreased the cost of the process by up to 16%, when theoretical conversion of pentoses to ethanol was assumed.
4.2 Future work

Throughout this study, the possibility of using corn fibre to demonstrate the technical and economical bioconversion of biomass to ethanol has been a main focal point. However, there are several unanswered questions in this area. Future experiments, described below, should help to reach the ultimate goal of one day establishing a commercially viable bioconversion of corn fibre to ethanol process.

4.2.1 Five carbon sugar fermentation

The economic analysis of the corn fibre to ethanol process including SO$_2$-catalysed steam explosion and SSF has shown that, by including the five carbon sugars in the fermentation, the final cost of the process could be decreased by about 16%, compared to the scenario when only six carbon sugars were utilized. Therefore, as part of any future work, five carbon sugar fermenting microorganisms should be tested for their ability to utilize the hemicellulose derived sugars from steam-pretreated corn fibre during the SSF process. Due to the low concentration of sugar degradation products obtained after pretreatment and the efficient utilisation of six carbon sugars by $S$. $cerevisiae$ with no additional nutrient or antibiotic supplementation, it is probable that pretreated corn fibre could be readily fermented by either ethanologenic bacteria or genetically modified yeast that can coferment pentose and hexose sugars.

4.2.2 The use of novel hydrolytic enzymes

During the comparisons of SSF with SHF, end-product inhibition was a significant factor hindering hydrolysis progress during separate hydrolysis and fermentation when commercial enzymes were applied. As mentioned in the previous section the economic analysis of the corn fibre to ethanol process has shown that the hydrolysis step contributed
the most to the final cost of the process. Thus, the various cellulolytic enzymes which have been improved by groups such as Novozymes, Genencor and our Forest Products Biotechnology group, characterized by better hydrolytic performance than commercial enzymes in the absence of additional supplementation with β-glucosidases, could be utilized during the bioconversion of corn fibre to ethanol, possibly reducing the total cost of the process. In addition, preparation of enzyme cocktails, including hemicellulases, which would be added to the cellulolytic enzymes, should improve the efficiency of hydrolysis.

4.2.3 Scale-up of subprocesses

During this study, it was shown that by maintaining a constant pH of 5 in the larger-scale reactors (Fleakers®), an increase in ethanol production was observed, compared to the control, when regular shake flasks were employed. However, when higher consistency solids (12% (w/v)) were used during SSF experiments, incomplete utilisation of the substrate occurred even though larger-scale Fleakers® were employed. This was likely due to the limitations in mixing. The construction of a plant demonstration unit (PDU) for the bioconversion of lignocellulosic biomass to ethanol at UBC will allow the use of larger scale fermenters (10 L), with pH and oxygen probes, especially during SSF experiments where higher consistency solids are used (more than 12%).

4.2.4 Continuous process

As mentioned previously, one of the problems associated with SSF is the possibility of bacterial contamination (Lactobacillus) and corresponding production of lactic acid, especially when continuous operation is applied. Even though the problem of bacterial contamination was not an issue in this study, when considering a continuous process, the conditions enabling lactic acid production should be investigated.
4.2.5 Co-products analysis

As stated in the Introduction, although corn fibre is mainly composed of carbohydrates, other components, primarily protein and lipids, account for 15% (w/w) of the original biomass. To make the corn fibre-to-ethanol process economically viable by employing steam explosion and SSF, the utilisation of residual biomass after SSF (microorganisms plus unhydrolysed solids) should be evaluated, for example, as potential animal feed.
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### Appendix 1

**Table 15 Base case model assumptions.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Units</th>
<th>Softwood</th>
<th>Corn SHF</th>
<th>Corn SSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feedstock price</td>
<td>Can./$/ODT</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Pretreatment temperature</td>
<td>°C</td>
<td>195</td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td>Pretreatment residence time</td>
<td>s</td>
<td>270</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>SO$_2$ concentration</td>
<td>% (w/w)</td>
<td>4.5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pretreatment recovery yield</td>
<td>% of input biomass</td>
<td>89.5</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Enzyme price</td>
<td>Can./$/million FPU</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Cellulase loading</td>
<td>FPU g cellulose$^{-1}$</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>C6 Hydrolysis time</td>
<td>h</td>
<td>48</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>C6 Hydrolysis yield</td>
<td>% of theoretical</td>
<td>98</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>C6 Fermentation time</td>
<td>h</td>
<td>48</td>
<td>12</td>
<td>28*</td>
</tr>
<tr>
<td>C6 Fermentation yield</td>
<td>% of theoretical</td>
<td>85</td>
<td>100</td>
<td>87**</td>
</tr>
</tbody>
</table>

* combined time for hydrolysis and fermentation during SSF process
** fermentation yield at the end of SSF
Appendix 2

Figure 33 Percentage of monomeric sugars in (A) water soluble fraction of steam exploded corn fibre following different pretreatment conditions, and (B) hydrolysates (unwashed solids = solids + water extracted sugars) generated following sequential steam explosion and enzymatic hydrolysis (Bura et al., 2002).
### Table 16: Concentration of total lignin in water insoluble fractions (acid soluble+insoluble) as percentage of the original dry material.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Severity of pretreatment (Log R₀)</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>170°C, 1 min, 3%</td>
<td>2.06</td>
<td>13.4</td>
</tr>
<tr>
<td>150 °C, 5 min, 3%</td>
<td>2.17</td>
<td>13.0</td>
</tr>
<tr>
<td>170°C, 5 min, 0%</td>
<td>2.76</td>
<td>17.0</td>
</tr>
<tr>
<td>170°C, 5 min, 6%</td>
<td>2.76</td>
<td>21.9</td>
</tr>
<tr>
<td>170°C, 9 min, 3%</td>
<td>3.02</td>
<td>26.6</td>
</tr>
<tr>
<td>190°C, 5 min, 3%</td>
<td>3.35</td>
<td>29.4 (0.9)</td>
</tr>
<tr>
<td>210°C, 2.2 min, 1%</td>
<td>3.58</td>
<td>29.8</td>
</tr>
<tr>
<td>210°C, 2.2 min, 5%</td>
<td>3.58</td>
<td>30.9</td>
</tr>
<tr>
<td>210°C, 7.8 min, 1%</td>
<td>4.13</td>
<td>33.6</td>
</tr>
<tr>
<td>210°C, 7.8 min, 5%</td>
<td>4.13</td>
<td>41.9</td>
</tr>
<tr>
<td>230°C, 5 min, 3%</td>
<td>4.53</td>
<td>41.3</td>
</tr>
<tr>
<td>190°C, 5 min, 0%</td>
<td>3.35</td>
<td>27.1</td>
</tr>
<tr>
<td>190°C, 5 min, 6%</td>
<td>3.35</td>
<td>29.0</td>
</tr>
</tbody>
</table>

Values in parentheses are standard deviation, where n=9
Appendix 4

The wood-to-ethanol-STEAM model has been developed based on the integration and refinement of the two inherited spreadsheet format models (Forintek and VPI) (Gregg, 1996). As a compromise, a new model (named STEAM) was developed from these past models, using a spreadsheet program that possessed a number of the elements (a complete structured programming language, drawing capabilities and object-oriented controls) that could provide most of the capabilities present in object-oriented flowsheeting format (Figure 34) (Gregg, 1996).

Within the STEAM model, each process/subprocess is build from graphical objects representing the various unit processes/major pieces of equipment and their associated flowstreams (Figure 34) (Gregg, 1996). Both the graphical elements representing the unit processes/pieces of equipment and the flowstreams have associated properties that are accessed though input/output dialog boxes. In this case, the chemical, physical, process-related and economic attributes of the feedstock are shown. Each graphical object is linked to the next lower level of detail thought simply “clicking” with the mouse on the graphical representation or icon of the object. In addition, one can also look at different subprocesses by choosing the appropriate “workbook” (“Feedstock Database”, “Front-end Database”, “Process configuration”, “Cost details”, etc.). Each unit process/piece of equipment within a subprocess has associated properties, including calculation routines. For example, details for the steam reactor contained within the steam-pretreatment subprocess (Figure 34) can be accessed through going to the appropriate “workbook”-“Steam pretreatment”. This brings up the equipment option dialogs with the currently calculated values for the balance
(material and energy) and cost estimations (operating and capital) for this particular piece of equipment.

The ability to link various "workbooks" provides us with the capacity to link the various subprocesses into a complete process scenario. Linkages can be fixed, through methods such as worksheet-row-cell referencing or worksheet-cell naming conventions. Alternatively they can be more flexible and dynamic, through development of an "executive workbook" and/or an executive program. Consequently, there is a substantial level of flexibility now available to the model developer for designing and implementing a structure that is modularized and reflects a structure similar to flowsheet simulators or previous high-level language models (Gregg, 1996).

Because most cost estimations are based on small lab-scale, it is difficult to use them to obtain an absolute production cost of ethanol. Keeping in mind the limitations of the model (not updated prices of equipment units, uncompleted energy balance for all of the subprocess, and lack of yeast recycling during SHF process), this study was not aimed at determining the absolute cost of ethanol production, nor an economic optimisation of its production, but rather at comparing the production cost of the two processes—SSF versus SHF.
Figure 34 STEAM Model generic wood-to-ethanol process flowsheet (Gregg, 1996).