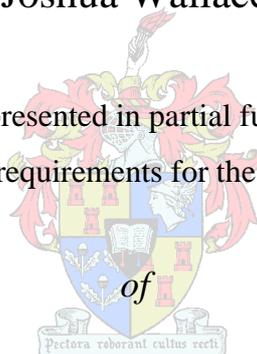


Enzymatic hydrolysis of steam pretreated
bagasse: Enzyme preparations for efficient
cellulose conversion and evaluation of
physicochemical changes during
hydrolysis

by

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of the requirements for the Degree



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Supervisor

Prof Johann Görgens

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ABSTRACT

Biomass recalcitrance represents the major technical obstacle in 2nd generation bio-ethanol production, as it impedes efficient enzyme action. Elucidating how the recalcitrant nature of lignocellulosic biomass inhibits the enzymatic degradation of cell wall polysaccharides could help to overcome these obstacles. This decrease in overall enzymatic hydrolysis performance in the industry, calls for the addition of different enzymes that work synergistically to alleviate slow cellulose to glucose conversion rates and low final glucose yields. Furthermore, high enzyme dosages (greater than 25FPU/g cellulose ~ approximately 75g enzyme/ kg cellulose) are required for rapid biomass conversion rates and complete hydrolysis, which have a negative effect on the viability of the process, as enzyme addition is a cost intensive part of biomass derived bio-ethanol production. In this regard, this study strived to improve the 2nd generation bio-ethanol industry by elucidating the slowdown phenomenon related to biomass recalcitrance during enzymatic hydrolysis.

The substrate and enzyme properties that affect enzymatic hydrolysis rate and yield were investigated. Additionally, screening and optimization of a commercially available enzyme cocktail for bio-ethanol production from steam pretreated sugarcane bagasse (SB) was performed. Therefore, in the present study, sugarcane bagasse was steam pretreated and subjected to enzymatic hydrolysis (Spezyme + Novozym 188) whereafter the kinetic of the conversion was studied. Furthermore, new commercially available cocktails (Cellic CTec2, Optiflow) were compared, and the dosage of the preferred cocktail (Cellic CTec2) optimized for the production of bio-ethanol from steam pretreated sugarcane bagasse. Additionally, xylanase (Cellic HTec2 and Multifect Xylanase), pectinase (Pectinex Ultra) and surfactant (Tween 80) supplementation was investigated for positive effects on glucose and xylose release, and on ethanol production.

Three distinctive phases of cellulose conversion rates were observed in succession of each other: (1) initial fast phase, (2) intermediate, slower phase, and (3) the slowest recalcitrant phase. The material was characterised by physicochemical analysis during each of the stages during enzymatic hydrolysis. The results indicated that the slowdown was caused by an increased lignin/cellulose ratio combined with changes in accessible surface area for enzymatic action (determined by Simons' staining). Xylanase supplementation was the only significant factor improving the cellulase cocktails. The positive effect of xylanase

on glucose and xylose release (rate and yield) was probably due to synergism between xylanases and cellulases, as well as the increased accessible surface area due to hemicellulose removal. Decreased solid loadings % (w/v) were also favorable for maximal cellulose conversion yield and rate with all enzymes studied. The optimized cocktail (0.15ml/g WIS Cellic CTec2 + 0.213ml/g WIS HTec2) resulted in 79.2% ethanol yield compared with 55% for the control cocktail, at equivalent volumetric dosages. The increased ethanol production yield and rate with the optimized cocktail was due to the presence of greater amounts of cellulase, xylanase, β -glucosidase and oxidative enzymes (GH61s). These enzymes possibly increased the accessible surface area for cellulose degradation vs. the control cocktail.

Altogether, the recalcitrance caused by increasing lignin/cellulose ratio with subsequent decreases in accessible surface area was the most important factors slowing down enzymatic hydrolysis rate and yield. However, it seems that newer generations of enzymes, such as CTec2, were less susceptible to the increasing biomass recalcitrance during enzymatic hydrolysis of lignocellulose resulting in higher product formation.

OPSOMMING

Die inherente weerstand van biomassa verteenwoordig die grootste tegniese probleem in 2^{de} generasie bio-etanol produksie. Deur te verstaan hoe die natuurlike weerstand van lignosellulosiese biomassa die ensiematiese hidroliese van die selwandsuikers vertraag kan help om hierdie weerstand te oorkom. In die lignosellulosiese bio-etanol indusitrie, lei hierdie vertraging in die ensiematiese reaksietempo en verlaagde suiker opbrengs, tot die gebruik van groot hoeveelhede ensieme. Bo en behalwe die vertraging van die reaksietempo, is die gebruik van hierdie hidrolitiese ensieme duur. Dus is die doel van hierdie projek, om die fisiese, asook chemiese verandering op die substraat wat tot die vertraging van die ensiematiese hidroliese lei, te ondersoek. Verder het hierdie studie daarna gestrewe om 'n kommersiële beskikbare optimale ensiem oplossing vir die produksie van bio-etanol, van stoom behandelde suikerriet bagasse, te identifiseer.

Dus is die stoom behandelde suikerriet bagasse ensiematies gehidroliseer (Spezyme + Novozym 188) waarna die reaksie-kinetieka bestudeer was. Nuwe generasie ensieme (Cellic CTec2, Optiflow) was ook met mekaar vergelyk en 'n optimale ensiem dosis was vasgestel vir die verkose ensiem (Cellic CTec2). Ondermeer was die voordele van die toevoeging van addisionele ensieme (Cellic HTec2, Multifect Xylanase, Pectinex Ultra) en Tween 80, op glukose, xylose en etanol produksie, bestudeer.

Drie onderskeidende fases van sellulose hidroliese tempo was opgemerk: (1) inisiële vinnige fase, (2) intermediêre fase en (3) verhardings fase. Die materie was onderworpe aan fisiese en chemiese analiese tydens al die stadiums gedurende ensiematiese hidroliese. Die resultate het aangedui dat die vertraging van ensiematiese hidroliese veroorsaak word deur verhoogde lignien/sellulose verhoudings, asook veranderings in die beskikbare oppervlakarea vir ensiem aksie. Xylanase toevoeging was die engste addisionele ensiem wat 'n voordelige uitwerking op sellulose hidroliese deur ensieme, veroorsaak het. Hierdie positiewe interaksie was heelwaarskynlik die gevolg van sinergie tussen die xylanase en sellulase ensieme, sowel as 'n vergrote oppervlakarea vir ensiem aksie as gevolg van hemisellulose hidroliese. Die geoptimeerde ensiem mengsel (0.15ml/g WIS Cellic CTec2 + 0.213ml/g WIS HTec2) het tot 79.2% etanol opbrengs gelei, teenoor 55% vir die kontrole. Hierdie verhoogde opbrengs was moontlik as gevolg van die verhoogte ensiem aktiwiteit in die geoptimeerde ensiem. Die addisionele ensieme teenwoordig in die geoptimeerde ensiem mengsel het waarskynlik gelei

tot groter oppervlakarea vir die sellulase ensieme, wat hoër etanol opbrengste tot gevolg gehad het.

Ten slotte, biomassa verharding was meestal toegeskryf aan die verhoogte lignien/sellulose verhouding soos ensiem hidroliese geskied, wat 'n verlaagde oppervlakarea vir ensiem-aksie tot gevolg gehad het. Die verlaagde oppervlakarea is verantwoordlik vir vertraagde ensiem reaksie-tempos en lae opbrengste. Nie te min, dit lyk asof nuwer ensieme, soos CTec2, hierdie verharding kan bestry, wat lei tot verhoogde produk vormasie in vergelyking met die kontrole ensiem mengsels.

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PREFACE

Although significant improvements in the bioconversion technologies for 2nd generation bio-ethanol production have been achieved during the last decades, its commercialization is still hampered by the cost and hydrolytic efficiency of the enzymes. In this context, this thesis addresses two aspects of enzymatic hydrolysis, which is presented in five chapters.

Chapter 1 introduces the physicochemical substrate characteristics and enzyme related factors that contribute to the slowdown of enzymatic hydrolysis rate and yield, as well as the importance of optimizing enzyme cocktails for the production of lignocellulosic bio-ethanol.

Chapter 2 comprises a literature review investigating biomass to bio-ethanol.

Chapter 3 contains the aims and objectives of this study identified by studying the literature.

Chapter 4 & 5 contains the experimental work aimed at addressing the aims and objectives of this study. The manuscript (**Chapter 4**) is introduced separately and written according to the style of the journal for which the manuscript was prepared and submitted.

Chapter 6 contains a general conclusion and remarks applying to the literature and experimental work, together with some suggestions for the future.

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ABBREVIATIONS AND NOMENCLATURE

| Abbreviation | Abbreviated Word |
|---------------------|---|
| ABF | α -Arabinofuranosidase |
| AFC | α -Fucosidase |
| AGL | α -1,4-Galactosidase |
| AGU | α -D-Glucuronidase |
| ASA | Accessible surface area |
| AXE | Acetyl (xylan) esterase |
| AXH | Arabinoxylan α -arabinofuranohydrolase |
| AXL | α -Xylosidase |
| AX | Arabinoxylan |
| BXL | β -1,4-Xylosidase |
| CBH | Cellobiohydrolyse |
| CBM | Carbohydrate-binding module |
| CBP | Consolidated Bioprocessing |
| CO | Carbon monoxide |
| CO ₂ | Carbon dioxide |
| CAC | Cellulose available for cellulase |
| CED | Cupriethylenediamine |
| CrI | Crystallinity index |
| CW | Cell wall |
| DB | Direct Blue 1 dye |
| DNS | Dinitrosalicilic acid |
| DO | Direct Orange 15 dye |
| DP | Degree of polymerization |
| DW | Dry weight |
| EC | Enzyme catalogue |
| EH | Enzymatic hydrolysis |
| EG | Endoglucanase |
| FAE | Feruloyl esterase |
| FPU | Filter paper units |
| FTIR | Fourier transform infrared spectrometry |

| | |
|--------------------------------|---|
| GH | Glucosidase hydrolase |
| GHGs | Greenhouse gasses |
| H ₂ SO ₄ | Sulphuric acid |
| HCA | Hydroxycinnamic acid |
| HFCs | Hydrofluorocarbons |
| HMF | Hydroxymethylfurfural |
| HPLC | High performance liquid chromatography |
| IDC | Industrial Development Corporation |
| IR | Infrared |
| IU | International units of β -glucosidase |
| KOH | Potassium hydroxide |
| LAC | β -1,4-Galactosidase |
| MAN | β -1,4-Endomannanase |
| MMgy | Million gallons per year |
| MND | β -1,4-Mannosidase |
| NaCl | Sodium chloride |
| NREL | National renewable energy laboratory |
| OD | Optical density |
| PCA | Principle component analysis |
| PFCs | Perfluorocarbons |
| rpm | Revolutions per minute |
| SB | Sugarcane bagasse |
| SEM | Scanning electron microscopy |
| SHF | Separate hydrolysis and fermentation |
| SO ₂ | Sulphur dioxide |
| SS | Simons' stain |
| SSF | Simultaneous saccharification and fermentation |
| UI | International unit of β -glucosidase activity |
| UN | United Nations |
| USA | United States of America |
| UV | Ultra violet |
| v/v | Volume per volume |
| w/v | Weight per volume |

| | |
|-----|--|
| w/w | Weight per weight |
| XDR | xylitol dehydrogenase |
| XEG | Xyloglucan-active β -1,4-endoglucanase |
| XI | Xylose isomerase |
| XLN | β -1,4-endoxylanase |
| XR | Xylose reductase |

| Abbreviation | Abbreviated Word | Units |
|---------------------|-------------------------|--|
| Å | Angstrom | 1×10^{-10} meter = 1×10^{-1} nm |
| nm | Nano meter | 1×10^{-6} meter |
| mL | Milliliter | 1×10^{-3} litre |
| kV | Kilovolt | 1×10^3 volts |
| mA | Miliampere | 1×10^{-3} ampere |
| cP | Centipoise | 1×10^{-2} poise = 1×10^{-2} P = 1×10^{-3} Pa.s |
| MPa | Mega Pascal | 1×10^6 Pascal = $1 \text{ N} / \text{mm}^2 = 10 \text{ bar} = 1000 \text{ kPa}$ |

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Chapter 1: Plant Biomass as Bioenergy

1.1 INTRODUCTION

The world runs on fossil derived energy such as oil (petroleum), coal and natural gas (Sarkar et al., 2012). These energy sources are used for the production of electricity, fuel and as chemical building blocks for the production of the vast majority of consumer goods (Uihlein & Schebek, 2009). The growing population on earth, which reached 7 billion in October 2011, has a growing demand for energy and fuel (United Nations, 2011). Combined with the rapid industrialization of emerging economies, energy consumption is increasing (da Costa Sousa et al., 2009). The combustion of fossil derived energies produces Greenhouse gasses (GHG) such as carbon dioxide (CO₂), nitrous oxide (N₂O) methane (CH₄), sulfur hexafluoride (SF₆), hydrofluorocarbons (HFCs), and perfluorocarbons (PFCs) (Cherubini & Jungmeier, 2009; Solomon, 2007). Additionally, carbon monoxide (CO), produced mainly due to incomplete combustion (partial oxidation) by motors, increases the lifetime of GHGs compounding their negative effect on the environment (Wang & Prinn, 1998).

Petroleum based fuels are the primary energy source driving the transportation sector, and it has been estimated that 61.5% of the world's oil consumption can be attributed to this sector (International Energy Agency, 2012). Furthermore, the transport sector contributes the majority of global CO and significantly to global CO₂ emissions (Goldemberg, 2008). Considering the increasing number of vehicles, subsequent increased GHG emissions, and depletion of fuel reserves merits investigation into sustainable fuel sources (Alvira et al., 2010a; Balat, 2011; CAPP, 2012; da Costa Sousa et al., 2009; EIA, 2012; Huang et al., 2011). Various governments, global and commercial organizations share this view (Bonass & Rudd, 2010). In 1997, 160 countries acknowledged that GHG are a problem and signed the Kyoto protocol in Japan. These countries, including South Africa, thereby committed themselves to take measures to reduce GHG emissions (United Nations, 1997). Various alternative fuel sources for the transportation sector are being investigated, including biomass based

fuels such as bio-ethanol and bio-diesel, Fischer-Tropsch fuels, natural gas reserves, solar fuels, boron, methanol, hydrogen and electricity (Balat, 2011).

However, biomass is currently the most viable substrate for the production of fuel and chemicals (Cherubini & Jungmeier, 2009; Lynd & Wang, 2003). Globally, policies encouraging the use of biomass for fuel have gained momentum (Sarkar et al., 2012). Biofuels show economic, environmental and sustainability advantages over fossil fuels on various levels. Some of the above mentioned advantages include: abundance of biomass material for fuel production, reduction of GHG, renewability and agricultural development (Demirbas, 2008).

The South African cabinet approved a national bio-fuels industrial strategy on 5 December 2007 (Department of Minerals and Energy, 2007; Esterhuizen, 2009; Van Zyl & Prior, 2009). The short term strategy aimed at 2% penetration of bio-fuels in the liquid fuel sector, which equates to estimated 400 million litres of bio-fuel per year (Esterhuizen, 2009; Van Zyl & Prior, 2009). If the target is met, bio-fuel would contribute to 30% of SA's renewable energy targets by 2013 (Esterhuizen, 2009; Van Zyl & Prior, 2009). The proposed blending of bio-fuel with petroleum based fuels is 8% (E8) and 2% (B2) for bio-ethanol and bio-diesel respectively (Esterhuizen, 2009; Van Zyl & Prior, 2009). Even though the white paper on mandatory blending has passed, presently the act has not been implemented (Department of Energy, 2012). Therefore, the bio-fuels industry in South Africa is still lagging. The most significant initiative to date involves two government owned institutions namely the Industrial Development Corporation (IDC) and the Central Energy Fund (CEF) (Esterhuizen, 2009; Van Zyl & Prior, 2009). They plan to invest R5.1 billion into the production of greener liquid fuels, with the construction of a 90 million litre sorghum to ethanol plant in Cradock, Eastern Cape, spearheading the developments (Buthelezi, 2012). There are additional prospects of constructing a 100 million litre sugarcane to ethanol plant in Mpumalanga and a 150 million litre sugar cane to ethanol plant in the Pondoland area between the Eastern Cape and KwaZulu-Natal (Esterhuizen, 2009; Van Zyl & Prior, 2009).

Bio-ethanol, which represents the majority of biomass-derived fuels, can be produced from the biochemical conversion of biomass to ethanol and used as a gasoline additive directly in cars (Balat, 2011). Ethanol is typically blended (10% ethanol, 90%

gasoline) for use in most cars; however, flex-fuel cars can use high ethanol concentrations (85% ethanol, 15% gasoline). Under the European Union quality standard (228), 10% bio-ethanol added to 90% gasoline is permitted (European Fuel Oxygenates Association, 2009). Global bio-ethanol production is set to reach 100 billion liters in 2015, a significant increase from the 84.6 billion liters produced in 2011 (Licht, 2006; Renewable Fuels Association, 2012). The major bio-ethanol producers, the United States (52.6 billion liters/year) and Brazil (21.1 billion liters/year), accounts for 87.1% of global bio-ethanol production (Renewable Fuels Association, 2012). The production of bio-ethanol on large scale is mostly derived from cornstarch in the United States and the sucrose rich sugarcane syrup in Brazil (Sarkar et al., 2012). Production of fuel ethanol from food crops such as starch (corn, sorghum, triticale) and sugar syrup is dubbed 1st generation bio-ethanol, which biggest disadvantage is that it competes with the food and animal feed industry (Cherubini & Jungmeier, 2009).

However, bio-ethanol can also be produced from lignocellulosic biomass such as crop residues, sawdust, wood chips, grasses, waste paper and municipal solid waste (Tomás-Pejó et al., 2008). Bio-ethanol derived from lignocellulose is termed 2nd generation- or cellulosic ethanol and it has been estimated that 491 billion liters of 2nd generation bio-ethanol could be produced annually (Kim & Dale, 2004). Presently, pilot plants for cellulosic ethanol production are in operation worldwide, while several commercial-scale plants are in construction, as shown by the summary of global industrial enzyme producer, Novozymes (**Figure 1.1**)

CELLULOSIC ETHANOL IS COMING

LARGE SCALE PRODUCTION PLANTS UNDER CONSTRUCTION (CAPACITY IN MILLION US GALLONS PER YEAR)



AND WE ARE JUST GETTING STARTED
 In 2014 the estimated production capacity of cellulosic ethanol is about 250 million US gallons. According to Bloomberg New Energy Finance there is enough biomass available to produce 93 billion US gallons of cellulosic ethanol in 2030.

Source:
 "Moving towards a next generation ethanol economy" Bloomberg New Energy Finance, 2012 and public information on large scale production plants (biochemical conversion only) under planned construction

Click here to see the report and other info-graphics at novozymes.com

Figure 1.1: Large scale cellulosic ethanol plants in construction. Reproduced with permission from Novozymes, 2012.

The production of lignocellulosic bio-ethanol involves 4 stages: pretreatment, enzymatic hydrolysis, fermentation and distillation (**Figure 1.2**) (Limayem & Ricke, 2012a). Lignocellulosic biomass is composed of three major components: cellulose, hemicellulose and lignin, with the polysaccharides (cellulose & hemicellulose), representing up to 75% of the total material (Zhao et al., 2012). However, lignocellulosic biomass is very recalcitrant to biological (enzymatic, microbial) degradation, as a result of its compact and rigid physicochemical matrix (Limayem & Ricke, 2012a). This recalcitrance negatively affects the saccharification of lignocellulosic biomass by decreasing the rate and yield of enzymatic hydrolysis (EH), which hinders the release of fermentable sugars, thereby representing one of the greatest obstacles to the commercial production of 2nd generation bio-ethanol (Himmel et al., 2007; Zhao et al., 2012). The physicochemical substrate factors implicated in hindering and slowing down the enzymatic hydrolysis are: lignin content, hemicellulose content, acetyl groups, crystallinity, degree of polymerization (DP) and the accessible- (ASA) and specific surface area (SSA) (Arantes & Saddler, 2011; Gupta & Lee, 2009; Hallac & Ragauskas, 2011; Rollin et al., 2011; Tejirian & Xu, 2011; Zhu et al., 2008). These factors and will be discussed in detail (**Chapters 2 - 4**).

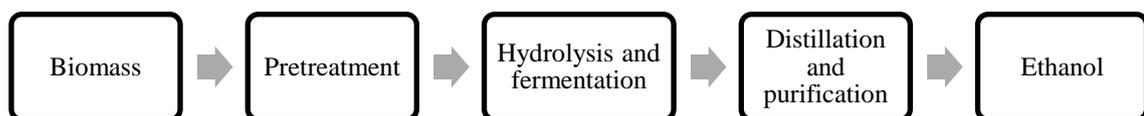


Figure 1.2: Simplified representation of bio-ethanol production from lignocellulosic biomass. Redrawn from (Seabra et al., 2010).

Therefore, one of the most important steps in the production of lignocellulosic bio-ethanol is pretreatment, which aims to improve enzymatic digestibility (defined as the maximal yield of EH at high enzyme dosages) of the feedstocks by decreasing physicochemical barriers implicated in biomass recalcitrance (Himmel et al., 2007). Even though pretreatment is vital in exposing the complex lignocellulosic sugars for degradation into monomers by enzymes, the release of these monomeric sugars needs to be balanced against possible inhibitor production during pretreatment (The most

significant inhibitors include furfural, hydroxymethylfurfural (HMF) and acetic acid, which are released from hemicellulose (Van Dyk & Pletschke, 2012).

Similar to the importance of the pretreatment method chosen, the addition of the right enzyme mixture is crucial to efficiently hydrolyse (high rate and ultimate yield) the recalcitrant lignocellulosic substrate, and depends on the feedstock and pretreatment method applied (Kazi et al., 2010; Kristensen et al. 2009). In addition to the physicochemical substrate features of lignocellulosic materials that hinder efficient EH, there are intrinsic difficulties with the enzymes themselves. Enzymes used in biomass degradation such as cellulases have low specific activity (compared with amylases, used in EH of cereal grain) (Merino & Cherry, 2007). The intrinsic recalcitrance of lignocellulose combined with inefficient enzyme mixtures, result in the saccharification rate falling off sharply as enzymatic hydrolysis proceeds (Arantes & Saddler, 2011). Therefore, it is necessary to use great amounts of enzymes, which can contribute up to (20 - 40%) of the overall production cost of bio-ethanol from lignocellulose (Duff & Murray, 1996; Klein-Marcuschamer et al., 2012; Nielsen 2012; Stefano Macrelli et al., 2012). The high enzyme production and utilization cost makes the EH step a critical point in the global cost of ethanol production (Brijwani et al., 2010; Fang et al., 2009). Numerous factors contribute to the reduction in the hydrolysis rate, and although there is no consensus on which factors are most influential, there is a general classification of factors into two groups/categories: those related to the substrate recalcitrance and those related to the mechanism and interactions of the cellulases (Arantes & Saddler, 2011). These factors were investigated in the present study (**Chapters 4 & 5**). Additionally, selecting feedstocks that are more digestible or less recalcitrant is favorable. Moreover, there is a wide variety of enzyme producers working towards releasing biocatalysts with increased performance and desirability. Therefore, choosing/developing the right enzyme cocktail for the feedstock used in the production is crucial towards economic feasibility.

Chapter 2: Literature review: Biomass to bio-ethanol

2.1 BIOMASS

2.1.1 Sugarcane bagasse as feedstock

Selecting a feedstock is an important parameter in the overall process development for the production of cellulosic ethanol (Sarkar et al., 2012). There are various feedstocks available, with individual advantages and disadvantages (Van Dyk & Pletschke, 2012). High sucrose containing feedstocks such as sugar cane, fruit, sugar beet, and sweet sorghum as well as starch containing material such as corn, milo, wheat, rice, potatoes, cassava, sweet potatoes, triticale and barley are commonly used in so-called first generation bio-ethanol production (Balat, 2011).

One major pitfall in bio-ethanol production from agricultural residue products is the availability of material (Tomás-Pejó et al., 2008). In an economy such as South Africa, the market price of farm produce varies greatly due to the volatile nature of international oil and currency fluctuations. Additionally the land availability, crop yields and environmental factors in the production of biomass feedstocks are very uncertain (Berndes et al., 2003). This cascades down to the agricultural sector and can therefore influence the amount of raw material available for bio-ethanol production. However, it is possible that SB can be obtained by improving sugar-milling processes or as the by-product of first generation bio-ethanol plants (Stefano Macrelli et al., 2012). Therefore, the conversion of SB to cellulosic bio-ethanol can potentially run parallel with first generation bio-ethanol plants and the food sector, contributing to value adding.

2.1.2 Sugarcane cultivation

The present study is part of a larger project looking at sugarcane development as a feedstock for new bio-ethanol production plants such as those planned by the IDC.

Sugarcane is a tall perennial grass that belongs to the genus *Saccharum*, family Poaceae. This inhomogeneous grass is composed of a soft central pith containing most of the valuable sucrose enclosed in peripheral fibers of lignocellulose (Nassar et al., 1996). Sugarcane belongs to the C4 group of plants giving it various advantages over other crops. Some of these advantages include higher CO₂ assimilation and far greater photosynthesis efficiency leading to greater sucrose and biomass production than other crops (Babu, 1990). These qualities lead to sugarcane being widely cultivated in the modern world. Relative to South Africa's size it produces competitive amounts of sugarcane, being the 14th largest sugarcane producer in 2010 (**Table 2.1**).

Table 2.1: Sugarcane production for various countries in 2010 (Food and agricultural organisation of the United Nations: Economic and Social Department: The statistical Division, 2010; South African Sugar Association, 2012)

| <i>Country</i> | <i>Production (Tons)</i> |
|----------------|--------------------------|
| Brazil | 717 462 000 |
| India | 292 300 900 |
| China | 111 454 359 |
| United States | 24 820 600 |
| South Africa | 16 015 600 |

The most productive sugarcane areas in South Africa are Kwazulu-Natal, Mpumalanga and the Eastern Cape (South African Sugar Association, 2012). The sugarcane industry plays a pivotal role in employment and economic growth in South Africa (South African Sugar Association, 2012). There are approximately 35 300 registered sugarcane growers in South Africa, producing an average of 20 million tons of sugarcane per annum (South African Sugar Association, 2012). The south African sugar industry directly employs approximately 79 000 people, mostly in cane production and processing (South African Sugar Association, 2012). Additionally, indirect industry support jobs are estimated at 350 000 (South African Sugar Association, 2012). The 14 sugar mills in South Africa are: Illovo Sugar Ltd and Tongaat Hulett Sugar Ltd each own four mills, Tsb Sugar RSA Ltd three mills with

Gledhow Sugar Company (pty) Ltd, UCL Company Ltd and Umfolozi Sugar Mill (pty) all possessing one mill respectively (South African Sugar Association, 2012).

Sugarcane bagasse is a term referring to the remnants of sugarcane after extraction of the sucrose rich juice for sugar or 1st gen ethanol production (Rasul et al., 1999). The fibrous SB consists mainly of 70% short fibers and 30% long fibers per dry biomass (Mwasiswebe, 2005). SB is a plentiful source of lignocellulosic material and the use of SB as lignocellulosic raw material for bio-ethanol production is well established (Cardona et al., 2010; Geddes et al., 2011a; Hernandezsalas et al., 2009; Sindhu et al., 2010a). The industrial SB used in the present study is depicted in **Figure 2.1**.



Figure 2.1: Photo showing a bag full of milled industrial SB used in the present study, before steam pretreatment.

Previously it has been shown that for every 1000 kg of sugarcane, 100 kg of raw sugar is produced as well as 270 kg of SB (Drummond & Drummond, 1996). Furthermore, South Africa produced an average of 20 million tons of sugarcane per

annum between 2005 and 2012 (South African Sugar Association, 2012) Therefore, it can be estimated that South Africa produces 5.4 million tons of SB annually. However, currently SB is mainly used for animal feed and energy production within the existing sugar mills and cannot be made available for bio-ethanol production without disrupting existing factories. Therefore, this project fits into research and development regarding the construction of new ethanol facilities with combined 1st and 2nd generation ethanol production on the same industrial site. However, there is a great potential for the expansion of sugarcane as a bioenergy crop in sub-Saharan Africa due to vast areas of available pasture and suitable cropland (Smeets et al., 2004; Watson, 2011). By using Geographical Information Systems (GIS), in combination with knowledge of the habitat requirements for sugarcane, 6 million hectares of land has been identified as suitable for sugarcane bioenergy crop expansion by The Cane Resources Network for Southern Africa (CARENSA) (Johnson & Matsika, 2006; Johnson et al., 2007; Watson, 2011). Additionally, the 6 million hectares identified excludes wetlands, closed canopy forests, protected lands, areas with (climate, terrain and soil) constraints and areas under food and crop production (Watson, 2011). Therefore, the potential growth for the bioenergy sugarcane industry is not at the cost of food security and biodiversity losses (Watson, 2011).

2.2 LIGNOCELLULOSE COMPOSITION

2.2.1 The plant cell wall

The plant cell wall (CW) comprises two phases: a microfibrillar phase as well as a matrix phase (Brett & Waldron, 1996). The highly crystalline microfibrillar phase consists of cellulose and has a relatively homogenous chemical composition (Brett & Waldron, 1996). Furthermore the matrix phase involves various components including pectins, hemicellulose, phenolics and proteins (Brett & Waldron, 1996). The different frameworks of lignocellulosic material can be seen in **Figure 2.2**. The figure shows the subdivisions of the plant CW between primary and secondary CWs. The primary and secondary CW differs considerably regarding the distribution of lignin, cellulose and hemicellulose within them (Menon & Rao, 2012). Cellulose is greatly distributed within the secondary CW (made up of secondary CW 1, 2 & 3), with secondary CW 2 being the thickest and richest in cellulose (**Figure 2.2**). On the other hand, lignin is the greatest component of the middle lamella, which connects the surrounding cells (Pandey,2009).

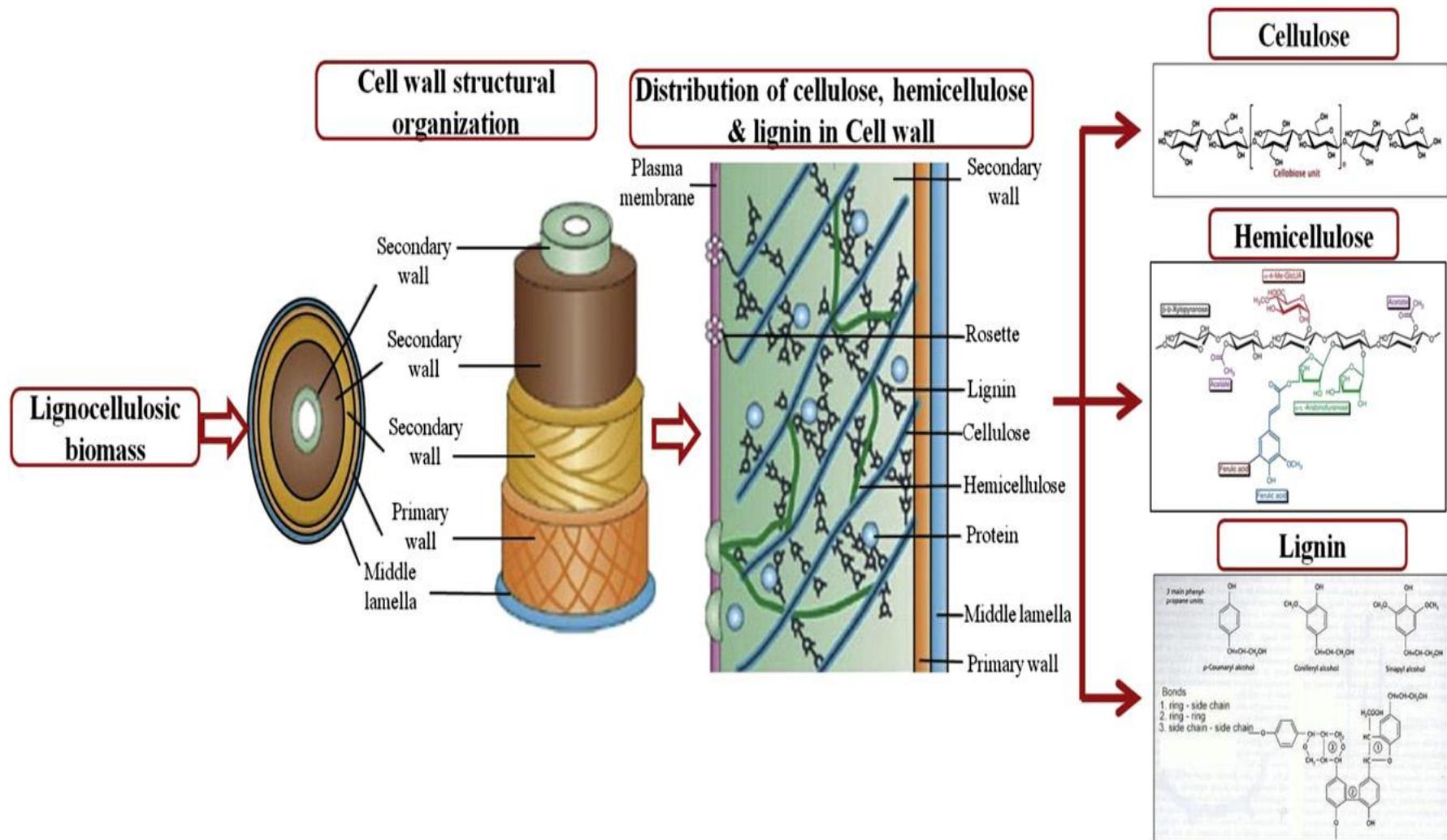


Figure 2.2: Representation of the structural order within lignocellulosic biomass (Menon & Rao, 2012). Reprinted with written permission from Elsevier and their Copyright Clearance Center (RightsLink service).

In addition, various groups have identified the major chemical composition of SB although results vary. A comparison of the chemical composition of raw SB found in literature and the raw SB used in the present study can be found in **Table 2.2**. Differences depicted in the results may be due to differences regarding sugarcane varieties, harvesting method, milling method, climate, shape, age, size, density and analytical procedures. Based on previous reports, the chemical composition of SB is approximately: 40% cellulose, 28% hemicellulose, 20% lignin and 12% extractives and ash.

Table 2.2: Comparison of SB chemical compositions previously reported

| <i>Cellulose*</i> | <i>Hemicellulose*</i> | <i>Lignin*</i> | <i>Extractives & Ash*</i> | <i>Reference</i> |
|-------------------|-----------------------|----------------|-------------------------------|---------------------------------|
| 34 | 27 | 18 | 21 | (Sindhu et al.,2010a) |
| 40 - 50 | 25 | 25 | - | (Pandey, 2000) |
| 25 -40 | 25-50 | 10 -30 | - | (Demirbaş, 2005) |
| 41.3 | 22.6 | 18.3 | 16.6 | (Zanzi, 2001) |
| 35 - 50 | 20 - 30 | 20 - 27 | 8 – 12 | (Garzia-Perez et al., 2001) |
| 45.0 | 31.8 | 20.3 | 2.9 | (Zhao et al., 2009) |
| 43.1 | 31.1 | 11.4 | 14.4 | (Martin et al., 2007) |
| 35.0 | 35.8 | 16.1 | 13.1 | (Sasaki, 2003) |
| 40.2 | 26.4 | 25.2 | 8.2 | (Neureiter et al., 2002) |
| 38.9 | 26.2 | 23.9 | 11.0 | (Aguilar, 2002) |
| 39.6 | 29.7 | 24.7 | 6.0 | (Teixeira et al., 2000) |
| 39.6** | 28.4** | 20.3** | 11.7** | Average from previous reports |
| 44.0 | 28.4 | 22.3 | 6.3 | This study (Chapter 4) |

*Results represent % w/w of dry SB. ** Represents calculated averages.

2.2.2 Cellulose

Cellulose, which consists of β -1,4 linked linear glucose molecules, is the most abundant polysaccharide occurring in nature (**Figure 2.3**) (Himmel, 2008). Although glucose is relatively simple in composition, its physical structure within the microfibrillar phase of the CW is complex. There is a high DP within the primary (6000 glucose units) and secondary (14 000 glucose units) CW. However, the DP vastly differs between hardwoods, softwoods, and grasses as well as between cellulose of different purities (Hallac & Ragauskas, 2011). Extensive intramolecular hydrogen bonding and van der Waals interactions between the glucan (polymeric glucose) chains allow the molecules to be tightly packed into a three dimensional microfibrillar aggregate (Himmel, 2008). Furthermore, electron micrograph studies have shown that plant cellulose microfibrils vary in width between 5 – 10 nm (Ha et al., 1998)

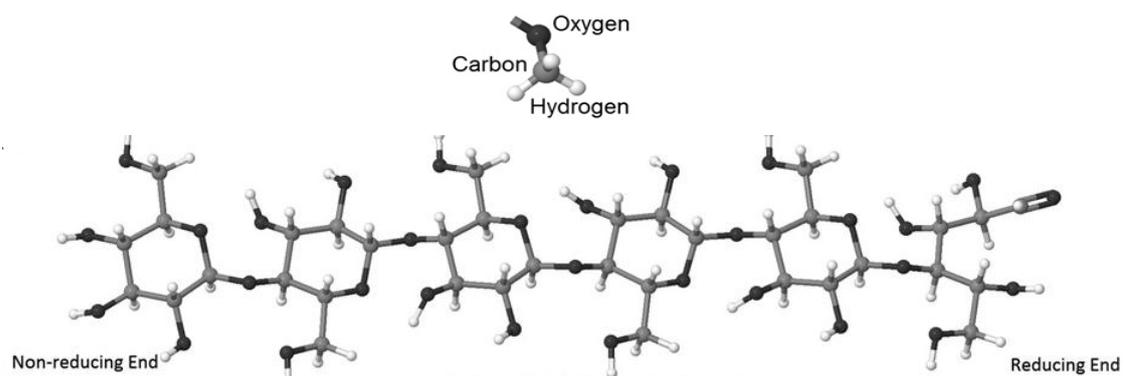


Figure 2.3: Structure of cellulose showing glucose units β -1,4 linked to each other (Zhang et al., 2012). Reprinted with written permission from Elsevier and their Copyright Clearance Center (RightsLink service).

2.2.3 Hemicellulose

Hemicelluloses are typically extracted from the CW by dilute alkali. This treatment disrupts the hydrogen bonding between cellulose and hemicellulose, allowing the hemicellulose to be extracted with little sugar depolymerization/degradation (Himmel, 2008). Different plant species exhibit differences in hemicellulose composition. The most common hemicellulose polymers are xylan, xyloglucan and mixed length glucan (Himmel, 2008). However, SB hemicellulose is dominated by the presence of xylan,

which contains both arabinose and 4-*O*-methyl-D-glucuronic acid side groups (Peng et al., 2010; Geng et al., 2006; Sun, 2004).

2.2.3.1 Xylan

Xylan polysaccharides consists of a backbone of β -1,4 linked D-xylose molecules (Brett & Waldron, 1996). Xylans are usually branched, however linear xylans have been identified (Eda et al., 1976). Xylans containing large amounts of L-arabinose, also called arabinoxylans, which are common in grasses and cereals. Additionally, SB xylan also contains glucuronic acid side groups (arabinoglucuronoxylan). The xylan backbone can be connected to arabinose via α -1,2- or α -1,3- bonds as single residues or side chains (de Vries & Visser, 2001), whereas glucuronic acid containing a 4-*O*-methyl ether attaches via α -1,2-linkage to xylan (Saulnier et al., 1995). Furthermore, xylose β -1,2 linked to arabinose as well as galactose either β -1,5 linked to arabinose or β -1,4 linked to xylose can be found in these side chains (de Vries & Visser, 2001). The xylan polymer and possible substitutes is depicted in **Figure 2.4**.

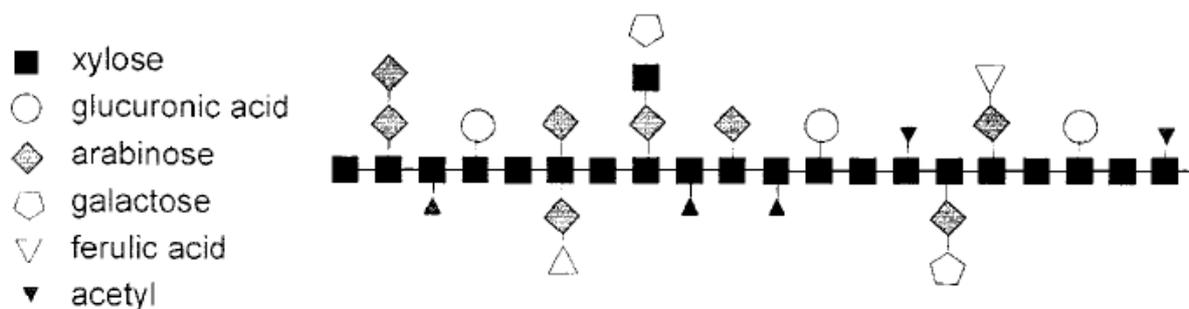


Figure 2.4: Structure of common xylans showing its heterologous nature (de Vries & Visser, 2001). Reprinted with written permission from American Society for Microbiology and their Copyright Clearance Center (RightsLink service).

The xylan backbone can also be acetylated at the O-2 and O-3 position of xylose (de Vries & Visser, 2001). Aromatic residues feruloyl and *p*-coumaroyl attach to arabinose residues on the O-5 terminal, resulting in covalent linkages between xylans and lignin present in SB (Peng et al., 2009; Saulnier et al., 1995; Spencer & Akin, 1980). Therefore, xylans have been classified as very heterologous polysaccharides

(Bajpai, 1997). Furthermore, xylan is covalently bound to lignin and hydrogen bonded to cellulose.

2.2.4 Lignin

After cell growth has ceased, the plant reinforces its CW to make the growing plant body more rigid, thereby adding strength. Lignin is nature's second most abundant polymer after cellulose, and is produced in the phenylpropanoid pathway (Himmel, 2008). Lignins are derived from the three hydroxycinnamyl alcohols named monolignols (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). The three monolignols: 4-coumaryl-, coniferyl- and sinapyl alcohol; are generally classified by their aromatic ring substitution pattern. Therefore, lignins are commonly identified as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) residues (**Figure 2.5**).

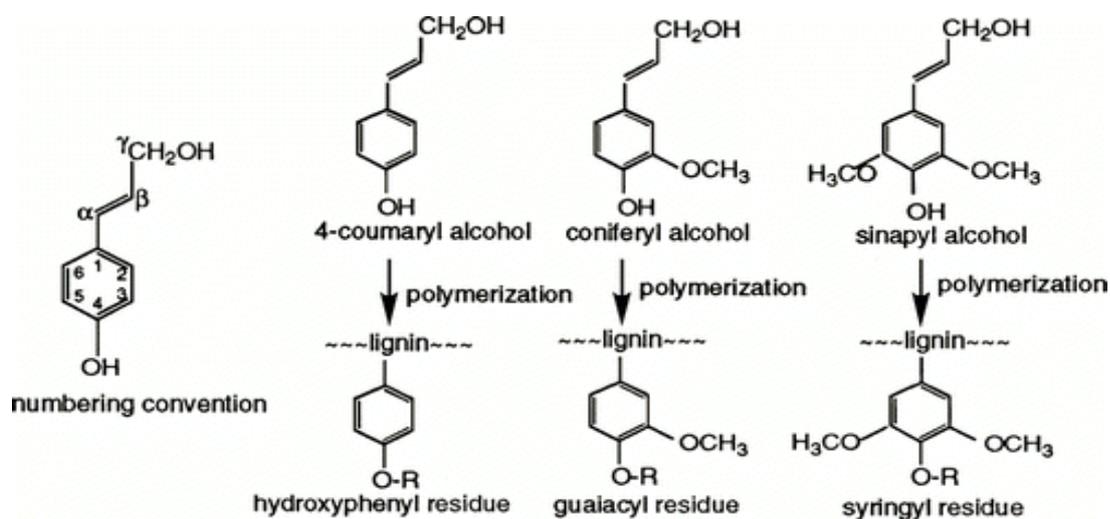


Figure 2.5: Structure of the three monolignols: 4-coumaryl-, coniferyl- and sinapyl alcohol with their respective residues (Whetten et al. 1998). Reprinted with written permission from Annual Review of Plant Physiology and Plant Molecular Biology and their Copyright Clearance Center (RightsLink service).

Although the lignin composition between different species may differ, lignins are considered complex, amorphous phenyl propane polymers in the CW's of plants (**Figure 2.6**) (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003;

Sun, 2004; Sun, 2011; Xu et al., 2005). The aromatic moiety in lignin residues makes lignin very hydrophobic, thereby waterproofing the xylem conducting cells. Aromatic molecules, such as lignins, show enhanced chemical and thermodynamic stability due to delocalized electrons (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). Aromatic molecules are also able to interact with each other in so-called “stacking” of p-orbitals, contributing to intramolecular forces within lignin.

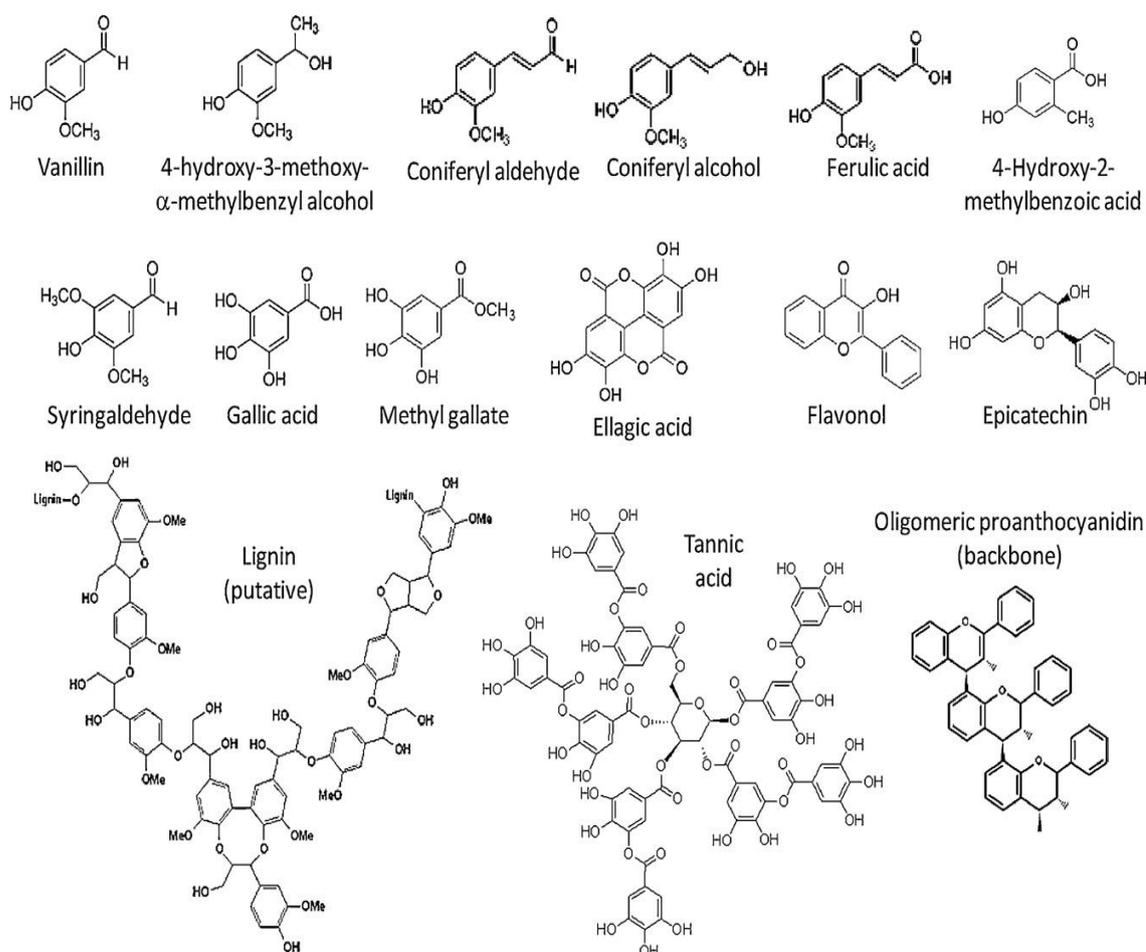


Figure 2.6: Lignin and its phenolic components (Tejirian & Xu, 2011). Reprinted with written permission from Elsevier and their Copyright Clearance Center (RightsLink service).

It is the evolution of plants that gave rise to the formation of tracheids, as well as the ability to enforce them with lignin (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). The ability to lignify cells gave plant cell walls increased strength; therefore, plants could grow higher and maximize photosynthesis, which vastly improved the globalization of plant

species. Lignins are deposited in the presence of previously formed CW polysaccharides (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). Therefore, the nature of the CW polysaccharides before lignin deposition has an influence on the ultimate structure of the lignin macromolecule as both covalent and hydrogen bonding is present in the lignin-carbohydrate complexes (LCCs) (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). However, these LCCs are difficult to isolate and quantify due to the complex relationship between CW components (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005).

2.2.5 Crosslinking between cellulose, hemicellulose, lignin and phenolics

In addition to cellulose, hemicellulose and lignin, the CW can also contain phenolic acids, tannins, lignans, flavonoids and stilbenes, which increase the mechanical stability of the plant and protects it against microbial attack (de Vries & Visser, 2001; Rose, 2003). Furthermore, the CW of monocots such as sugarcane can contain hydroxycinnamic acid (HCA) crosslinks (Rose, 2003). A schematic of some of the suggested crosslinking within grasses is represented in **Figure 2.7**. The HCA's: ferulic, coumaric and sinapic acids have been shown to be involved in cross-linking between polysaccharides and lignin (**Figure 2.7a**) (Himmel, 2008; Iiyama et al., 1994; Rose, 2003). Similarly, grasses such as sugarcane can contain direct ester linkages between the hydroxyl groups of lignin monomers with carboxyl groups of xylan and hydroxyls on surrounding polysaccharides (Himmel, 2008; Iiyama et al., 1994; Rose, 2003). The xylans in grasses can contain monomeric and dimeric HCA esters (Himmel 2008; Rose 2003). Therefore, HCA esterified xylan can esterify to surrounding polysaccharides or lignin (**Figure 2.7c**) (Himmel, 2008; Iiyama et al., 1994; Rose, 2003). Furthermore, these HCA esterified xylans can then be directly etherified by the hydroxyls on the lignin surface resulting in ester-ether crosslinks between the CW hemicelluloses and lignin (**Figure 2.7b**) (Himmel, 2008; Rose, 2003).

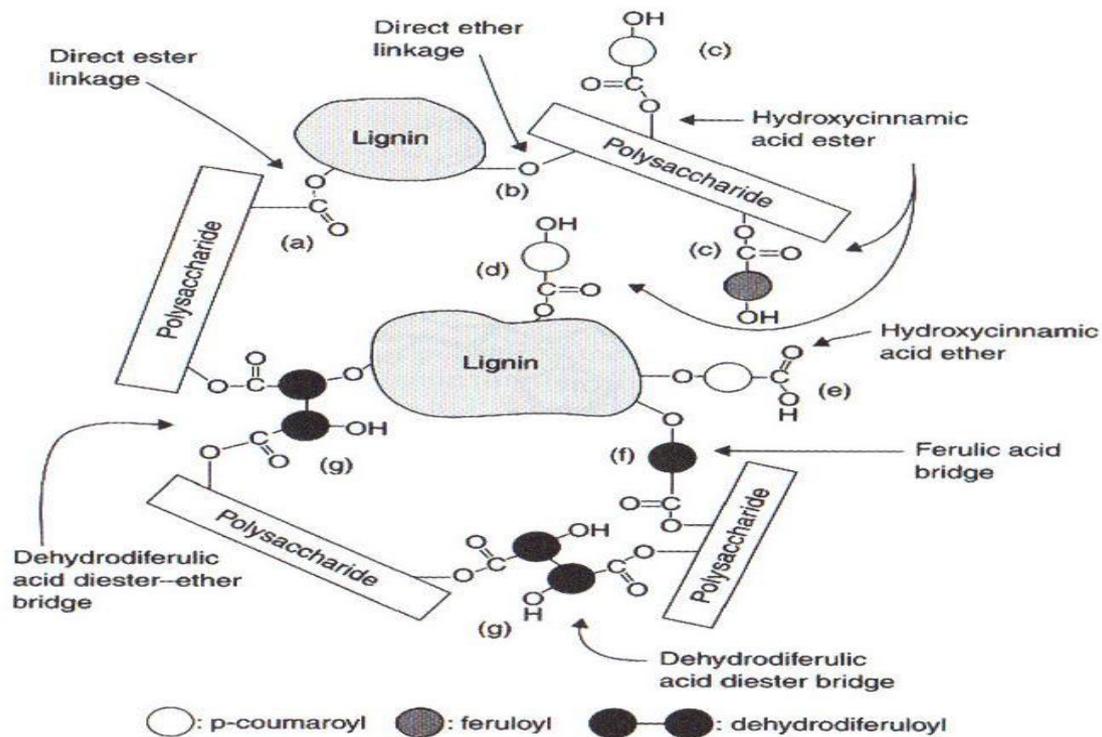


Figure 2.7: Schematic representation of possible crosslinks in CW of grasses. (a) direct ester linkage; (b) direct ether linkage; (c) HCA esterified to polysaccharides; (d) HCA esterified to lignin; (e) HCA etherified to lignin; (f) ferulic acid ester-ether bridge; (g) dehydrodiferulic acid diester-ether bridge (Iiyama et al., 1994). Reprinted with written permission from American Society of Plant Biologists and their Copyright Clearance Center (RightsLink service).

Cellulose microfibril surfaces are hydrogen bonded to non-cellulosic polysaccharides such as xyloglucan, contributing to microfibril adhesion and CW strength (de Vries & Visser, 2001; Rose, 2003). Additionally, different hemicellulose components interact differently with cellulose and binding seems non-specific (Rose, 2003). For that reason, various bonds between polysaccharides have been reported (Himmel, 2008; Iiyama et al., 1994; Rose, 2003; Saulnier, 1999). Saulnier *et al.* (1999) reported that hemicellulose contains esterified ferulic acid components, enabling hemicellulose to form covalent inter-molecular cross-linking with surrounding polysaccharides (**Figure 2.7f, g**) (Saulnier, 1999). Iiyama *et al.* (1994) also suggested that similar cross-linking gives the lignocellulosic CW its mesh-like network (Iiyama et al., 1994). Similar work by Encina *et al.* (2005) showed that arabinoxylans are extensively feruloylated allowing cross-linking between CW components (Encina, 2005). Furthermore, Encina *et al.* (2005) suggested that the bound hydroxycinnamates

contribute to bonding between cells via the formation of covalent bridges (Encina, 2005). Therefore, the HCA molecules seem to add to the simple hydrogen and covalent binding model that exists between cellulose and hemicellulose. Therefore feruloyl-esterases have been receiving interest as these enzymes can hydrolyze phenolic cross-links, which could make CW polysaccharides more accessible (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). Altogether, it is clear that bagasse CW components partake in various interactions adding complexity to complete extraction of any one component without altering the others. In this regard, one of the aspects studied was the presence of these compounds and their change in the pretreated material during EH (**Chapter 4**). It was possible that these CW interactions were increasing the substrate recalcitrance as EH progressed, influencing the EH rate and ultimate EH yield.

2.3 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

2.3.1 Introduction: Pretreatment

One of the most important factors influencing the viability of second generation bio-ethanol production is the pretreatment process (Menon & Rao, 2012). Pretreatment is crucial in improving the digestibility of the lignocellulosic substrate, to release fermentable sugars for bioconversion to bio-ethanol (Sarkar et al., 2012). It has been stated that an effective pretreatment method should: minimize energy requirements, increase cellulose accessibility, preserve the hemicellulose stream, include lignin recovery, recycle catalysts, minimize inhibitors and be cost effective (Banerjee et al., 2010; Van Dyk & Pletschke, 2012). Together with improved access to the sugars, pretreatment should avoid the generation of inhibitors that could affect downstream hydrolysis and fermentation (Laser et al., 2002). Therefore, the choice of pretreatment should consider various factors such as: feedstock characteristics, enzymes utilized, as well as the organisms used in fermentation. Moreover, pretreatment and its effects on the downstream process contribute considerably to the economic viability of the process (Zhang et al., 2009).

The most common pretreatments include: steam explosion (Kumar et al., 2010), ammonia fiber explosion (Sun & Cheng, 2002), acid hydrolysis (Canilha et al., 2011), alkaline hydrolysis (Carrillo et al., 2005), and mechanical comminution (Kumar et al., 2009a). There are various studies published in the literature indicating the advantages or disadvantages of the different pretreatment methods (Kumar et al., 2009a). However different feedstocks can respond differently to varying pretreatments, impacting the economic viability of the pretreatment method chosen (Balat, 2011; Canilha et al., 2011; Kumar et al., 2009; Kumar et al., 2010; Menon & Rao, 2012; Zabihi et al., 2010). In the present study, steam explosion was used to improve the EH and fermentation of industrial SB. This method is the most widely used pretreatment method and has shown to be effective for herbaceous materials without the need of adding catalyst (McMillan, 1994).

2.3.2 Steam explosion

Steam explosion is a physicochemical pretreatment method whereby the material is exposed to saturated steam at 160 – 260°C with a corresponding pressure of 0.69–4.83 MPa for seconds to minutes, depending on the severity of the treatment (Balat, 2011; Kaar et al., 1998; Kovacs et al., 2009; Kumar et al., 2010; Menon & Rao, 2012; Wu et al., 1999; Zabihi et al., 2010). After the desired pretreatment time, the material is exposed to atmospheric pressure which causes the material to undergo explosive decompression. The steam explosion equipment used in the present study is depicted in **Figure 2.8**.



Figure 2.8: Photo of the Steam explosion pretreatment equipment used in the present study.

2.4 ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS

2.4.1 Introduction: History of enzymatic hydrolysis

In the second world war, the U.S. Army formed the U.S. Army Natick Laboratories to investigate the degradation of military clothing and tents in the jungles of the South Pacific (Augustine, 1976). It was here in the Solomon Islands that Elwyn T. Reese and Mary Mandels isolated a mold, *Trichoderma viride*, which was causing havoc on the military equipment (Maheshwari, 2008). The isolation of *T. viride* sparked research on how insoluble polymers could be broken down by microorganisms. Furthermore, it was investigated if these organisms were using enzymes for the breakdown, and if so, which enzymes. It was in the 1960's and 1970's that the military realised that the breakdown of cellulose to sugars could contribute to food and fuel supplies, which increased the research done in these areas (Mandels & Reese, 1999; Peterson et al., 1945; Reese, 1976).

Advances in the fields of protein engineering, molecular- and structural biology, as well as computational methods, have significantly increased the knowledge surrounding the study of enzymes and their hydrolytic ability, started in the 1950s (Viikari et al., 2012). Therefore, *Trichoderma* quickly became a model organism for genetic manipulation responsible for a great amount of industrially used enzyme-producing fungi, with cellulase from *Trichoderma* valued at 125 million US\$ in 1999 (Maheshwari, 2008). The research into the cellulase producing *T. viride* combined with the oil crisis following the 1970's Gulf war lead to increasing funding for ethanol fuels based on the hydrolysis of cellulose material by enzymes such as cellulase from *T. viride*. The visions of cellulosic ethanol by Elwyn T. Reese lead to the renaming *T. viride* to *T. reesei*. Today the understanding of enzymes such as the cellulase system has improved with the great advancements in available technologies.

2.4.2 Classification of lignocellulose degrading enzymes

Chemical- and EH are the two main approaches for hydrolysis of lignocellulosic material after pretreatment has decreased the substrate recalcitrance (Balat, 2011). Chemical hydrolysis techniques such as dilute and concentrated acid hydrolysis are

effective in the breakdown of polysaccharides. However, chemical hydrolysis has certain disadvantages such as high costs, lower selectivity, and corrosion (Menon & Rao, 2012). Therefore, EH is preferred owing to milder conditions, reduced inhibitor formation, and the ability to improve with advances in biotechnology (Balat, 2011). The most important EH products, for successful fermentation, are glucose from cellulose and xylose from hemicellulose (Tomás-Pejó et al., 2008). For that reason, selecting the correct enzymes to hydrolyse the complex sugars in the pretreated material to monomeric sugars for microbial fermentation is crucial.

There are a vast number of enzymes, with a wide spectrum of activities and applications. Therefore, the International Union of Biochemistry and Molecular Biology (IUBMB) classify enzymes in six major classes based on the type of reactions they catalyze and substrate specificity (**Figure 2.9**). The enzyme commission (EC) of the IUBMB classifies these enzymes by giving them a 4 digit number that represents: 1 → class, 2 → subclass, 3 → chemical groups involved in reaction, 4 → serial number for the enzyme in its sub-subclass (www.iubmb.org, 2012). However, each class has their own principles which dictate the classification of their respective sub class and sub-subclasses (www.iubmb.org 2012). In the case of enzymes involved in lignocellulose degradation, such as cellulase (EC.3.2.1.4), the enzymes are mostly glycoside hydrolases (EC.3.2.1.x). The first digit number (3) indicates that it is part of the hydrolase class of enzymes. The second number (2) indicates the nature of the bond hydrolysed (hydrolysis of glycosidic bonds). The third number (1) specifies the nature of the substrate (O-glycosidases). The last number is specific for each member of the sub-subclass (www.iubmb.org 2012).

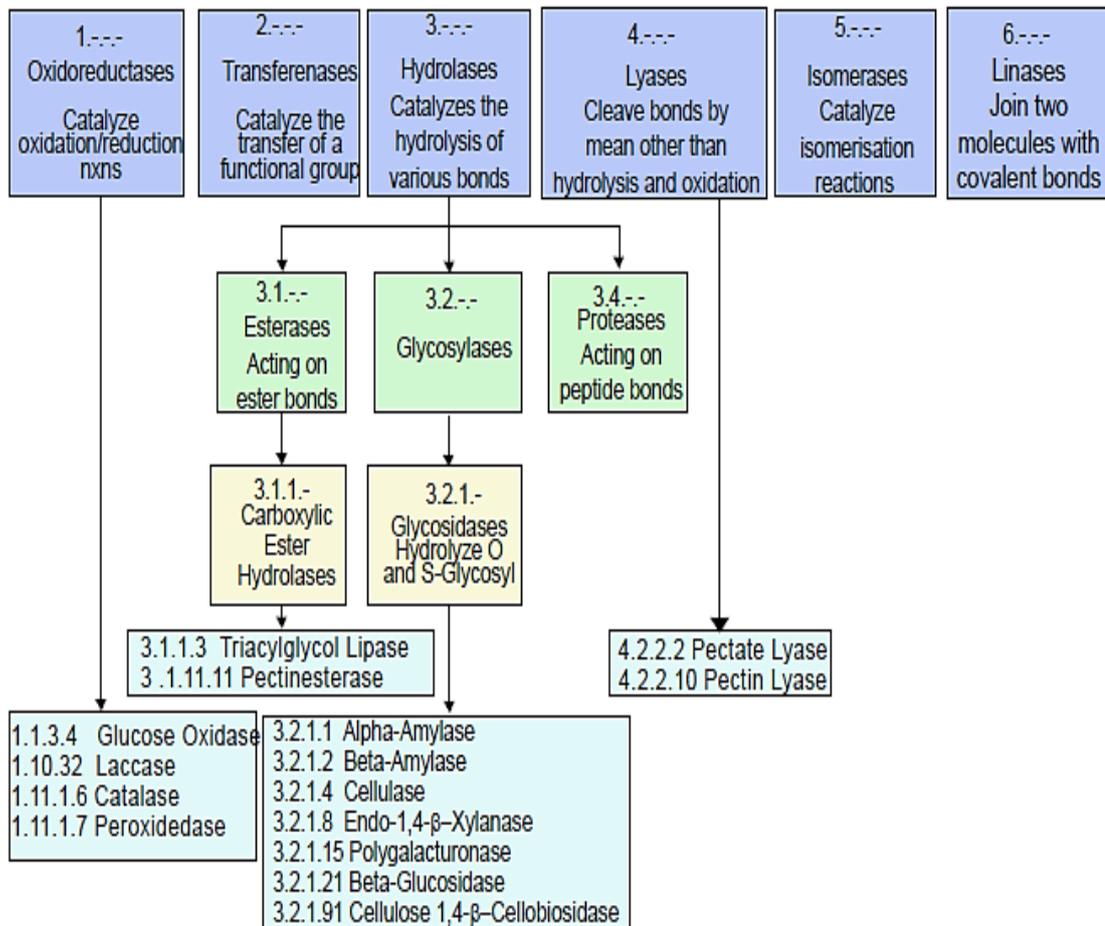


Figure 2.9: The general classification of enzymes based on the NC-IUBMB system (Sarrouh et al., 2012). Reprinted under the terms of the OMICS group's Creative Commons Attribution License.

However, this classification does not provide information about the structure and phylogenetic relations among the enzymes (Withers & Williams, 2009). That is why nowadays, a complementary classification assigns sequences to various families using algorithms (Cantarel et al., 2009). This system has allowed for the catalogue of 131 families of glycoside hydrolases permanently available through the Carbohydrate Active Enzyme Database (www.cazy.org).

Moreover, various bacteria and fungi produce enzymes capable of degrading lignocellulose. Bacteria of the genus *Avetovibrio*, *Bacillus*, *Bacteroides*, *Clostridium*, *Cellulomonas*, *Erwinia*, *Microbispora*, *Ruminococcus*, *Streptomyces* and *Thermomonospora* are able to produce cellulase (Sun, 2002). Species of fungi including: *Aspergillus*, *Penicillium*, *Schizophyllum* as well as mutant strains of *Trichoderma* (*T.*

longibrachiatum, *T. reesei*, *T. viride*) are also capable of hydrolyzing cellulose (de Vries & Visser, 2001). *Trichoderma reesei* is widely used for the production of potent commercial cellulases (Kubicek et al., 2011). In the study, enzymes of *Trichoderma reesei* and *Aspergillus niger* were used (**Chapter 4 & 5**) as they have been widely applied in bio-ethanol production processes and are commercially available (Bansal et al., 2012; Cannella et al., 2012; Pribowo et al., 2012; Romaní et al., 2012). The degradation of lignocellulosic biomass by fungal enzymes, such as those from *Trichoderma reesei* and *Aspergillus niger*, can be classified in at least 35 glucosidase hydrolyse (GH), three carbohydrase esterases (CE) and six polysaccharide lysase (PL) families (Coutinho et al. 2009). The filamentous fungi *Trichoderma reesei* and *Aspergillus niger* have different amounts of putative genes expressing enzymes involved in lignocellulose degradation, as the complex substrate requires a pool of different enzymes to be degraded (**Table 2.3**).

Most cellulases produced by fungi such as those used in the present study, have a two-domain structure (Carbohydrate Active Enzymes database <http://www.cazy.org/>; Cantarel et al., 2009). This structure contains a cellulose-binding module (CBM) that is linked to the catalytic domain by a flexible linker, allowing the enzyme to progress down the insoluble substrate (Carbohydrate Active Enzymes database <http://www.cazy.org/>; Cantarel et al., 2009). The following sections will describe the specific enzyme complexes within these fungal enzyme families responsible for the degradation of specific biomass components.

Table 2.3: Carbohydrate-active enzymes involved in the degradation of lignocellulose from the fungal genomes of the industrial organisms used in this research (Chapter 4 and 5) where An = *Aspergillus niger* and Tr = *Trichoderma reesei* (Brink & Vries, 2011). Reproduced and adapted under Springer's Creative Commons Attribution Non-commercial License.

| Total putative genes involved in plant polysaccharide degradation | | | | An | Tr | |
|---|---------------------------------------|-----------------------------|-------------|---------|--------|----|
| Glycoside hydrolases (GHs) | | | | 178 | 113 | |
| Carbohydrate esterases (CEs) | | | | 9 | 6 | |
| Polysaccharide lyases (PLs) | | | | 13 | 0 | |
| Substrate | Enzyme activity | Code | CAZy fam. | An | Tr | |
| Cellulose | β -1,4-endoglucanase | EGL | GH5,7,12,45 | 15 (6) | 13 | |
| | Cellobiohydrolase | CBH | GH6,7 | 4 (4) | 3 | |
| | β -1,4-glucosidase | BGL | GH1,3 | 20 (15) | 15 | |
| Xyloglucan | Xyloglucan β -1,4-endoglucanase | XEG | GH12,74 | 4 | 3 | |
| | α -arabinofuranosidase | ABF | GH51,54 | 5 | 2 | |
| | α -xylosidase | AXL | GH31 | 7 | 4 | |
| | α -fucosidase | AFC | GH29,95 | 3 | 4 | |
| | α -1,4-galactosidase | AGL | GH27,36 | 7 | 10 | |
| | β -1,4-galactosidase | LAC | GH2,35 | 11 (5) | 8 | |
| Xylan | β -1,4-endoxylanase | XLN | GH10,11 | 5 | 5 | |
| | β -1,4-xylosidase | BXL | GH3,43 | 28 (6) | 15 | |
| | α -arabinofuranosidase | ABF | GH51,54 | 5 | 2 | |
| | Arabinoxylan arabinofuranohydrolase | AXH | GH62 | 1 | 1 | |
| | α -glucuronidase | AGU | GH67,115 | 1 | 2 | |
| | α -1,4-galactosidase | AGL | GH27,36 | 7 | 10 | |
| | β -1,4-galactosidase | LAC | GH2,35 | 11 (5) | 8 | |
| | Acetyl xylan/feruloyl esterase | AXE/FAE | CE1 | 5 | 3 | |
| | Galactomannan | β -1,4-endomannanase | MAN | GH5,26 | 11 (2) | 8 |
| | | β -1,4-mannosidase | MND | GH2 | 6 (3) | 7 |
| | | β -1,4-galactosidase | LAC | GH2,35 | 11 (5) | 10 |
| | | α -1,4-galactosidase | AGL | GH27,36 | 7 | 8 |
| Pectin | Endo-/exo-(rhamno)galacturonases | | GH28 | 22 | 4 | |
| | α -rhamnosidase | RHA | GH78 | 8 | 1 | |
| | α -arabinofuranosidase | ABF | GH51,54 | 5 | 2 | |
| | Endoarabinanase | ABN | GH43 | 11 (5) | 2 | |
| | Exoarabinanase | ABX | GH93 | 1 | 0 | |
| | β -1,4-endogalactanase | GAL | GH53 | 1 | 0 | |
| | Unsaturated glucuronyl hydrolase | UGH | GH88 | 1 | 0 | |
| | Unsat.-rhamnogalacturonan hydrolase | URH | GH105 | 2 | 1 | |
| | β -1,4-xylosidase | BXL | GH3,43 | 28 (6) | 14 | |
| | β -1,4-galactosidase | LAC | GH2,35 | 11 (5) | 8 | |
| | Pectin lyase | PEL | PL1 | 6 | 0 | |
| | Pectate lyase | PLY | PL1,3,9 | 10 | 0 | |

2.4.3 Cellulases and their cellulose degradation mechanism

The information regarding the cellulase complex first identified in the 1950's has changed drastically from the earlier mechanisms where cellulase was crudely described as a combination of a non-hydrolytic enzyme (C_1), which de-crystallizes cellulose, with hydrolytic enzymes (C_x), which hydrolyze the cellulose to simple sugars (Reese et al., 1950). The classical view of cellulose hydrolysis depends on the synergistic action of three of enzymes (Balat, 2011):

1. Endoglucanases (EG, endo-1,4- β -D-glucanases or EC 3.2.1.3.)
2. Exoglucanases (CBH, exo-1,4- β -D-glucan cellobiohydrolases or EC 3.2.1.91.)
3. β -glucosidases (BGL, cellobiases or EC 3.2.1.21.)

These 3 enzyme are divided into 8 GH enzyme families (de Vries et al., 2011). The industrial cellulase-producing workhorse used in the present study, *T. reesei*, does not express the greatest amount of cellulases; however, the cellulases produced by *T. reesei* are efficient at achieving good polysaccharide EH rates and yields. The characterized cellulose degrading enzyme classes within *T. reesei* are (Martinez et al., 2008):

- 5 EGs in GH families (5, 7, 12 and 45)
- 2 CBHs in GH families (6 and 7) which are highly expressed
- 2 BGLs in GH families (1 and 3)

The enzymes can also be described by the substrate they hydrolyze, family number and order of identification. For example: CHB2 is also called (Cel6a) as it hydrolyses cellulose, is part of GH family 6 and was the first enzyme of this family to be reported with the specific activity (de Vries et al., 2011). As the knowledge of these cellulases grows, so does our understanding of their mechanics. The current view (Cannella et al., 2012; Horn & Vaaje-Kolstad et al., 2012) of cellulose hydrolysis by a fungal enzyme set (**Figure 2.10**), such as the set from *T. reesei*, is improved form the classical view and is described by:

1. EGs cleave the internal and amorphous regions of the cellulose chains at random, forming more regions for CBHs. EGs can act processively by threading a single polysaccharide chain through the active site cutting it consecutively, however non-processive action also occurs (Horn & Sørli et al., 2012). Of the 5 EGs produced by *T. reesei*, EG1 (Cel7b) and EG2 (Cel5a) accounts for the greatest amount of expressed EGs (Vlasenko et al., 2010). Furthermore, these EGs possess carbohydrate-binding modules (CBM) which allow these enzymes to successfully bind to the cellulose during hydrolysis (Guillén et al., 2009).
2. CBHs hydrolyze the reducing and non-reducing cellulose chain ends releasing cellobiose and cellodextrin. CBHs act predominantly processively, which is deemed beneficial, as being closely associated with the crystalline cellulose substrate aids hydrolysis (Teeri, 1997). The CBHs that are the most significant components of the total *T. reesei* cellulase mixture are CBH1 (Cel7a), which prefers reducing ends, and CBH2 (Cel6a), which prefers non-reducing ends. CBH1 and CBH2 make up 60% and 20% of the cellulose enzyme complex, respectively (Rosgaard et al., 2007; Teeri, 1997). Therefore, CBHs are the most abundant enzyme components in natural and commercial cellulase mixtures, which could be attributed to the fact that the chain ends are more accessible than the internal regions (Rosgaard et al., 2007; Teeri, 1997). Additionally, the greater amount of crystalline cellulose (hydrolysed by CBHs), relative to the amorphous fraction (hydrolysed by EGs), present in lignocellulose could also contribute to the abundance of CBHs. However, CBHs are highly susceptible to product inhibition (Kristensen et al., 2009), which could also explain why great amounts of these enzymes are present.
3. GH61 is a group of fungal derived proteins that have recently been associated with the hydrolysis of lignocellulose by the fungal kingdom (Phillips et al., 2011). Currently GH61s can accurately be called copper-dependent polysaccharide monooxygenases (PMOs) (Beeson et al., 2012). PMOs use oxygen and reducing equivalents from cellobiose dehydrogenase (CDH) to oxidatively cleave glycosidic bonds on the cellulose surface without needing separate sugar strands (Langston et al., 2011). Oxidative cleavage occurs at the

C1 and C4 position which generates non-reduced sugars for CBHs (Beeson et al., 2012; Horn & Vaaje-Kolstad et al., 2012). Even though *T. reesei* expresses PMOs, with GH61B comprising the majority action in *T. reesei*, their activity is low. The addition of PMO (GH61B and GH61E) genes from *Thielavia terrestris* to *T. reesei* improves the hydrolytic ability of the resulting cellulase mixture by 1.6 – 2 fold, which drastically improves the economics of lignocellulose digestion (Harris et al., 2010; Phillips et al., 2011).

4. CDH (EC 1.1.99.18) is a secreted flavocytochrome produced by many cellulose-degrading fungi such as *T. terrestris* and catalyses the oxidation of cellodextrins or cellobiose to their corresponding lactones (Henriksson et al., 2000; Westermark & Eriksson, 1975). CDHs are usually co-expressed with PMOs (GH61) whereby it contributes to the synergistic degradation of cellulose by the hydrolytic cellulase complex (Langston et al., 2011). CDHs also contribute to the enzymatic action by donating electrons to the PMOs (Phillips et al., 2011), although non-enzymatic electron donors, such as gallic acid, ascorbic acid and reduced glutathione, have also been identified (Horn & Vaaje-Kolstad et al., 2012).

5. BGLs from families GH1 and GH3 act by converting the major product from EGs and CBHs, cellobiose and cellodextrins, to glucose. However, BGL1 and BGL2 from *T. reesei* are not very robust, as these BGLs are expressed in low levels and are susceptible to product inhibition from glucose (Chen et al. 1992). Therefore, commercial cellulase mixtures are commonly supplemented with BGLs from *A.niger* as these are more resistant to glucose inhibition and are expressed in great quantities (Réczey et al., 1998). In the present study cellulase mixtures from *T. reesei* were supplemented with BGLs from *A.niger*.

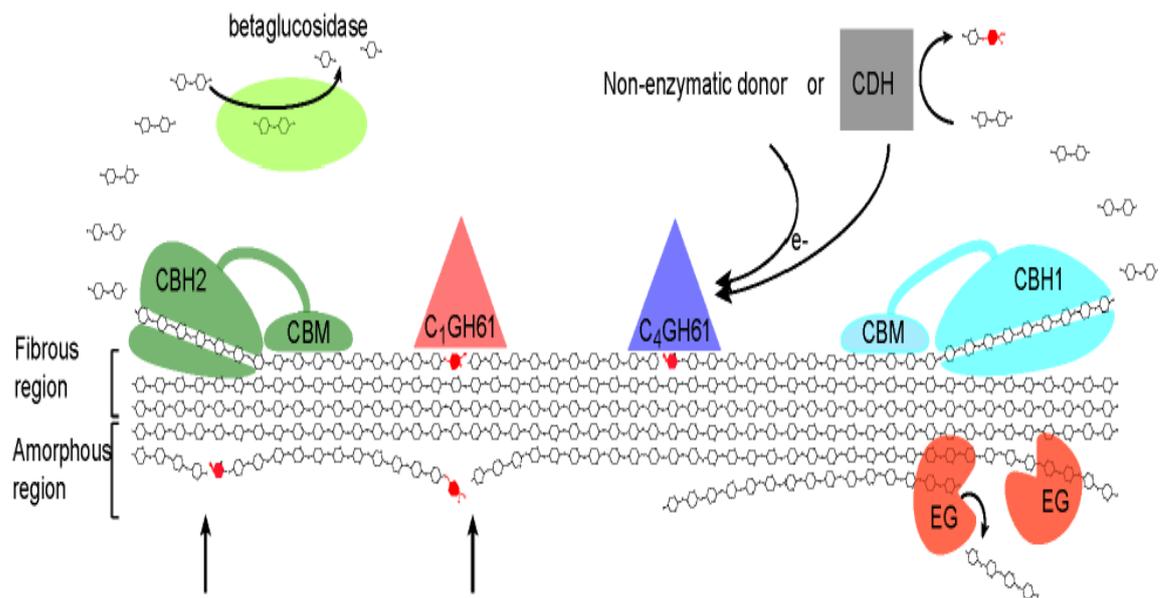


Figure 2.10: Current view on fungal enzymatic degradation of cellulose (Horn & Vaaje-Kolstad et al., 2012). The arrows indicate new points of action for the hydrolytic enzymes, generated by PMOs (GH61) action. Sugars that have been oxidised by the PMOs (GH61) are shown in red. Reprinted under the terms of The American Association for the Advancement of Science's and their Copyright Clearance Center (RightsLink service).

One of the enzymes used in the present study was Cellic CTec2 from Novozymes. This enzyme is widely used in the degradation of lignocellulose and benefits from the presence of PMOs (GH61) (Cannella et al., 2012; Horn & Vaaje-Kolstad et al., 2012). Therefore, it is possible that Cellic CTec2 follows the mechanics described and shown in **Figure 2.10**. However, the different polysaccharides within the lignocellulosic biomass require different enzyme complexes and the following section will describe the specific enzymes sets required for efficient hemicellulose degradation.

2.4.4 Enzymatic degradation of hemicellulose

Various organisms produce hemicellulose degrading enzymes, including bacteria, fungi, yeasts and protozoa (Whitaker, 2003). These include the previously mentioned cellulase-producing organisms, *T. reesei* and *A. niger*. Xylanases have a number of interesting potential applications such as biofuel production, ruminal digestion, paper production, agricultural-, water-, and industrial waste treatment

(Whitaker, 2003). The complex heterologous structure of hemicellulose requires a multi-enzyme system for saccharification (Balat, 2011). However the 3 major backbone structures of hemicellulose (**Figure 2.11 A-C**) are degraded by specific carbohydrate active enzymes (de Vries et al., 2011):

A. Xylan → β -1,4-endoxylanase (EC 3.2.1.8) + β -1,4-xylosidase (EC 3.2.1.37)

- β -1,4-endoxylanases (XLN), which belong to the families GH10 and GH11, cleave the internal β -1,4-xylan backbone, producing oligomers (Balat, 2011; Whitaker, 2003). However, GH10 has a broader activity than GH11 and can also degrade smaller xylan oligomers and a broad arrange of backbone substitutions (Pollet et al., 2010).
- β -1,4-xylosidase (BXL) is responsible for degrading the released xylo-oligosaccharides (XOS) to simple sugars such as xylose. Fungal BXL such as those from *T.reesei* mostly belong to the family GH3 (Carbohydrate Active Enzymes database <http://www.cazy.org/>; Cantarel et al., 2009).

B. Xyloglucan → xyloglucan-active β -1,4-endoglucanase + β -1,4-glucosidases (BGL, cellobiases or EC 3.2.1.21.)

- xyloglucan-active β -1,4-endoglucanase (XEG) (GH12 & 74) cleaves either branching glucose residues or XOS depending on the fungal origin of the enzyme (Desmet et al., 2007).
- β -1,4-glucosidases (BGL) work as previously described for the cellulosic enzymes complex.

C. Galactomannan ---> β -1,4-endomannanase (E.C. 3.2.1.78), + β -1,4-mannosidase (EC 3.2.1.25)

- β -1,4-endomannanase (MAN) is responsible for the degradation of mannan backbones and mannan-oligosaccharides with greater than 3

residues. Even though MAN belongs to GH5 & 26, it is predominantly GH5 in fungal expression systems such as those from *T.reesei* (Tenkanen et al., 1997) and *A.niger* (Do et al., 2009).

- β -1,4-mannosidase (MND) is important in the degradation of mannotriose and mannobiose to mannose. It is an exoglucosidase that belongs to the GH2 family (Flieđrová et al., 2012).

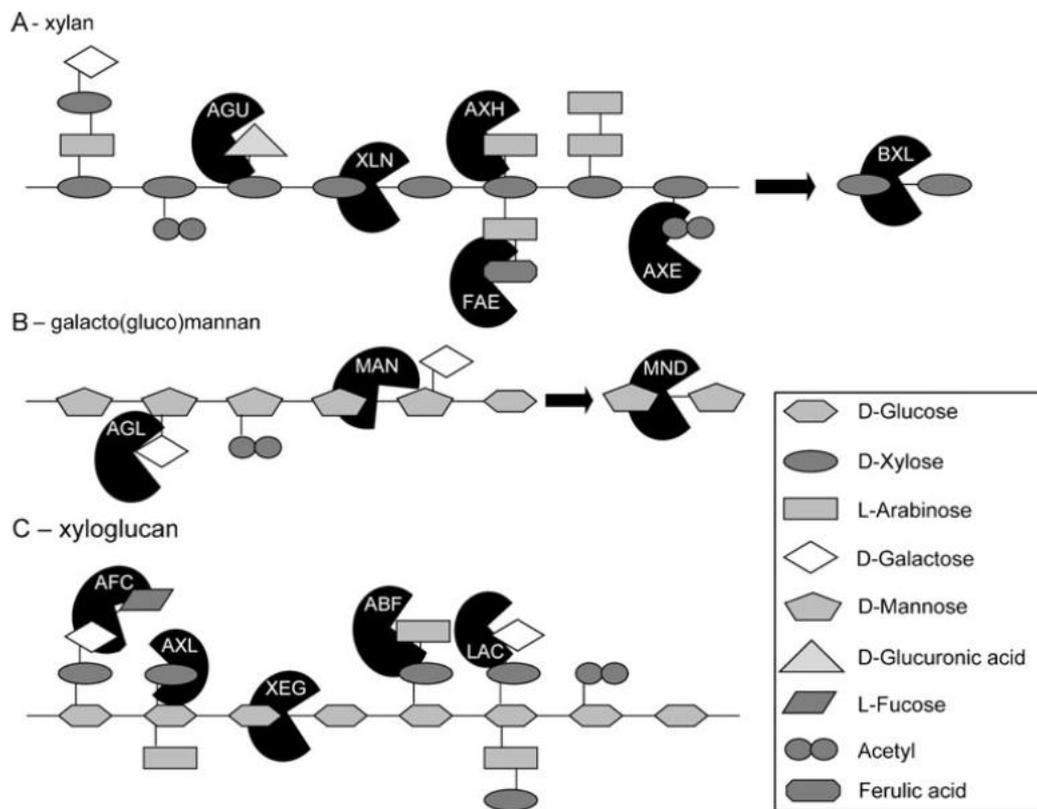


Figure 2.11: Schematic of the enzymes involved in the breakdown of the three major hemicellulose backbones: xylan (A), galacto (gluco) mannan (B) and xyloglucan (C) and their respective side chains. AGU α -D-Glucuronidase, XLN β -1,4-endoxylanase, AXH arabinoxylan α -arabinofuranohydrolase, FAE feruloyl esterase, AXE acetyl (xylan) esterase, BXL β -1,4-xylosidase, AGL α -1,4-galactosidase, MAN β -1,4-endomannanase, MND β -1,4-mannosidase, AFC α -fucosidase, AXL α -xylosidase, XEG xyloglucan-active β -1,4-endoglucanase, ABF α -arabinofuranosidase, LAC β -1,4-galactosidase (de Vries et al., 2011). Reproduced and adapted under Springer's Creative Commons Attribution Non-commercial License.

However, the backbones of the three major hemicellulosic structures can be highly substituted with a variety of molecules and the degradation of backbone side chains is summarized by (Balat 2011; de Vries et al., 2011; Whitaker, 2003):

1. L-Arabinose → is hydrolysed by arabinoxylan α -arabinofuranohydrolase (AXH) and α -arabinofuranosidase (ABF)(EC 3.2.1.55), which is important when taking into consideration that the major hemicellulose in SB is the arabinoglucoxyllans.
2. D-xylose → is released from the xyloglucan backbone by α -xylosidase (AXL).
3. L-fucose → which can be found in the side chains of xyloglucans are released by α -fucosidase (AFC)
4. Alpha linked D-galactose and β -galactose → is released from the galacto(gluco)mannan and xyloglucan backbone by the hydrolytic activity of α -1,4-galactosidase (AGL)(EC 3.2.1.22) and β -1,4-galactosidase (LAC) respectively.
5. D-glucuronic acid → α -D-Glucuronidase (AGU) (EC 3.2.1.139) releases the 4-O-methyl glucuronic acid from the xylan backbone.
6. Acetyl → Acetyl (xylan) esterase (AXE)(EC 3.1.1.72) releases the acetyl subunits linked to the xylan backbone, subsequently acetic acid is produced.
7. Ferulic acid → feruloyl esterase (FAE)(EC 3.1.1.73) hydrolyses the arabinoferulic acid bond between hemicellulose and lignin, producing ferulic acid.

Therefore, it is clear that there is a wide array of enzymes used in the degradation of hemicellulose. These enzymes can act synergistic or non-synergistically to efficiently hydrolyze the heterologous nature of biomass into its building blocks. However, multiple enzymes in a cocktail working by itself will require higher total enzyme dosages compared to enzymes working synergistically. This will be described in more detail in the following section.

2.4.5 Enzyme synergism

Enzyme synergism is defined as enzyme mixtures in an appropriate ratio that can produce a greater number of EH products than if each of the individual enzymes within the mixture were applied individually (Kumar & Wyman, 2009a; Van Dyk & Pletschke, 2012). Synergism is different to additive effect and quantified as the ratio of the yield or rate of EH product when these enzymes are used together vs. the sum of the yield or rate of EH product when enzymes are used separately in the same amounts as they were employed in the mixture (Kumar & Wyman, 2009a; Van Dyk & Pletschke, 2012). Synergy is influenced by the substrate and enzyme characteristics, as well as the ratio of enzymes in the mixture (Nidetzky et al., 1994).

Synergism between combinations of EGs and CBHs, between CBHs, and between CBHs, EGs and BGLs in the cellulase system are proven examples (Boisset et al., 2001; Zhang & Lynd, 2004). However, synergistic cellulase action is usually observed for hydrolysis of crystalline cellulose (Van Dyk & Pletschke, 2012). It has been suggested that synergism in the cellulase system only occurs when the different enzymes attack different regions of the substrate as creating more substrate for each other (Himmel, 2008). Additionally, it seems that as long as individual cellulases are efficient, they work synergistically (Himmel, 2008).

The more complex xylanase system has also been shown to exhibit synergism (de Vries et al., 2000; Sorensen et al., 2005). However, synergy in the xylanase system can be classified as either homesynergistic, heterosynergistic or anti-synergistic (Kovacs, 2009; Van Dyk & Pletschke, 2012). Synergism between backbone cleaving enzymes, such as XLNs and exoxylanase, is dubbed homeosynergy (Gasparic et al., 1995). Heterosynergy occurs between backbone cleaving and side chain debranching enzymes, such as XLNs, BXLs and ABFs (Shi et al., 2010). This can be explained as the XLNs degrades the un-branched backbone, which is a product of the debranching xylanases such as ABFs and AXHs (Wyman, 1996). Complimentary to this, XLNs decreases the substrate viscosity thereby increasing the substrate accessible to debranching xylanases. Therefore, the individual xylanase enzymes create a loop where one enzyme improves the work of another. However, in a phenomenon called anti-synergy, enzymes can inhibit the work of each other by removing substituents required

by the other enzymes in the mixture (Kovacs, 2009; Van Dyk & Pletschke, 2012; Wyman, 1996). Although synergistic studies are mostly conducted on substrates such as cellulose or hemicellulose, synergy studies on the more complex lignocellulose have been done. Enzyme synergy on SB between MANs and XLNs (2.85), between ABFs, MANs and XLNs (1.87) and between XLNs and EG (3.59) has been reported (Beukes et al., 2008; Beukes & Pletschke, 2011; Beukes & Pletschke, 2010). The numbers in the brackets indicate the degree of synergism with a degree of synergism equal or greater than one indicating significant synergism (Van Dyk & Pletschke, 2012). Altogether, it is clear that the exact composition of the enzyme mixture is vital as minor alterations could have a drastic effect on the overall hydrolysis profile. For that reason, the synergism between different enzyme preparations classed as cellulases, xylanases and pectinases were investigated in the present study (**Chapter 5**).

2.5 FACTORS THAT IMPEDE OVERALL ENZYMATIC HYDROLYSIS PERFORMANCE OF LIGNOCELLULOSIC BIOMASS

2.5.1 Introduction

The most important mechanisms hampering overall EH performance (rate and yield) include the feedstock chosen, pretreatment method, nature of the enzymes and process configuration. Similarly, there are various specific enzyme and substrate related factors that have been implicated in the slowdown in the conversion rate and decreased yields of lignocellulose EH to fermentable sugars (Arantes & Saddler, 2011). As a result, high enzyme loadings are required to overcome the inhibitory effect of the enzyme and substrate-related factors that inhibit complete and rapid biomass hydrolysis (Merino & Cherry, 2007).

The enzyme related factors include: end-product inhibition (mono and oligosaccharides), unproductive enzyme adsorption/inactivation, spherical hindrances by enzyme components, loss of synergism between individual enzymes and thermal- and mechanical deactivation (Gunjekar et al., 2001; Kumar & Wyman, 2009a; Kumar & Wyman, 2009b; Yang et al., 2010). These enzyme related factors that influence the overall EH performance were experimentally addressed in **Chapter 4** and **Chapter 5** of this theses.

Yang *et al.* (2006) set out to determine if the cellulose substrate is responsible for the slowdown phenomenon observed during EH (Yang et al., 2006). After complete hydrolysis on Avicel (97 % cellulose) the solid from EH was washed to remove all protein and resubmitted to so-called “restart” experiments (Yang et al., 2006). In order to remove the enzyme from the substrate, proteinase treatment followed by proteinase inhibitors and a water-sodium chloride (NaCl)-water wash sequence was used whereafter nitrogen testing methods indicated that this technique removed all enzymes relative to the Avicel control (Yang et al., 2006). The results suggested that the Avicel substrate did not lose reactivity as the reaction proceeded (Yang et al., 2006). Furthermore, addition of β -glucosidase had no effect on the conversion of the solids; therefore, product inhibition was not the cause of slowdown. It was suggested that the slowdown in the rate of hydrolysis may be due to the size of the cellulases providing steric hindrance to enzyme-substrate binding (Yang et al., 2006). It was hypothesized

that slowdown is more likely due to factors involved in slowing or sterically hindering the enzymes than a decrease in cellulose reactivity (Yang et al., 2006).

Work from the same group suggested that xylooligomers (XOS) were inhibiting the cellulases resulting in the slowdown phenomenon (Qing et al., 2010). The effect that xylose, xylan and xylooligomer addition and concentration has on the EH of Avicel and their influence on cellulase and β -glucosidase action, was investigated (Qing et al., 2010). The results suggest that xylose, xylan and xylooligomers inhibits the EH rate and yield of Avicel by cellulases and that increasing concentrations of these compounds increase their inhibitory role (Qing et al., 2010). Furthermore, xylooligomers exhibited greater inhibition of cellulose hydrolysis than xylose, xylan, glucose and cellobiose respectively (Qing et al., 2010). Therefore, the removal of the hemicellulose fraction before EH could be more important than previously described (Qing et al., 2010). Hemicellulose removal not only increases the ASA, but simultaneously reduces the inhibitory effect that xylose, xylan and xylooligomers have on EH (Qing et al., 2010). However, in both instances Avicel was used as the substrate, which does not represent the complexity of lignocellulosic biomass. Therefore, evaluating the increased complexity that the different substrate characteristics in lignocellulose introduce, and how these characteristics influence cellulose conversion rate and yield, is important to understand biomass recalcitrance and the factors hampering fast rates and complete hydrolysis during the EH of lignocellulosic biomass.

The substrate related factors include: Cuticle and epicuticular waxes in the epidermal tissue of the plant, vascular bundle arrangement and density, high degree of lignification and phenolics, the thick wall of the sclerechymatous tissue, microfibril and matrix polymer heterogeneity (acetyl and hemicellulose), insoluble substrates, crystalline cellulose and degree of polymerisation of the cellulose fraction (Arantes & Saddler, 2011; Gupta & Lee, 2009; Hallac & Ragauskas, 2011; Himmel et al., 2007; Rollin et al., 2011; Tejirian & Xu, 2011; Zhao et al., 2012; Zhu et al., 2008). The following section focusses on the physicochemical substrate factors involved in decreasing the overall performance during EH of lignocellulosic, which were experimentally addressed in **Chapter 4**.

2.5.2 Physicochemical factors that influence the overall EH performance

2.5.2.1 Introduction

The breakdown of CW sugars represents a major process bottleneck in the bioconversion of lignocellulose to various derived products (Zhao et al., 2012). The bottleneck stems from the fact that plants have evolved for millions of years to strengthen their cell walls against microbial attack, as well as giving them the ability to grow in more challenging environments (Himmel et al., 2007). This resilience is referred to as biomass recalcitrance and the factors involved in lignocellulosic recalcitrance continue to be an interesting subject for investigation. The specific physicochemical characteristics and their interactions within the lignocellulosic substrate are directly related to the recalcitrance of the substrate. The individual properties and their subsequent influence on enzymatic digestibility of the substrate are summarized in **Table 2.4**. These factors will be individually discussed below especially considering that one of the goals of this study was investigating the physicochemical changes that the substrate undergoes after steam explosion and at various stages during the course of EH, and how these changes influence the slowdown of EH rate.

Table 2.4: Physicochemical substrate properties and the influence it has on saccharification.

| Structural feature | | Influence on saccharification | Reference |
|--------------------|--|-------------------------------|---|
| Physical | Crystallinity | - | (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Gupta & Lee, 2009; Lynd et al., 2002; Puri, 1984; Zhang & Lynd, 2004; Zhu et al., 2008) |
| | Degree of polymerization | +/- | (Chandra et al., 2007a; Gupta & Lee, 2009; Hallac & Ragauskas, 2011; Pan et al., 2007; Puri, 1984; (Sinitsyn et al., 1989; Zhang & Lynd, 2004) |
| | Accessible surface area, specific surface area, pore size and volume | + | (Zhu et al., 2008); Burns et al., 1989; Chandra et al., 2009; Chiara Piccolo et al., 2010; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Grous et al., 1986; Mooney, 1998; Mooney et al., 1999; Rollin et al., 2011; Sinitsyn et al., 1989; Sinitsyn et al., 1991; Thompson et al., 1992; Yeh et al., 2010; Zhang & Lynd, 2004; Zhu et al., 2009) |
| Chemical | Lignin, hemicellulose and acetyl content | - | (Akin, 1982; Akin, 1990; Akin et al., 1990; Akin & Chesson, 1989; Chandra et al., 2007a; Chandra et al., 2009; Chang & Holtzapple, 2000; Draude, 2001; Grabber et al., 2009; Grohmann et al., 1989; Laureano-Perez et al., 2005; Mooney, 1998; Pan, 2008; Tejirian & Xu, 2011; (Zhu et al., 2008); Zhu et al., 2008) |

2.5.2.2 Chemical composition (lignin, hemicellulose and acetyl)

Pretreatment alters the chemical and structural composition of the lignocellulose, which has an array of effects on EH. Furthermore, these effects are compounded as the enzymes that degrade the material contribute to further structural changes. The major influential chemical components that influence cellulose conversion are discussed below.

Lignin is responsible for decreased saccharification performance (yield and rate) due to decreased enzyme access to sugars (Chandra et al., 2009; Chang & Holtzapple, 2000; Draude, 2001; Mooney, 1998; Zhu et al., 2008). Lignin has also been shown to bind irreversibly to cellulase decreasing the available enzyme activity at a particular dosage. Additionally, substrate recalcitrance is also increased depending on the amount of ferulate cross-links within lignin, lignin subunit composition, and degree of carbohydrate/lignin ester bonds (Chandra et al., 2007a; Van Dyk & Pletschke, 2012). Consequently, the overall EH performance is negatively affected, as increased recalcitrance greatly decreases EH rate and yield. Moreover, previous work has indicated that ferulate cross-linking accounts for 50% of the recalcitrant impact, of the lignin sheath and complexes with polysaccharide, on EH performance (Grabber et al., 2009). Therefore, breeding plants with less ferulate cross-linking would aid downstream saccharification and fermentation to a greater extent than general lignin alteration (Grabber et al., 2009).

Lignin removal is therefore a major focus in pretreatment of lignocellulosic material as it has been shown to drastically improve digestibility (Banerjee et al., 2010; Chang & Holtzapple, 2000; Van Dyk & Pletschke, 2012; Zhu et al., 2008). However, removal of all the lignin may cause substrate pores to collapse decreasing the surface area available to enzymes (Sun, 2011). In the present study, steam explosion was utilized as it modifies and partially removes lignin, which leads to increased saccharification (Chandra et al., 2009; Chang & Holtzapple, 2000; Draude, 2001; Mooney, 1998; Zhu et al., 2008). Zhu *et al.* (2008) reported the effect that various structural features have on the digestibility of poplar wood (Zhu et al., 2008). Lignin removal from poplar with per-acetic acid did not affect the crystallinity or acetyl content of the raw material, but significantly improved the digestibility of

lignocellulose. Removal of 50% of the lignin fraction increased EH yield from 20% for the untreated raw material to 85 – 90% for the treated material after 72h (Zhu et al., 2008). Furthermore, no significant increase in the substrate digestibility was observed when lignin removal was increased beyond 50% of the content of the raw material, thereby negating the impact of lignin content. This was attributed to the collapse of pores in the lignocellulose due to excessive lignin removal, subsequently decreasing the ASA for EH. A range of lignin-removal levels were also investigated with varying crystallinity samples of poplar. The lignin content increases the steric hindrance of the substrate to enzymes thereby decreasing the ASA, while the crystallinity decreases the EH rate of the enzymes once they have reached the cellulose fraction (Chang & Holtzaple, 2000; Zhu et al., 2008). Therefore, in the case of the high lignin, low crystallinity material the enzymes struggle to reach the cellulose fraction. However, due to their efficiency on low crystalline cellulose, the EH rate is high. Additionally, in this case, the ultimate EH yield is low possibly due to the high lignin content blocking certain areas of cellulose for enzyme action. On the other hand, in the case of the low lignin, highly crystalline material, the initial EH rates are low. However, the final EH yield is high given enough time, as a great amount of enzymes can slowly degrade the highly accessible crystalline material. Therefore, different chemical factors simultaneously contribute to the overall hydrolysis profile (Arantes & Saddler, 2011; Himmel, 2008; Zhao et al., 2012). Although lignin exhibited the greatest inhibitory effect on EH yield and rate, high crystallinity was also shown to inhibit EH rate (Zhu et al., 2008).

In a study by Chandra *et al.* (2009) different lignin reduction techniques were employed to improve EH yields at low enzyme loadings (10 FPU/g cellulose). An ethanol-organosolv pretreatment dissolved the lignin and part of the hemicellulose fraction yielding a cellulose rich fraction which resulted in 100% EH yield after 48h. However, steam explosion, which enriches the lignin fraction, due to solubilizing the hemicellulose fraction, resulted in 53% EH yield after 72h. The ASA of the samples were analysed by SS, which indicated that samples from ethanol-organosolv pretreatment had a greater ASA than steam pretreated samples. Therefore, lignin removal is effective in decreasing the substrate recalcitrance to EH as it increases the ASA which results in greater EH yields and rates (Chandra et al., 2009).

Similarly, Draude *et al.* (2001) investigated the effect of oxygen delignification on the EH of softwood pulp with low enzyme loadings (2 – 10 FPU/g substrate) (Draude, 2001). Oxygen delignification of the pulp removed up to 64% of residual lignin, which increased EH rate by 111% and EH yields by 174%. However, in samples from another mill, decreased lignin removal resulted in a lower (43%) increase in EH yield. The data indicated that lignin removal was directly correlated to EH yield and significantly increased the EH rate in the range studied. The magnitude of lignin removal could be increasing the ASA as shown by (Chandra *et al.*, 2009) and resulting in less irreversible enzyme-lignin binding, thereby increasing the EH yield and rate. Mooney *et al.* (1998) investigated 4 Douglas fir pulps to determine the effect of pore volume and lignin content on EH yield and rate (Mooney, 1998). Literature suggests that the inhibitory role of lignin in EH, is due to its inhibitory role in fibre swelling and enzyme accessibility (Chandra *et al.*, 2007a; Chandra *et al.*, 2009; Chang & Holtzapple, 2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu *et al.*, 2008). However the data indicated that lignin also inhibits EH even in swollen fibers (Mooney, 1998). Although the protein absorption of the samples was similar, the delignified pulp showed much greater EH yields. This could be due to lignin-enzyme associations in the lignin rich samples indicating increased substrate recalcitrance as EH proceeds, which decreased the EH rate and yield. Additionally, lignin removal contributed to the formation of pores within the fibers thus creating more ASA, resulting in greater EH yields.

As previously mentioned hemicellulose is involved in various cross-links with CW polymers, including lignin, and contributes to the physical barrier that encloses cellulose, thereby increasing substrate recalcitrance (Scott *et al.*, 1984). Therefore, the effect that hemicellulose removal has on cellulose digestibility has been investigated (Schell *et al.*, 1989; Scott *et al.*, 1986a). However, SB hemicellulose is mostly xylan, which is highly acetylated and contains arabinose and *O*-methyl-D-glucuronic acid side groups. (de Vries & Visser, 2001; Grohmann *et al.*, 1989; Geng *et al.*, 2006; Peng *et al.*, 2010; Sun, 2004; Wyman, 1996). Grohmann *et al.* (1989) showed that xylan removal and deacetylation by hydroxylamine pretreatment had a strong impact on cellulose to glucose conversion yield of aspen wood and wheat straw at high enzyme dosages (75 FPU/g substrate). Although lignin removal was important, removal and deacetylation of the xylan fraction was sufficient for increasing EH conversion of xylan to xylose 7 fold. Thereafter, the cellulose to glucose yield increased 3 fold, indicating that the acetylated

xylan represents a physical barrier to cellulose digestibility (Grohmann et al., 1989). Removal of these fractions within the substrate possibly also increases ASA, similar to lignin removal, subsequently decreasing substrate recalcitrance and increasing overall EH performance. Additionally, the acetic acid released during the EH of acetylated xylan could have an inhibitory effect on the microorganisms used in fermentation. Therefore, deacetylation could represent a viable target in pretreatment optimization for grasses such as sugarcane. However, Wyman states that the positive results seen from hemicellulose removal could be partially attributed to the melting and coagulation of lignin by pretreatments such as dilute sulphuric acid, sulphur dioxide (SO₂), steam explosion and hydrothermolysis, making cellulose more accessible (Wyman, 1996). Zhu *et al.* (2008) treated poplar wood with potassium hydroxide (KOH) to deacetylate the xylan at varying cellulose loadings (1-30 FPU/g dry substrate). Even though deacetylation increased the EH rate and ultimate yield, deacetylation had a minor effect when compared to lignin removal and crystallinity decrease. However, grassy feedstocks such as SB, could possibly benefit more from deacetylation owing to the greater acetylated xylan content in the feedstock (de Vries & Visser, 2001; Grohmann et al., 1989; Wyman, 1996). This has previously been shown for different corn stover varieties (Chen, et al., 2012a; Chen, et al., 2012b). The change in the chemical features of SB during EH, was experimentally addressed in the present thesis (**Chapter 4**).

2.5.2.3 Physical properties: Crystallinity

Cellulose crystallinity is an important factor in the recalcitrance of the substrate, significantly inhibiting the EH rate, increasing the EH time required to reach high yields. (Chang & Holtzapple, 2000; Fan et al., 1981; Zhu et al., 2008). Various studies have shown that crystalline cellulose is less accessible to cellulase compared to amorphous cellulose (Chang & Holtzapple, 2000; Fan et al., 1981; Zhu et al., 2008).

Fan *et al.* (1981) compared the effect of crystallinity and ASA on EH rate of Solca Floc and cellulose. The results suggest that EH rate increases linearly with decreasing crystallinity and increased ASA after ball milling, as both these factors are changed simultaneously during ball milling (Fan et al., 1981). Similarly, Zhu *et al.*

(2008) compared the effect of crystallinity and digestibility in low-lignin vs. high-lignin poplar wood. Ball milling was used to decrease the crystallinity, whereafter the pretreated biomass was subjected to EH (5-10 FPU/g dry pretreated biomass). Lower crystallinity in high-lignin wood substantially increased EH rate at 1 and 6h and moderately at 72 hours (Zhu et al., 2008). Therefore, lowering crystallinity leads to lower enzyme loadings and faster EH rates (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008). Additionally, even though CBHs can hydrolyse crystalline cellulose, it occurs at very low rates (Teeri, 1997). These results support the belief that crystalline cellulose is more recalcitrant and resistant to enzymatic degradation than amorphous cellulose (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008). However, hydrolysis of low crystalline low-lignin wood indicated that crystallinity does not have as great effect on EH yield in low-lignin, as in high-lignin containing wood (Zhu et al., 2008). This can be explained as various structural features exhibit a combined impact on overall EH performance. In the case of low-lignin wood, the removal of lignin seems sufficient in increasing EH yield. However, due to the high crystallinity, EH occurs at slower EH rates (Zhu et al., 2008). Moreover, the lignin and hemicellulose fraction contributes significantly to the amorphous nature of lignocellulosic material (Brett & Waldron, 1996; Bidlack et al., 1992; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005; Zhu et al., 2008). This complicates accurate determinations of the influence of crystallinity on the overall EH performance, but more specifically the EH rate, of biomass by cellulases. Therefore, in the present study the crystallinity of steam pretreated SB was investigated at various steps during enzymatic hydrolysis to determine its effect on overall EH performance (**Chapter 4**).

2.5.2.4 Physical properties: Degree of polymerisation

Various studies examine the effect that pretreatments have on the DP of cellulose, as reduction in the DP increases the downstream EH rate and yield (Hallac & Ragauskas, 2011; Pan et al., 2007; Puri, 1984, Sinitsyn et al., 1989; Zhang & Lynd, 2004). The change in DP is a result of the pretreatment used, as well as the nature of the lignocellulose feedstock. A high DP indicates greater hydrogen bonding (intra- and

intermolecular), which increases the substrate recalcitrance and decreases ASA (Pan et al., 2007; Puri, 1984). Therefore shorter cellulose fragments (lower DP), are not as resistant to solvents and enzymes, due to the decreased hydrogen bonding and increased ASA (Pan et al., 2007; Puri, 1984). As a result, more cellulose reducing ends are available for CBHs at low DP, resulting in higher cellulose to glucose conversion rates and yields (Hallac & Ragauskas, 2011; Pan et al., 2007; Puri, 1984, Sinitsyn et al., 1989; Zhang & Lynd, 2004).

Puri (1984) determined the DP of SB as 925 glucose units using the viscometric method (Puri, 1984). The DP of SB cellulose falls to 800 with ozone (O_3), 572 with carbon-dioxide explosion and 550 with alkaline explosion pretreatments (Puri, 1984). The decrease in DP increased cellulose digestibility by 58%, 50% and 57% respectively vs. untreated SB (Puri, 1984). Although it is clear that a decrease in DP leads to an increase in the saccharification yield, it is important to keep in mind that pretreatment may alter the lignin and hemicellulose fraction. Therefore, increased saccharification may be due to factors other than DP. Research suggests that ionic liquids can be used to determine accurately DP as it dissolves cellulose without altering DP (Hallac & Ragauskas, 2011). Consequently, in the present study the DP of steam pretreated SB was investigated at various steps during enzymatic hydrolysis (**Chapter 4**).

2.5.2.5 Physical properties: Accessible surface area, internal/external area, pore size and volume

Accessible surface area (ASA) is the most critical factor involved in the enzymatic conversion of biomass to sugar, as the EH rate and yield is directly limited by the adsorption of enzymes onto the substrate (Zhao et al., 2012). Various studies have indicated that increased surface area leads to better saccharification, increasing both the hydrolysis rates and yields, in particular with smaller fibres that have increased specific surface area (SSA) (Burns et al., 1989; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Mooney, 1998; Mooney et al., 1999; Sinitsyn et al., 1991; Thompson et al., 1992; Yeh et al., 2010; Zhu et al., 2009). The total SSA of a substrate is related to exterior surface area (particle size i.e. length and width) and interior surface (porosity, pore volume, lumen size), with the internal surface area up to 2 orders greater than the external surface area (Chandra et al., 2007a; Chang et al., 1981; Zhao et al., 2012).

Various works have showed the importance of the interior surface area, which has been associated as the most influential biomass characteristic inhibiting or improving EH rate (Cosgrove, 2005; Grous et al., 1986; Huang et al., 2009; Shevchenko et al., 2000). It has been shown that the porous interior surface area accounts for more enzyme accessibility than the exterior surface, with more than 94% of the substrate hydrolysis yield related to accessible interior pore surfaces (Hong et al., 2007; Luo & Zhu, 2011; Wang et al., 2011). In brief, less interior pores result in lower EH yields (Hong et al., 2007; Luo & Zhu, 2011; Wang et al., 2011). However it is important to note that ASA and SSA are distinctly different in that SSA is the total surface area/unit of bulk or solid volume and ASA specifically refers to surface that is accessible to enzyme (Zhao et al., 2012). Therefore, increasing SSA does not necessarily lead to increased ASA.

Additionally, the pore diameter is important to allow cellulase access, with the rate-limiting pore size for lignocellulose at 40 -51Å (4 - 5.1nm), which correlates to the size of the *T. reesei* cellulase (**Table 2.5**) (Chandra et al., 2007a; Grethlein, 1985; Stone et al., 1969; Tanaka et al., 1988; Thompson et al., 1992; Weimer & Weston, 1985; Zhang & Lynd, 2004). It has been shown that the initial rate of substrate hydrolysis, regardless of the substrate origin, is linearly correlated to the pore volume of the substrate accessible to particles with a 51 Å (5.1nm) diameter (Grethlein, 1985). Various works indicate the importance of ASA and suggest increase porosity/pore accessibility increases cellulose conversion (Burns et al., 1989; Chandra et al., 2008; Fierobe et al., 2002; Grethlein et al., 1984; Thompson et al., 1992; Weimer et al., 1990; Wong et al., 1988). Piccolo et al (2012) showed that increased ASA leads to increased protein adsorption and increased conversion rates (Piccolo et al., 2010). Previous work by Sinitsyn et al. (1991) showed a linear relationship between increased SB hydrolysis and greater ASA (Sinitsyn et al., 1991). Increasing the ASA is more important than decreasing lignin content for efficient EH of recalcitrant lignocellulose (Rollin et al., 2011). ASA impacts the EH rate and yield, as increased ASA results in greater overall EH performance. At the initial phases of biomass degradation, enzymes need to reach the cellulose; therefore ASA is the rate limiting step. Even though the physicochemistry and subsequent recalcitrance of the material and remaining cellulose fraction changes as EH proceeds, ASA remains the most important factor for high yields at the final phases of EH (Burns et al., 1989; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Hong et al., 2007; Luo & Zhu, 2011; Mooney, 1998; Mooney et al., 1999;

Sinitsyn et al., 1991;Thompson et al., 1992; Wang et al., 2011; Yeh et al., 2010; Zhu et al., 2009).

Table 2.5: Dimensions of the cellulases.

| <i>Enzyme</i> | <i>Size</i> | <i>Reference</i> |
|---------------|--|-------------------------------------|
| CBH1 | Core (4.5– 6.7nm), tail (3.2 – 12.9nm) | (Abuja et al., 1988; Kubicek, 1992) |
| CBH2 | Core (5-6nm), tail (3.5 – 15.2nm) | (Abuja et al., 1988) |
| Cellulases | Hydrodynamic radius varies between (1.8 – 6.5nm) | (Ghaouar & Gharbi, 2011) |
| CBH1 | Globular (6.5nm diameter), if spherical (5.9nm) | (Li et al., 2001) |
| CBH1 | (6 x 5 x 4nm overall dimensions) 4nm active site | (Divne et al., 1994) |
| CHB1 & 2 | Core (6-7nm long, 4nm diameter), tail (15nm) | (Bergfors et al., 1989) |

Various methods can be used for determining the ASA and SSA of a substrate. In 1950, a two colour differential stain technique was used and described for investigating microscopic changes in the fibrillation and mechanical damage of beaten fibre (Simons, 1950). The so-called “Simons’ stain” (SS) is sensitive to the interior accessibility of interior fibre structures (Chandra et al., 2008; Chandra & Saddler, 2012; Esteghlalian et al., 2001; Yu et al., 1995). The 2 dyes, Direct blue 1 (DB) and Direct orange 15 (DO) (**Figure 2.12**) have previously been used to investigate the effects of enzyme treated fibres. The two dyes differ in size, with the dimensions of the bigger DO consist of a 5-7nm and 14-36nm sections and 1nm diameter for DB (Yu et al., 1995). It was shown that the absorption of DB and DO to the fibre is a measure of the accessibility of the interior surface area for the dyes, with pore widths of larger than 5nm needed to accommodate DO (Yu et al., 1995). Furthermore, the SS method is independent of the type of fibres, lignin content, cellulose content, as it has been shown that the DO always has a higher affinity for the material than DB regardless of the fibre properties (Simons, 1950). The SS techniques provides similar information to that of water

retention and solute exclusion however, the information is of a more qualitative nature (Yu et al., 1995).

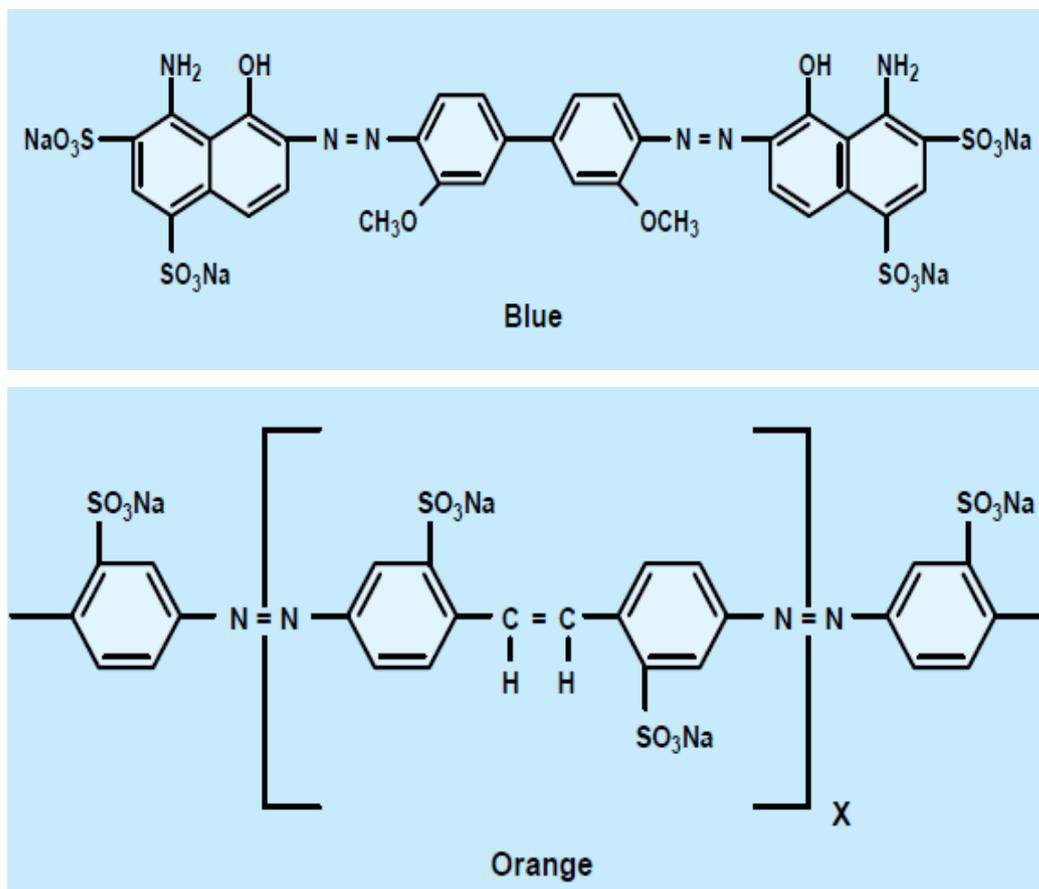


Figure 2.12: Chemical structure of DB and DO dyes (Yu et al., 1995). Reprinted with permission from Tappi journal.

Chandra *et al.* (2009) showed that increasing the ASA of lodge pole pine samples with steam and ethanol pretreatment increased the EH yield of the material (Chandra et al., 2009). The SS method was used to differentiate between pretreatments and found that greater ASA lead to the observed greater EH yield. Therefore, ASA can be used as a screening tool to predict the EH yields of pretreated lignocellulosic substrates. Additionally, the total amount of enzyme that can adsorb per gram of material is a useful indication of the amount of accessible cellulose (Chandra et al., 2009). Arantes *et al.* (2011) used SO_2 catalyzed steam explosion combined with the SS method according to Chandra *et al.* 2009 to investigate a variety of agricultural and industrial lignocellulosic materials and found that the amount of ASA was the limiting factor in efficient hydrolysis (Arantes & Saddler, 2011). The minimum amount of protein loading needed to achieve 70% hydrolysis is linear to the amount of ASA

(Arantes & Saddler, 2011). These results emphasize the importance of ASA initially stated by Chandra *et al.* (2009). Mooney *et al.* (1998) also showed that interior pore size, which is directly related to SSA and to a lesser degree ASA, is the rate-limiting step in EH (Mooney, 1998). In comparisons between Kraft pulp containing 4% lignin and delignified pulp containing 8.2% lignin, greater hydrolysis yields were observed in the delignified pulp, although it had greater lignin content. The ASA was greater due to the finer fibers present in the sample versus the larger heterologous fibers found in the Kraft pulp (Mooney, 1998). Additionally, delignification in pulp samples increased the formation of small pores in the fibers. These pores subsequently increased the SSA and therefore ASA. These combined factors lead to increase in EH yields indicating the complex relationship that various physicochemical characteristics exhibit regarding enzymatic saccharification (Mooney, 1998). Considering the importance of SSA [exterior surface area (particle size i.e. length and width) and interior surface (porosity, pore volume, lumen size)] and ASA in overall EH performance, these factors were experimentally addressed in this thesis (**Chapter 4**).

2.5.2.6 Interaction between chemical and physical properties

Even though ASA and SSA represent the most important factors for efficient enzymatic hydrolysis, the other physicochemical substrate characteristics can directly influence these parameters and decrease overall EH performance due to their individual and combined recalcitrant effects on the biomass (**Figure 2.13**). Chang and Holtzapple (2000) did an extensive study on the effect that lignin, crystallinity and acetyl content has on EH of 147 samples and how these different characteristics interact to influence EH yield (Chang & Holtzapple, 2000). Delignification was done by peracetic acid treatment, deacetylation by KOH treatment and decrystallisation by ball milling. Acetyl removal however had a minor impact. At low lignin and high crystallinity, sufficient enzyme is able to adsorb to cellulose, however the EH rate is low due to the crystalline nature of the cellulose (Bansal *et al.*, 2010; Chang & Holtzapple, 2000; Fan *et al.*, 1981; Lynd *et al.*, 2002; Zhu *et al.*, 2008). However, after 72 h EH high yields can be obtained even though the EH rate is low, due to the long EH period (Chang & Holtzapple, 2000). low crystallinity and high lignin, enzymes struggle to adsorb to cellulose because they are blocked by lignin (Banerjee *et al.*, 2010; Chandra *et al.*, 2009; Chang & Holtzapple,

2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu et al., 2008). However, the available cellulose is easily digestible by the few enzymes that adsorb due to the low crystallinity of the cellulose resulting in a high EH rate (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008). And even though high initial EH yields are achieved, the negative impact of the high lignin content could reduce the ultimate EH yield by decreasing ASA and sterically blocking enzyme/substrate interaction (Banerjee et al., 2010; Chandra et al., 2009; Chang & Holtzapple, 2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu et al., 2008). However, by reducing the lignin in this scenario high initial EH rates and ultimate EH yields can be achieved. Thus, both low lignin and low crystallinity results in high EH yields albeit for different reasons (Chang & Holtzapple, 2000). In the control experiment with high lignin and high crystallinity, very low EH rates and yields were observed even at very long hydrolysis times (Chang & Holtzapple, 2000). It is possible that the lignin barrier decreased the ASA and enzyme access (Banerjee et al., 2010; Chandra et al., 2009; Chang & Holtzapple, 2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu et al., 2008), which combined with the low EH rate due to the highly crystalline cellulose (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008), is responsible for the poor overall EH performance. Additionally, the data indicated that delignification, deacetylation and crystallinity have different effects on hemicellulose than on cellulose (Chang & Holtzapple, 2000). Delignification and deacetylation play a greater role in the hydrolysis of the hemicellulose fraction because lignin and acetyl are attached to hemicellulose within the CW matrix phase, while cellulose is in the microfibrillar phase (Brett & Waldron, 1996; Himmel, 2008; Iiyama et al., 1994; Menon & Rao, 2012; Pandey, 2009; Rose, 2003; de Vries & Visser, 2001). Cellulose saccharification rate is greatly improved by lowering crystallinity due to its crystalline nature (Bansal et al., 2010; Chang & Holtzapple, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008), however hemicellulose is not crystalline and therefore lower crystallinity plays no role in improving hemicellulose EH rate (Chang & Holtzapple, 2000).

2.5.2.7 Summary of the physicochemical factors affecting enzymatic hydrolysis of biomass

It is clear that the enzymatic hydrolysis of lignocellulose is a complicated by various substrate factors, each playing their own role in the biomass recalcitrance. However, these physicochemical substrate characteristics often overlap and interact, thereby increasing the difficulty of determining the weight of a specific factor in biomass recalcitrance (**Figure 2.13**).

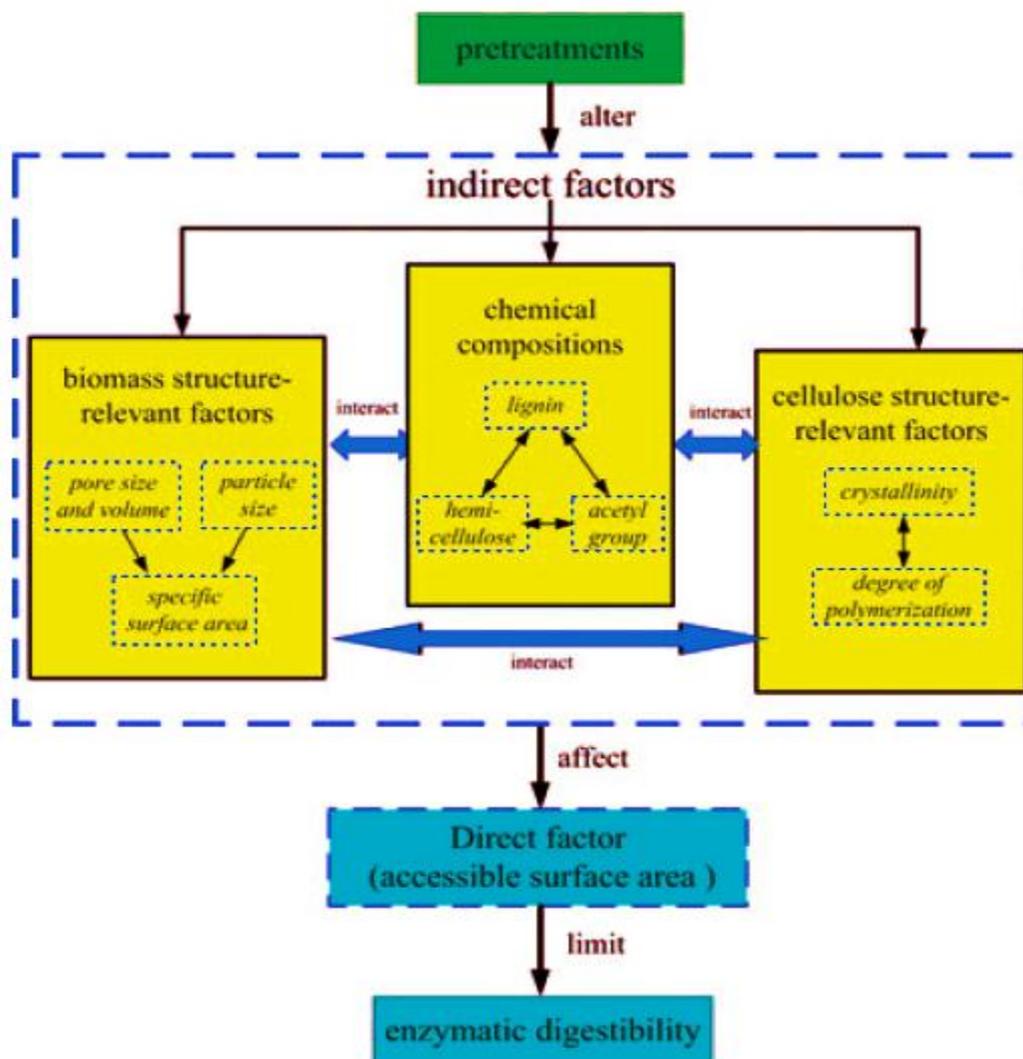


Figure 2.13: The factors influencing the enzymatic hydrolysis of lignocellulosic material as well as their interactions with each other (Zhao et al., 2012). Reprinted with written permission from John Wiley and Sons and their Copyright Clearance Center (RightsLink service).

The single most important factor for efficient enzymatic hydrolysis is ASA (Zhu et al., 2008). The physicochemical substrate characteristics of the material represent indirect factors influencing the direct factor: ASA. However, it is clear that there are various indirect factors each with their own interactions. Therefore, complete studies investigating various factors are required to elucidate biomass recalcitrance and “slowdown” of EH rate and decreased overall EH yields. In that regard, (**Chapter 4**) comprises a paper that shows the changes in the physicochemical substrate characteristics of steam pretreated SB during enzymatic hydrolysis. The individual physicochemical characteristics discussed in this section are summarized below:

- Lignin: → Lignin origin and structure has a unique impact on the level of inhibition such as irreversible cellulase binding. Lignin decreases enzyme access to cellulose by acting as a physical hindrance, decreasing ASA (**Figure 2.13**) (Banerjee et al., 2010; Chandra et al., 2009; Chang & Holtzapfle, 2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu et al., 2008). Enzyme/lignin adsorption is influenced by hydrogen bonding, electrostatic and hydrophobic interactions. The soluble and insoluble phenolic content of the biomass contributes to enzyme inhibition and substrate recalcitrance (Akin, 1982; Akin, 1990; Akin et al., 1990; Akin & Chesson, 1989). Furthermore, the reactivity of lignin depends on the feedstock and pretreatment method utilized. Lignin decreases the EH rate and yield (Banerjee et al., 2010; Chandra et al., 2009; Chang & Holtzapfle, 2000; Draude, 2001; Mooney, 1998; Van Dyk & Pletschke, 2012; Zhu et al., 2008). Lignins contribution to substrate recalcitrance and the EH of SB was addressed in **Chapter 4** of this study.
- Hemicellulose: → Similar to lignin, hemicellulose also acts as a physical barrier inhibiting cellulase action by decreasing the ASA (**Figure 2.13**). Therefore, hemicellulose decreases cellulose conversion to glucose yield and rate (de Vries & Visser, 2001; Grohmann et al., 1989; Schell et al., 1989; Scott et al., 1986a; Wyman, 1996). However, the recalcitrant effect of hemicellulose is overshadowed by the negative effects of lignin (de Vries & Visser, 2001; Grohmann et al., 1989; Schell et al., 1989; Scott et al., 1986a; Wyman, 1996). Additionally, most pre-treatments, such as steam explosion used in the present study, remove/reduce the

hemicellulose, decreasing its inhibitory role (Alvira et al., 2010b) (**Chapter 4 & 5**).

- Acetyl: → It has been shown that the acetyl content of the biomass can obstruct cellulase/cellulose interaction, impact ASA (**Figure 2.13**) and sugar EH yield (de Vries & Visser, 2001; Grohmann et al., 1989; Wyman, 1996). However, due to the relatively small impact and amount of acetyl relative to lignin and hemicellulose, its effects on substrate recalcitrance have been described as negligible (Zhu et al., 2008). On the other hand, acetyl content could lead to the formation of inhibitors, such as acetic acid, which can inhibit enzymes and fermentative microorganisms (de Vries & Visser, 2001; Grohmann et al., 1989; Wyman, 1996). However, the acetyl content of the WIS in the present study was low, diminishing its inhibitory role (**Chapter 4 and 5**).
- Crystallinity: → The effect of crystallinity is very often reported for pure cellulose substrates where it is shown that amorphous cellulose is significantly faster to hydrolyse than crystalline cellulose (Bansal et al., 2010; Chang & Holtzapfle, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008). Similarly, decreasing the CrI leads to increased initial cellulose conversion rates and higher EH yields, especially in the absence of lignin (Bansal et al., 2010; Chang & Holtzapfle, 2000; Fan et al., 1981; Lynd et al., 2002; Zhu et al., 2008). However, in lignocellulosic biomass the crystallinity of the overall material is relatively small compared to the amorphous regions such as lignin. Furthermore, changes in crystallinity increases the ASA, thereby making CrI a dependent factor (**Figure 2.13**). Changes in the crystallinity of the SB during EH were investigated in the present study (**Chapter 4**).
- Degree of polymerization: → Lower DP allows increased saccharification due to more available cellulose reducing ends available for CBHs and greater ASA resulting in higher EH rates (Gupta & Lee, 2009; Hallac & Ragauskas, 2011; Pan et al., 2007; Puri, 1984, Sinitsyn et al., 1989; Zhang & Lynd, 2004). However, the initial DP of a material has little impact on final EH yield after long EH periods as EH is in fact just the reduction of the glucose units of the cellulose chain, which is

termed DP (Nazhad et al., 1995; Zhang et al., 2012). Therefore, the varying DPs could have an impact on the rate of EH due to greater ease of access for the enzymes, however DP does not have a recalcitrant effect on the maximal EH yield as DP refers to the number of glucose units available for EH (Nazhad et al., 1995; Zhang et al., 2012). Furthermore, changes in DP are usually brought by changes in CrI and ASA of the material making DP a dependent factor (Nazhad et al., 1995; Zhang et al., 2012). Changes in the DP of the SB during EH were investigated in the present study (**Chapter 4**).

- Specific surface area and pore volume: → SSA is related to the porosity, pore volume and particle size of the substrate. These factors in turn have an impact on the surface area for potential enzymatic hydrolysis. However, SSA is not equivalent to ASA as physicochemical substrate characteristics could still hinder enzyme action within these areas. It is also believed that the interior surface area within the lignocellulosic substrate is more important for efficient enzymatic hydrolysis than the exterior surface area (Abuja et al., 1988; Arantes & Saddler, 2011; Bergfors et al., 1989; Chandra et al., 2009; Divne et al., 1994; Ghaouar & Gharbi, 2011; Kubicek, 1992; Li et al., 2001; Mooney, 1998; Simons, 1950; Yu et al., 1995). Furthermore, it has been shown that the bulk of microbial cellulase used in biomass hydrolysis is in the 5-7nm size thereby requiring pores of 5.1nm or larger for enzyme access (Abuja et al., 1988; Arantes & Saddler, 2011; Bergfors et al., 1989; Chandra et al., 2009; Divne et al., 1994; Ghaouar & Gharbi, 2011; Kubicek, 1992; Li et al., 2001; Mooney, 1998; Simons, 1950; Yu et al., 1995). Therefore, porosity, pore volume and particle size and SSA size measurements, which interact with the physicochemical factors such as lignin content and crystallinity, give an indication of the ASA. Therefore, it is an important factor in the overall EH performance (Abuja et al., 1988; Arantes & Saddler, 2011; Bergfors et al., 1989; Chandra et al., 2009; Divne et al., 1994; Ghaouar & Gharbi, 2011; Kubicek, 1992; Li et al., 2001; Mooney, 1998; Simons, 1950; Yu et al., 1995). In the present study SS was used to determine the internal and external surface area for enzyme action (**Chapter 4**).

- Accessible surface area: → By definition enzymes need access to their respective substrates (Burns et al., 1989; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Hong et al., 2007; Luo & Zhu, 2011; Mooney, 1998; Mooney et al., 1999; Sinitsyn et al., 1991; Thompson et al., 1992; Wang et al., 2011; Yeh et al., 2010; Zhu et al., 2009). This makes accessible surface area arguably the most important factors that could aid or limit enzymatic hydrolysis (**Figure 2.13**). It is believed that most other physical and chemical substrate characteristics that negatively influence enzymatic hydrolysis are involved in modifying or decreasing the ASA for enzymes (Burns et al., 1989; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Hong et al., 2007; Luo & Zhu, 2011; Mooney, 1998; Mooney et al., 1999; Sinitsyn et al., 1991; Thompson et al., 1992; Wang et al., 2011; Yeh et al., 2010; Zhu et al., 2009). Therefore, one of the most important factors in the pretreatment of raw lignocellulosic biomass is modification of the physicochemistry of the material to increase ASA which is critical in fast EH rates and high ultimate EH yields (Burns et al., 1989; Converse et al., 1990; Fan et al., 1981; Grethlein, 1985; Hong et al., 2007; Luo & Zhu, 2011; Mooney, 1998; Mooney et al., 1999; Sinitsyn et al., 1991; Thompson et al., 1992; Wang et al., 2011; Yeh et al., 2010; Zhu et al., 2009).

Altogether, it is clear that there is great interaction between the physicochemical substrate characteristics, affecting the overall ASA (**Figure 2.13**). The ASA is ultimately the most important factor for efficient EH rate and yield but the ASA is comprised of all the different physicochemical substrate characteristics, thereby linked each characteristic to the overall EH performance. However, even if the physicochemistry of a specific substrate is understood, a good EH rate and yield is still dependent on the right mixture of enzymes. Therefore, the following section introduces the commercial enzymes available and used in the present study as well as the importance of efficient EH (fast rates & high yields) on downstream fermentation and bio-ethanol process economy.

2.6 COMMERCIAL ENZYMES AND THE IMPORTANCE OF EFFICIENT HYDROLYSIS

2.6.1 Commercially available enzymes tested in the present study

In the present study, commercially available enzymes from Dyadic, Genecor and Novozymes were compared and tested for use in EH and fermentation optimization (**Table 2.6; Chapter 4 & 5**). The activities identified for these enzymes (cellulases, β -glucosidase and xylanase) are mostly what would be expected for the efficient degradation of lignocellulose (**Table 2.6**) (Van Dyk & Pletschke, 2012). It is also clear that the new generation CTec2 mixture from Novozyme has a good balance between cellulase and β -glucosidase (**Table 2.6**).

2.6.2 The cost involved in enzymatic hydrolysis & the importance of optimum dosages

Companies such as Novozymes, Genecor, DSM and Verenium have implemented intensive research schemes which have contributed to gradually decreasing enzyme prices. Companies such as Novozymes (www.novozymes.com; www.biosciences.dupont.com; www.dsm.com; www.verenium.com). However, enzyme cost is still a major economic hurdle in the production of bio-ethanol from lignocellulose (Hettenhaus, 2000; Hinman et al., 1992; Huang et al., 2011; Klein-Marcuschamer et al., 2012; Petiot, 2008). The criteria important in the selection and production of efficient biocatalysts can be summarized by **Figure 2.14**, and represents the major research areas for improving enzyme quality (Jørgensen et al., 2007b; Sarrouh et al., 2012; Viksø-Nielsen, 2009). Properties such as pH-, byproduct- and solvent stability, enzyme activity (k_{cat}) and enzyme space-time yield efficiency seem to be the most important factors in the production of novel biocatalysts (**Figure 2.14**) (Jørgensen et al., 2007b; Sarrouh et al., 2012; Viksø-Nielsen, 2009).

Table 2.6: Commercially available enzyme mixtures used in the present study and their respective main activities and protein concentrations, reported in literature or determined in the present study.

| Commercial mixture | Company | Protein (mg/ml)* | Main activity (U/ml) unless otherwise indicated.* Source indicated in brackets |
|------------------------------|----------------|-------------------------|---|
| Alternafuel CMax (liquid) | Dyadic | 130 | 37FPU cellulase, 213 β -glucosidase (Chapter 4 & 5; Pengilly et al., 2012) |
| Spezyme CP | Genencor | 140 | 59FPU cellulase, 5263 xylanase (Chapter 4 & 5; Pengilly et al., 2012) 58.2FPU cellulase, 2622 xylanase (Dien et al., 2008) |
| Optiflow | Genencor | 130 | 130FPU cellulase (Chapter 4 & 5 of this study) 220 β -glucosidase, 2850 xylanase (Pengilly et al., 2012) |
| Multifect Xylanase | Genencor | 47.7 | 12FPU cellulase, 60855 xylanase (Chapter 4 & 5; Pengilly et al., 2012) |
| Pectinex Ultra | Genencor | 183 | 176 xylanase, 29 mannanase (Chapter 4 & 5; Pengilly et al., 2012) |
| Novozym 188 | Novozymes | 114 | 700 β -glucosidase (Chapter 4 & 5 of this study) 665 β -glucosidase (Dien et al., 2008) |
| Cellic CTec2 | Novozymes | 161.2 160 | 120FPU cellulase, 2731 β -glucosidase (Cannella et al., 2012) 132FPU cellulase (McIntosh et al. 2012) |
| Cellic HTec2 | Novozymes | - | Endo-xylanase with strong cellulase background and high specificity toward soluble hemicellulose (www.novozymes.com) |

*All activities and protein concentrations were determined as described by Ghose (Ghose, 1987) and may vary depending on assay conditions (Van Dyk & Pletschke, 2012).

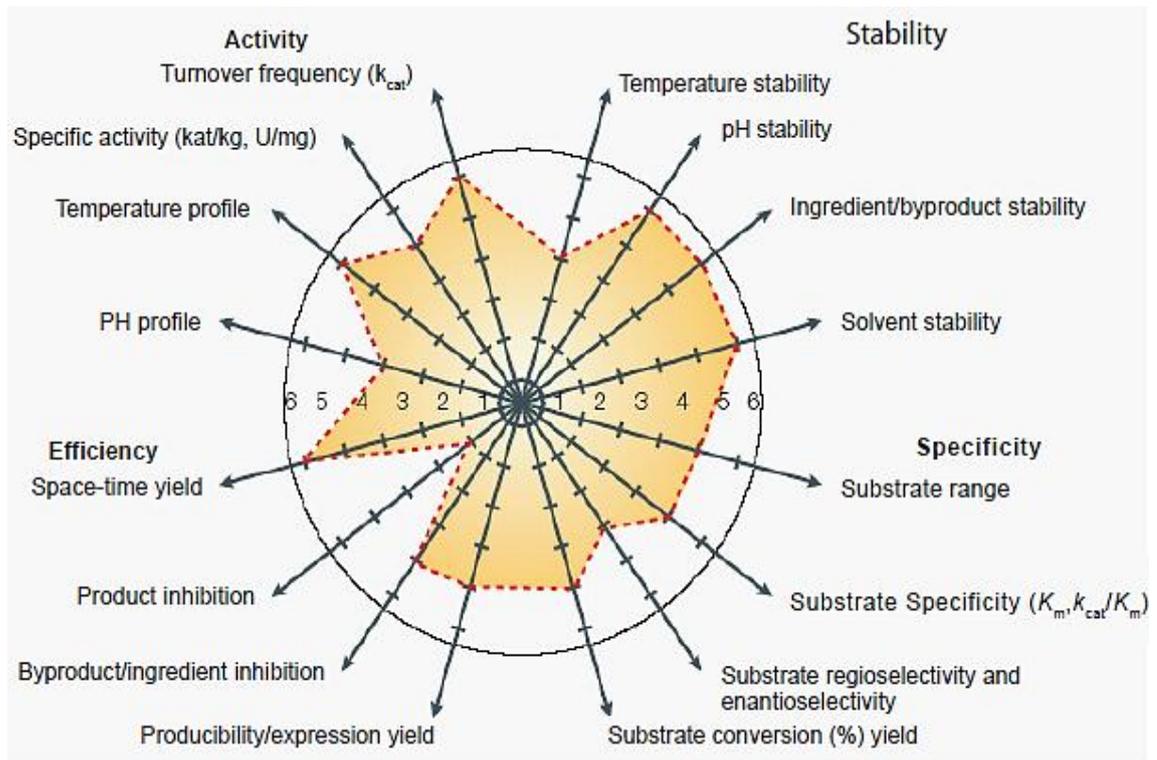


Figure 2.14: Schematic representation of the ideal enzyme properties and areas of improvement. The yellow area represents how a specific enzyme is performing in the overall selection criteria. The numbers range from 1 – 6 with 6 being the most desirable with, catalytic reaction rate (k_{cat}), catalytic constant (K_m) and Michaelis constant (U) (Sarrouh et al., 2012). Reprinted under the terms of the OMICS group's Creative Commons Attribution License.

At a recent National Ethanol Conference in Orlando, Novozymes executive vice president Peder Holk Nielsen talked about Novozymes new product Cellic CTec3 as well as the current costs of producing cellulosic ethanol (Nielsen, 2012). According to Novozymes, it is clear that enzyme cost is still a major factor hindering competitive cellulosic ethanol compared to gasoline (**Figure 2.15**), however due to advancements in the technology the overall contribution of enzyme cost is diminishing.

A recent in depth techno-economic evaluation of cellulosic ethanol from SB showed that ethanol could be produced for between (3.7 – 2.9\$/gal), which agrees with the projections of Novozymes (**Figure 2.15A**) (Macrelli et al., 2012). Furthermore, Marcuschamer et al., (2012) showed that the price of enzyme usage equates to \$0.68/gal - \$1.47/gal, which is a similar range that Novozymes reported (**Figure 2.15B**) (Klein-Marcuschamer et al., 2012).

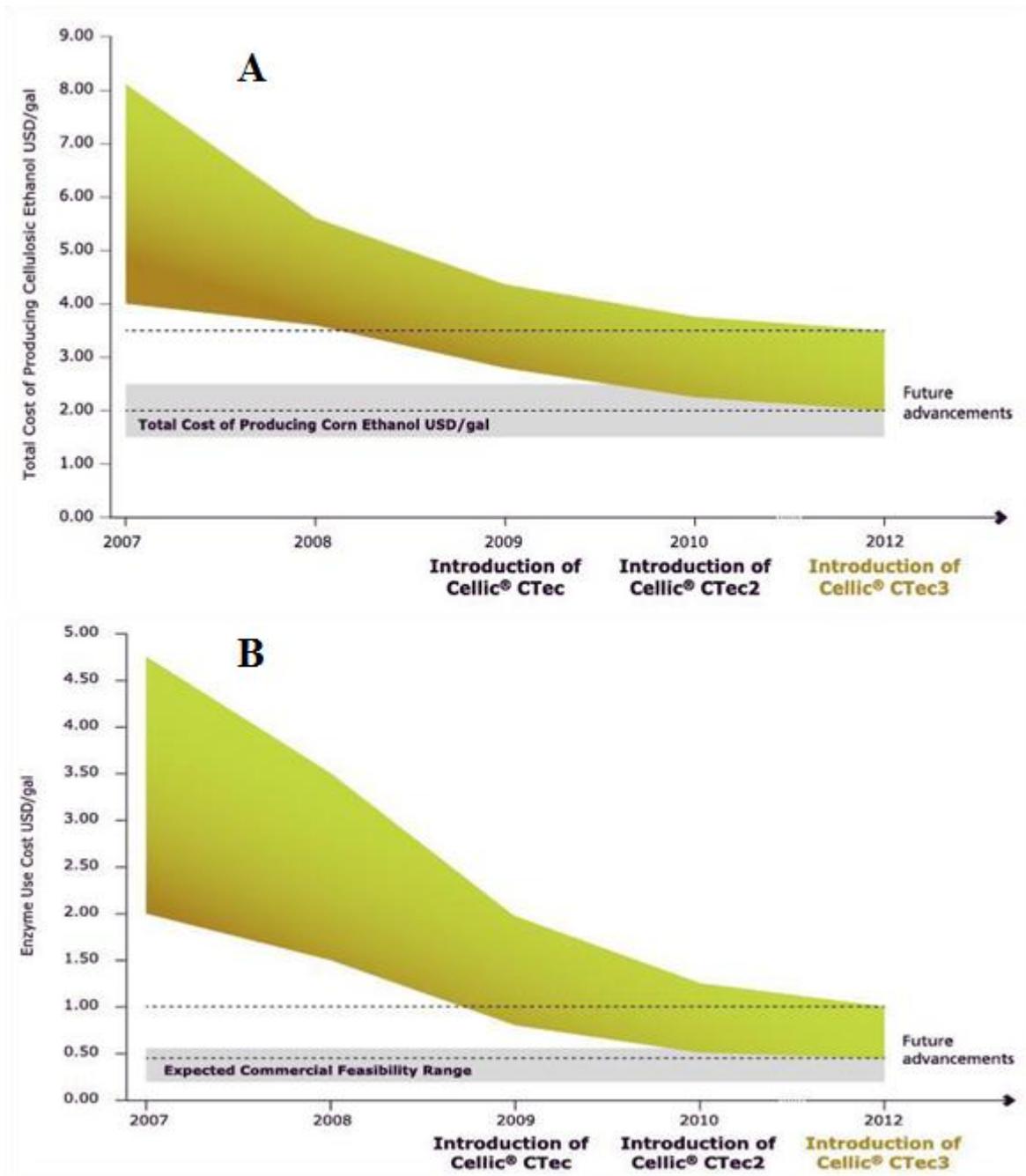


Figure 2.15: Current cost of producing cellulosic ethanol with Novozymes Cellic CTec3 enzyme technology (A) and the enzyme use cost (B). The graphs also indicate the decrease in ethanol production price and enzyme usage price by using the newest Cellic CTec3 enzyme from Novozymes. (Nielsen, 2012). Reproduced with permission from Novozymes.

Additionally, this range could be decreased by cheaper feedstocks, reduced residence times during fermentation and efficient saccharification at low enzyme dosages. In brief, enzyme prices can be roughly 33% of the total 2nd generation ethanol

production cost. However, establishing how much enzyme production contributes to the overall production of cellulosic ethanol is still debated which ranges from (0.5 - 1.5\$/gal) (Hettenhaus, 2000; Hinman et al., 1992; Klein-Marcuschamer et al., 2012; Petiot, 2008). Therefore, it is clear that advancements within the enzyme sector would increase the profits of the biotech companies producing them as well as their clients who use the industrial enzymes within their processes (Sarrouh et al., 2012). Additionally, yeast related factors are also important to consider for economic 2nd generation biofuel production as fermentation costs can be significant (\$0.5/gal) due to high cell counts for inhibitor tolerance and C5 utilization (Bryant, 2011).

2.7 FERMENTATION TO PRODUCE BIO-ETHANOL

2.7.1 Organisms used in the production of bio-ethanol: fermentation

After the efficient hydrolysis of the lignocellulosic biomass to fermentable sugars, a microorganism is required to produce ethanol. However, the ideal industrial microorganism should be able to utilize the resulting hexoses (glucose) as well as pentoses (xylose) from enzymatic hydrolysis although most organisms do not have the ability of fermenting pentoses (Huang et al., 2011). There are some basic considerations when choosing a microorganism for industrial use such as: high ethanol yield and productivity, ethanol tolerance, temperature and inhibitor tolerance as well as a broad substrate utilization (Sarkar et al., 2012).

Wild type microorganisms used in fermentation include *Escherichia coli* (Geddes et al., 2011b), *Saccharomyces cerevisiae* (Antoni et al., 2007), *Pichia stipitis* (Rudolf et al., 2008), *Candida brassicae*, *Candida shehatae* (Balat, 2011), *Pachysolen tannophilus*, *Zymomonas mobilis* (Sánchez & Cardona, 2008) *Mucor indicus* and *Rhizopus oryzae* (Abedinifar et al., 2009). These organisms have specific substrate preferences (**Table 2.7**). The best known ethanol producing microorganisms are the yeast *Saccharomyces cerevisiae* and the bacteria *Zymomonas mobilis*; however *S. cerevisiae* and *Z. mobilis* can't utilize pentose sugars (Balat, 2011; Olofsson et al., 2008). Furthermore, after pretreatment there is an abundance of pentose (xylose) available in the liquid hydrolysate, therefore fermentation of pentoses improves the process economy (Sarkar et al., 2012).

Table 2.7: Summary of industrial microbes used in fermentation (Hahn-Hägerdal et al., 2007). Reprinted with written permission from Springer and their Copyright Clearance Center (RightsLink service).

| Organism | Natural sugar utilization pathways | | | | | Major products | | Tolerance | | | O ₂ needed | pH range |
|----------------------|------------------------------------|-----|-----|-----|-----|----------------|--------|-----------|-------|-------------|-----------------------|----------|
| | Glu | Man | Gal | Xyl | Ara | EtOH | Others | Alcohols | Acids | Hydrolysate | | |
| Anaerobic bacteria | + | + | + | + | + | + | + | - | - | - | - | Neutral |
| <i>E. coli</i> | + | + | + | + | + | - | + | - | - | - | - | Neutral |
| <i>Z. mobilis</i> | + | - | - | - | - | + | - | + | - | - | - | Neutral |
| <i>S. cerevisiae</i> | + | + | + | - | - | + | - | ++ | ++ | ++ | - | Acidic |
| <i>P. stipitis</i> | + | + | + | + | + | + | - | - | - | - | + | Acidic |
| Filamentous fungi | + | + | + | + | + | + | - | ++ | ++ | ++ | - | Acidic |

Natural xylose utilizing yeast such as *P. stipitis* and *C. shehatae* convert D-xylose to xylitol by the action of xylose reductase (XR) and converts xylitol to xylulose with xylitol dehydrogenase (XDR) (Balat, 2011; Hahn-Hägerdal et al., 2007; Huang et al., 2011; Sarkar et al., 2012). Heterologous expression of XR and XDR in other hosts does enable the fermentation of D-xylose into ethanol but with low yields. This fact is due to incomplete recycling of redox co-substrates in the catalytic steps of the NADPH-preferring XR and the NAD⁺-dependent XDH, which in turn results in the formation of xylitol reducing the ethanol yield from xylose. However, bacteria use the action of xylose isomerase (XI) converting xylose into xylulose which is used in the pentose phosphate pathway after phosphorylation (**Figure 2.16**) (Balat, 2011; Hahn-Hägerdal et al., 2007; Huang et al., 2011; Sarkar et al., 2012). The expression of XI does not have the constraints of XR-XDH, but given its bacterial origin, its functional expression in yeast is challenging.

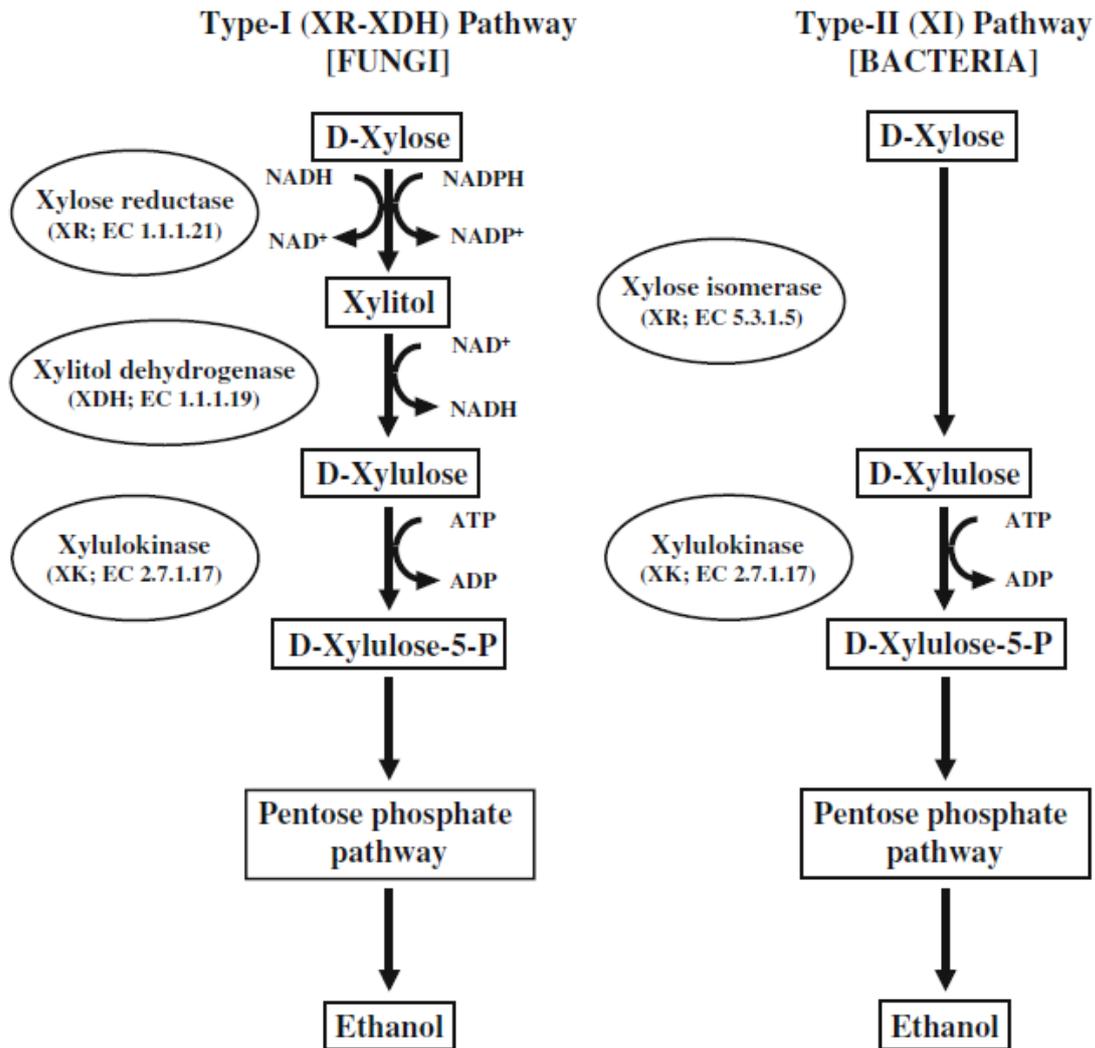


Figure 2.16: Outline showing the different metabolic pathway for D-xylose in fungi and bacteria (Matsushika et al., 2009). Reprinted with written permission from Springer and their Copyright Clearance Center (RightsLink service).

Therefore, recombinant strains for xylose fermentation is produced by introducing XR and XDH (encoded by *XYL* and *XYL2* genes) from organisms such as *P. stipitis* (Ha et al., 2010) or XI (encoded by *xylA* genes) from bacteria such as *E. coli* (Zhang et al., 1995). However, the xylokinase (XK) activity in wild type *S. cerevisiae* is very low and overexpression of the endogenous gene (*XKS1*) (Toivari et al., 2001) or introduction of the *P. stipitis* XK gene (*XYL3*) (Jin et al., 2003) is required for efficient xylose utilization. Other strategies include genetic modifications to increase xylose transport, as the glucose transporters in native strains such as *S. cerevisiae* have very low xylose affinities ($K_m = 49 - 300$ mM) compared to glucose ($K_m = 1 - 28$ mM)

(Kotter & Ciriacy, 1993) As a result, genetic modification and metabolic engineering has produced strains with the ability to utilize pentose such as *S. cerevisiae* TMB3400 (Rudolf et al., 2008), *Z. mobilis* 8b (Zhang & Lynd, 2010) and *E. coli* ATCC11303 (pLOI297) (Dien et al., 2003). However, in the present study the focus was on fermenting the sugars resulting from the efficient hydrolysis of the hexose stream by optimised enzyme cocktails. Therefore, the robust industrial ethanol producing yeast strain *S. cerevisiae* MH-1000 was utilized (Van Zyl et al., 2011). It is possible that if MH-1000 performs well it could be used for genetic manipulation in order to co-ferment pentoses and hexoses.

2.7.2 Fermentation methods and strategies

There are currently various strategies being investigated for the fermentation of lignocellulose to bio-ethanol. The options vary on the degrees of process integration; each strategy includes advantages and disadvantages (Gírio et al., 2010; Margeot et al., 2009). The general process flow involves the pretreatment of the lignocellulosic material producing two streams. One stream consists of the water insoluble solids (WIS), which mostly comprises cellulose and lignin, and the other stream is the solubilized hemicellulose sugar stream also called hydrolysate (Palmqvist & Hahn-Hägerdal, 2000) or pretreatment liquor (Kim & Holtzaple, 2005). Depending on the nature of the pretreatment used, the sugars in the hemicellulose fraction could be in monomeric or oligomeric form, with oligomers requiring subsequent acid/EH to monomers (Gírio et al., 2010).

One of the process options, where the WIS stream containing mostly hexose sugars are hydrolysed with commercial enzymes in a separate step from the fermentation conversion, is called separate hydrolysis and fermentation (SHF) (Sarkar et al., 2012). However, a big limitation with SHF is end product inhibition prevalent in SHF minimizes the ethanol yield (**Table 2.8**) (Erdei et al., 2012; Gírio et al., 2010; Jin et al., 2012; Sarkar et al., 2012). However, when the hemicellulose liquid fraction undergoes separate EH from the WIS stream, but both fractions are fermented together it is referred to as separate hydrolysis and co-fermentation (SHCF), requiring the use of a micro-organism that can convert xylose to ethanol under anaerobic conditions (Erdei et al., 2012). Even though SHCF also experiences end product inhibition, it has an

advantage over SHF in maximal utilization of sugar streams from biomass pretreatment (**Table 2.8**) (Erdei et al., 2012; Gírio et al., 2010; Jin et al., 2012; Sarkar et al., 2012). When the cellulose fraction is hydrolysed and fermented simultaneously in the same reactor it is called simultaneous saccharification and fermentation (SSF) (Rudolf et al., 2008). In “whole slurry” SSF the hexose and pentose sugar streams from pretreatment and hydrolysed and co-fermentation in a single reactor vessel, in a process designated as simultaneous saccharification and co-fermentation (SSCF) (Zhang & Lynd, 2010). Lastly, when the organisms used to ferment all the sugars present from pretreatment, also express the enzymes needed for hydrolysis, and do so in one step it is termed consolidated bio processing (CBP) (Olofsson et al., 2008; Olson et al., 2012) or direct microbial conversion (DCM) (Sarkar et al., 2012). CBP has a great advantage in the low capital cost as the whole reaction occurs in one vessel and no external enzymes are required (**Table 2.8**) (Erdei et al., 2012; Gírio et al., 2010; Jin et al., 2012; Sarkar et al., 2012).

Table 2.8: Important process factors when considering a biomass to ethanol process. Compiled from (Erdei et al., 2012; Gírio et al., 2010; Jin et al., 2012; Sarkar et al., 2012).

| Fermentation process | Features and advantages | Limitations |
|--|--|---|
| Simultaneous saccharification and fermentation (SSF) | <p>Low costs.</p> <p>Higher ethanol yields due to removal of end product inhibition of saccharification step.</p> <p>Reduces the number of reactor required.</p> | <p>Difference in optimum temperature conditions of enzyme for hydrolysis and fermentation.</p> <p>Problems with microbial re-use.</p> |
| Separate hydrolysis and fermentation (SHF) | <p>Each step can be processed at its optimal operating conditions.</p> <p>Separate steps minimize interaction between steps.</p> <p>Yeast recycle possible</p> | <p>End product inhibition minimizes the yield of ethanol. Chance of contamination due to long period process</p> |

| | | |
|--|---|---|
| Simultaneous saccharification and co-fermentation (SSCF) | High potential ethanol yield. Minimal investment costs. Potential for fed-batch xylose with low glucose consumption development. | Whole slurry fermentation has a high WIS composition and inhibitor content. Discrepancies between temperature optimum for enzyme and microorganism. Problems with microbial re-use. |
| Separate hydrolysis and co-fermentation (SHCF) | Each step can be processed at its optimal operating conditions. Maximal utilization of sugar stream from biomass pretreatment. Yeast recycle possible | High capital cost. Contamination risks. Sugar inhibition from saccharification. Long process times. |
| Consolidated bioprocessing (CBP) | Low capital cost as no external enzymes required. Single reactor for enzyme production, biomass hydrolysis and fermentation. | Poor ethanol yields. Long fermentation times. |

Currently the two main strategies remain SHF and SSF however, SSF has become preferred due to advantages when compared to SHF (**Table 2.8**) (Erdei et al., 2012). In the present study SSF was chosen to diminish the effect of product inhibition of the enzymatic hydrolysis of the material, which is one of the advantages of the SSF strategy (**Table 2.8**; Erdei et al., 2012; Gírio et al., 2010; Jin et al., 2012; Sarkar et al., 2012; **Chapter 5**). The latter can have a critical impact on the optima of enzyme cocktails and dosages for efficient hydrolysis, thus requiring the assessment of enzyme options under conditions of SSF (**Chapter 5**).

Chapter 3: Aims and objectives of this study

This master thesis “**Evaluation of enzyme preparations and physicochemical changes during enzymatic hydrolysis of steam pretreated sugarcane bagasse**” has been carried out as part of a larger project on the conversion and development of sugarcane as a feedstock for new bio-ethanol production plants such as those planned by the IDC. This master thesis was conducted at Stellenbosch University within the research interest of the Biofuels Research Chair and the Department of Process Engineering and funded by the South African Sugar Research institute (SASRI).

Commercial scale bio-ethanol production is mostly derived from cornstarch in the United States and the sucrose rich sugarcane syrup in Brazil (Sarkar et al., 2012). Fuel ethanol from food crops such as starch (corn, sorghum, triticale) and sugar syrup is dubbed 1st generation bio-ethanol. However, a big disadvantage of 1st generation bio-ethanol is that it competes with the food and animal feed industry and competes with arable land available (Cherubini & Jungmeier, 2009). However, bio-ethanol can also be produced from lignocellulosic biomass such as crop residues, sawdust, wood chips, grasses, waste paper and municipal solid waste (Tomás-Pejó et al., 2008). Bio-ethanol derived from lignocellulose is termed 2nd generation- or cellulosic ethanol and it has been estimated that 491 billion liters of 2nd generation bio-ethanol could be produced annually (Kim & Dale, 2004).

However, the lignocellulosic biomass needs to be efficiently converted (high EH rate and yield) to monomeric sugars for microbial fermentation to make 2nd generation bio-ethanol economically feasible (Huang et al., 2011; Klein-Marcuschamer et al., 2012; Sarkar et al., 2012) The problem is that the plant CW is a matrix consisting of cross-linked polysaccharides, lignin and glycosylated proteins. As a result of this complex heterogeneous structure, lignocellulosic biomass is very recalcitrant to biological degradation slowing down the EH rate and decreasing the ultimate EH yield (da Costa Sousa et al., 2009) (**Figure 3.1**). Recalcitrance is an

important evolutionary mechanism protecting the structural sugars from degradation by microbes, animals and enzymes (Himmel et al., 2007).

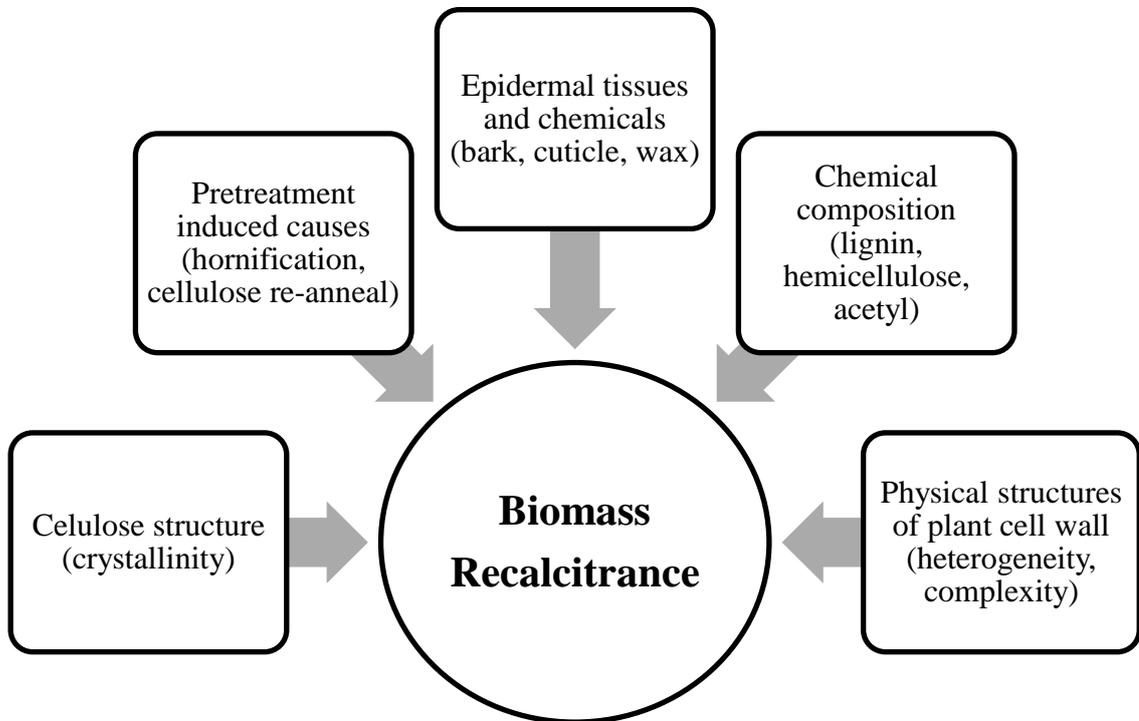


Figure 3.1: Biomass factors that are involved in recalcitrance. Redrawn from (Zhao et al., 2012).

Previously, Himmel et al. (2007 and 2008) summarized the natural characteristics of lignocellulosic biomass that could contribute to recalcitrance:

- Epidermal tissue of the plant body (cuticle and epicuticular waxes)
- Vascular bundle arrangement and density
- Sclerenchymatous tissue (thick wall)
- High degree of lignification
- Complexity and structural heterogeneity of microfibrils and matrix polymers
- Insoluble substrate hinders enzymes
- Fermentation inhibitors generated during conversion or naturally occurring in cell walls

Additionally, cellulose crystallinity, degree of polymerisation, phenolics, cell wall proteins, hemicellulose, acetyl groups as well as lignin deposits from

pretreatment increases recalcitrance. These factors were experimentally addressed (**Chapter 4**). In addition to the physicochemical characteristics implicated in biomass recalcitrance, geographical location and origin of the biomass also influence lignocellulosic composition. The feedstock chosen has an important impact on the pretreatment and downstream process (Olofsson, Bertilsson, et al., 2008). Moreover, pretreatment and enzyme production/usage contribute considerably to the economic cost of the process (Limayem & Ricke, 2012a). Even though various physical and chemical factors have been implicated in biomass recalcitrance, the modifications achieved during pretreatment and EH remain relatively unknown. A better understanding of the key factors involved in the EH rate and yield of pretreated lignocellulose can be used for the development and optimization of low-cost and advanced 2nd generation bio-ethanol production processes. In light of the above mentioned biomass recalcitrance and its impact on the EH rate and yield, the aim of this study was to **investigate the specific physicochemical substrate changes during the EH of steam pretreated SB and how these changes contribute to slowing down the EH rate**. The following objectives were realized to complete the aim:

1. Investigate the kinetic profile of enzymatically hydrolysed steam pretreated SB.
2. Determine the changes in the chemical composition of the substrate during EH.
3. Analyse the enzyme adsorption profile during EH.
4. Characterising the functional group changes in the substrate as EH proceeds.
5. Investigate the impact of phenolic compounds as EH proceeds.
6. Determine the change in the degree of polymerisation and crystallinity of the substrate during EH.
7. Analyse the interior and exterior fibre surface area of the substrate during EH.

Similar to the inhibitory effect that biomass recalcitrance has on the EH rate and yield, the problems with the inefficient enzyme cocktails used for saccharification still represent one of the major economic hurdles (Klein-Marcuschamer et al., 2012). Genetic engineering, bioprospecting and strain improvements have identified thousands of enzymes such as cellulases, hemicellulases, ligninases and pectinases that could contribute towards greater process economics (Zhang et al., 2012). Therefore, understanding the mechanism of action of the enzyme cocktails used, as well as optimizing their impact on biomass hydrolysis, is crucial in selecting the right biocatalyst to ensure maximal process economy.

Previous studies have investigated the effect of various supplementary enzymes and chemicals to improve the efficiency of the commercially available enzymes. Supplementary addition of xylanase, pectinase (Berlin et al., 2007), expansin (Suwannarangsee et al., 2012), PEG 6000, Tween 20, BSA (Kumar & Wyman, 2009d) and Tween 80 (Chen et al., 2008) have all been shown to improve the efficiency of cellulase cocktails. Moreover, studies have shown that the ratios of commercial cocktail to supplementary factors depend on the feedstock and pretreatment. Additionally, predicting the optimum ratios is hindered by the lack of fundamental mechanistic understanding of enzyme components (Banerjee et al., 2010). Therefore, it has been stated that meaningful statistical models can help identify and select enzyme cocktails, components and ratios for an economically advantageous process (Billard et al., 2012; Suwannarangsee et al., 2012; Van Dyk & Pletschke, 2012; Zhang et al. 2012). These factors were experimentally addressed (**Chapter 5**).

However, another problem with producing efficient industrial enzymatic mixtures is the compounding variance introduced by combining different feedstocks and different pre-treatments (Billard et al., 2012). Therefore, industrial SB was the only feedstock used in the present study, and it can be used as a “baseline” feedstock in order to compare with the behavior of other modified materials. Additionally, pretreatment optimization and process configuration also improve EH. In the present study, optimized steam explosion pretreatment conditions were employed and simultaneous saccharification and fermentation (SSF) process option was chosen as it has shown to alleviate end-product inhibition, helping to reduce the required enzyme dosage (Van Dyk & Pletschke, 2012).

Considering the above mentioned limitations with identifying efficient enzymes mixtures for biomass degradation, an additional aim of this master thesis was to **evaluate and optimize a commercially available enzyme cocktail for efficient substrate hydrolysis and fermentation to produce bio-ethanol from steam pretreated SB**. The best enzymes performers were combined and studied by experimental design during a SSF process to reach a target ethanol yield of 80%. The following objectives were realized to complete the aim:

- Evaluation of different commercial preparations for EH and fermentation of steam exploded SB
- Determine the effect that xylanase, pectinase and Tween 80 on cellulose conversion by cellulase of steam pretreated SB with a factorial design.
- Investigate the effect of increasing solid loadings (%) on the digestibility during EH of steam pretreated SB.
- Evaluate the effect of combinations of preferred enzyme preparations, selected by central composite design (CCD) and steepest ascend, on ethanol production rate and yield during SSF.

PUBLICATION IN PREPARATION FOR THIS STUDY

This thesis is based on the following paper, which has been written as a chapter.

Chapter 4

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Authors' contributions:

I planned the study and executed the experimental work. The results were evaluated in collaboration with M. Brienzo and M.P. García-Aparicio. M. Brienzo, M.P. García-Aparicio and J.F. Görgens contributed to draft the final manuscript. All authors provided input to the manuscript and read and approved the final manuscript.

Chapter 4: Physicochemical substrate changes of steam pretreated sugarcane bagasse during enzymatic hydrolysis

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ABSTRACT

Steam pretreated sugarcane bagasse (SB) water insoluble solids (WIS) was subjected to enzymatic hydrolysis and the physicochemical substrate changes were investigated. The profile of the enzymatic hydrolysis identified an initial fast, intermediate and recalcitrant phase, with cellulose to glucose conversion yields of 61.7, 81.6 and 86% respectively. Enzyme adsorption studies suggested that non-specific enzyme/lignin binding was not significant. Chemical composition, phenolic content and FT-IR analysis of the solid residue indicated that lignin (mass and phenolics content) increased while cellulose decreased as hydrolysis progressed. In the initial fast phase of enzymatic hydrolysis, the WIS was characterised by more internal pore surface area. Thereafter, in the intermediate and recalcitrant phases, enzyme action enriched the external surface area of the WIS. Altogether, decreased cellulose available for cellulase and the increased lignin/cellulose ratio was responsible for the slowdown in the rate and decreased yield of hydrolysis as enzymatic digestion progressed.

Keywords: Bio-ethanol, enzymatic hydrolysis, slowdown, internal/external surface area, scanning electron microscopy.

INTRODUCTION

The use of lignocellulosic agricultural residues such as SB for the production of 2nd generation biofuels represents an important development into renewable energy, and is receiving international interest (Arantes & Saddler, 2011). The transportation sector is one of the major focus areas in which 2nd generation biofuels, especially bio-ethanol, will contribute. The major bio-ethanol producers, the United States (52.6 billion liters) and Brazil (21.1 billion liters), accounts for 87.1% of global bio-ethanol production (Renewable Fuels Association, 2012; Sarkar, Ghosh, Bannerjee, & Aikat, 2012). The production of bio-ethanol on large scale is mostly derived from corn starch in the United States and the sucrose rich sugarcane syrup in Brazil. Additionally, it has been proposed that 51 billion liters of lignocellulosic bio-ethanol can be produced from SB (Kim & Dale, 2004).

Bio-ethanol can be produced from lignocellulosic biomass by pretreatment, enzymatic hydrolysis and sugar fermentation processes (Balat, 2011). Advances in the enzyme technology used for bioconversion of biomass to fermentable sugars have significantly improved the viability of the process. However, the recalcitrance of the lignocellulosic biomass as well as the cost of enzymatic conversion still represents major process bottlenecks for industrial application of the process.

The β -1,4 orientation of the glucosidic bonds within crystalline cellulose results in the formation of intra- and intermolecular hydrogen bonds between glucose monomers, making the microfibrils highly resistant against biological and chemical breakdown (Nishiyama, Langan, & Chanzy, 2002). Lignin, which is a complex cross-linked polymer of phenyl propane units, plays a major role in the recalcitrance and structural rigidity of the substrate fibers (Zhao, Zhang, & Liu, 2012). Furthermore lignin, nature's second most abundant substance, encrusts the carbohydrate polymer matrix of cellulose and hemicellulose by forming covalent as well as hydrogen bonding in the lignin-polysaccharide complex (Rose, 2003).

The heterogeneous nature of lignocellulosic biomass, such as SB, must be pretreated to increase the accessibility and porosity of the substrate to enzymes. Therefore, one of the most important factors influencing the viability of second

generation bio-ethanol production is the pretreatment process (Menon & Rao, 2012). In the present study steam explosion was used to improve the digestibility of industrial SB. Steam pretreatment has been used widely and has shown to be effective for herbaceous materials without the need of adding catalyst (McMillan, 1994).

Although the cellulase complex is able to hydrolyse lignocellulose, the kinetic profile during hydrolysis is characterised by a recalcitrant or slowdown phase (Arantes & Saddler, 2011; Bansal et al., 2012). This slowdown of the sugar release rate and decreased ultimate yield during the enzymatic hydrolysis of lignocellulose represents one of the major technological barriers impeding 2nd generation bio-ethanol (Himmel et al., 2007; Stephen, Mabee, & Saddler, 2012). Therefore, understanding the slowdown of the rate and decreased ultimate yield of enzymatic hydrolysis of lignocellulose as the reaction proceeds is important and a frequent topic of debate. Although there is no consensus on which factors are most influential, there is a general classification of the factors that lead to the slowdown observed in enzymatic hydrolysis of lignocellulose into two groups/categories: those related to the substrate structure, which are heavily influenced by the feedstock chosen and the pretreatment conditions (1), and those related to the mechanism and interactions of the cellulases with cellulose, which depend on the nature and source of the enzyme complex (2).

The reduction in the reaction rate and yield caused by lignocellulosic substrate changes (1) could be explained by: changes in the heterogeneous substrate composition (Whitaker, 2003), cellulose crystallinity (Chang & Holtzapfle, 2000), degree of polymerisation (Hallac & Ragauskas, 2011) and surface accessibility (Arantes & Saddler, 2011). On the other hand, the slowdown in the reaction rate and yield caused by the enzymes (2) could be explained by: thermal- and mechanical deactivation (Yang, Zhang, Yong, & Yu, 2010), unproductive binding (Kumar & Wyman, 2009a), end-product inhibition (Kumar & Wyman, 2009b), steric hindrances and loss of synergism between the individual cellulases.

Various studies have indicated the effects that changing individual parameters has on efficient enzymatic hydrolysis. However, it is most likely a combination of factors that contributes to the observed rate and yield of enzymatic hydrolysis. In this context, the aim of this work was to study the physicochemical changes that occur during enzymatic hydrolysis of SB. Furthermore, elucidating the substrate

characteristics that contribute to slowing down the rate and decreasing the yield could contribute to the implementation of successful enzyme recycling, recovery and substrate feeding strategies for optimal enzymatic hydrolysis procedures. Ultimately, a better understanding of the difficulties of the hydrolysis steps would contribute to the cost reduction of 2nd generation biofuels from lignocellulosic material.

MATERIALS AND METHODS

Material

Industrial SB from an industrial plant in Malelane, Mpumalanga, South Africa was supplied by TSB Sugar. The samples were packed in zipped plastic bags and stored in a temperature and moisture controlled room (20°C and 65% humidity) until used for chemical composition, pretreatment and enzymatic hydrolysis. The steam pretreated bagasse was donated by CIEMAT biomass unit (CIEMAT- Renewable Energies Department, Av. Complutense, 22, 28040 Madrid, Spain). The material was pretreated at 210°C for 5min. The resulting pretreated material (slurry) was pressed to obtain a separate liquid and solid fraction. The solid fraction was washed 10x (10g water/g pretreated solid) and the resulting water insoluble solids (WIS) were used in the enzymatic hydrolysis experiments.

Chemical composition analysis

Carbohydrate, lignin and ash content of the raw, steam pretreated, solid and liquid residues from enzymatic hydrolysis were determined according to the National Renewable Energy Laboratory (NREL, Golden, Colorado USA) methods (Sluiter et al., 2008).

Enzymes, activities and protein determinations

The commercial enzyme preparations used for the study were Spezyme CP and Novozym® 188, kindly donated by Genencor (Genencor International, Rochester, NY, USA) and Novozymes A/S (Bagsværd, Denmark), respectively. Spezyme CP is mainly a cellulase produced by *Trichoderma reesei* with an activity of 60 FPU/mL, residual xylanase activity of 5263IU/mL and protein concentration of 188 mg/ml. Novozym® 188 is a beta-glucosidase produced by *Aspergillus niger* with an activity of 700IU/mL

and protein concentration of 114 mg/ml. The Cellulase and β -glucosidase activity of the enzyme preparations were determined with Cellulase and β -glucosidase assays as previously described (Ghose, 1987). The protein concentration of the enzyme preparations as well as the supernatant resulting from enzymatic hydrolysis was determined by Bicinchoninic acid (BCA) assay (Thermo Scientific Pierce BCA Protein Assay) using bovine serum albumin as protein standard.

Enzymatic hydrolysis of WIS

Approximately 2g dry weight (DW) of WIS was transferred to a 250mL Erlenmeyer flask containing 100ml of Sodium-citrate buffer, pH 5. Mixing was provided by orbital shaker set at 100rpm and temperature at 50°C. After an equilibration period of approximately 15min, cellulase was added to ensure an activity of 15FPU/g WIS (44mg/g) and 15U/g of WIS (2.44mg/g). Samples were taken at 0, 3, 6, 9, 12, 18, 24, 48 and 72h for analysis of glucose, xylose and cellobiose by HPLC. The material was washed as previously described (Material) to ensure soluble sugar removal before hydrolysis and the sugar concentration of the enzyme preparation was deducted from all HPLC data. The cellulose conversion to glucose rate (g/L*h) was determined using the slope function of the change in glucose released over time. The cellulose to glucose conversion yield (%) was calculated according to Eq. (1)(Dowe & McMillan, 2001):

$$\text{Cellulose conversion (\%)} = \frac{[\text{Glucose}] + 1.053 [\text{Cellobiose}]}{1.111 f [\text{Biomass}]} \times 100\% \quad (1)$$

Where:

| | |
|---------------------|--|
| <i>[Glucose]</i> | Glucose concentration (g/L) released during enzymatic hydrolysis |
| <i>[Cellobiose]</i> | Cellobiose concentration (g/L) released during enzymatic hydrolysis |
| <i>[Biomass]</i> | Dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L) |
| <i>f</i> | Cellulose fraction in dry biomass (g/g) |
| <i>1.053</i> | Correction factor of cellobiose to equivalents of glucose. |
| <i>1.111</i> | Conversion factor of cellulose to equivalents of glucose |

Phenolic content in the solid residue

Samples of 50mg (DW) of the solid residue from enzymatic hydrolysis were treated with HCl in butylated hydroxytoluene according to Govender et al. (2009) (Govender, Bush, Spark, Bose, & Francis, 2009).

FT-IR analysis of solid residue from enzymatic hydrolysis

The Fourier Transform -Attenuated Total Reflectance Spectroscopy (FT-ART) analysis was performed with samples (1mg) of solid residue after enzymatic hydrolysis as well as un-hydrolysed materials, which were dried and milled passing through a 100 mesh-screen and stocked with phosphorous pentoxide (P₂O₅). The samples were analysed by Perkin Elmer Spectrum GX, where infrared spectra were obtained by using 32 scans in the range of 400 cm⁻¹ to 4000 cm⁻¹. FT-ATR spectra were baseline corrected at 1850, 1190 and 790 cm⁻¹.

Crystallinity of solid residue from enzymatic hydrolysis

The samples of the solid residue from enzymatic hydrolysis were defragmented into a powder with a mortar and pestle. X-ray diffraction (XRD) data were obtained with a LabX - XRD-6000 Shimadzu (X-ray diffractometer), accelerating voltage of 40 kV and current of 30 mA. The scan was done with a 2θ range between 8° and 28° with a step of 0.05° and a scan rate of 2°/min. The crystallinity index (CrI) was determined as the percentage of crystalline material in the biomass (Segal & Conrad, 1959), according to Eq. (2):

$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100 \quad (2)$$

Where: CrI= relative degree of crystallinity; I₀₀₂= intensity of the diffraction from the 002 plane at 2θ= 22.5°; I_{am}= intensity of the background scatter at 2θ= 18.7°.

Internal/external surface of solid residue from enzymatic hydrolysis

The samples were subjected to Simon's staining (SS) pore size method modified by Chandra et al. (2008) (Chandra, Ewanick, Hsieh, & Saddler, 2008). The amount of dye adsorbed onto the fibre was determined as the difference in the concentration of initial dye added minus the concentration dye in the supernatant. For all samples, the

concentrations of direct orange (DO) and direct blue (DB) in the dye-stripping solution as well as in the supernatant were determined using the Beer-Lambert law for binary mixtures that were solved simultaneously:

$$A_{455\text{nm}} = \epsilon_{\text{O}/455} LC_{\text{O}} + \epsilon_{\text{B}/455} LC_{\text{B}} \quad (3)$$

$$A_{624\text{nm}} = \epsilon_{\text{O}/624} LC_{\text{O}} + \epsilon_{\text{B}/624} LC_{\text{B}} \quad (4)$$

Where A = Absorption of mixture at 455 or 624nm, ϵ = extinction coefficient of each component at the respective wavelength (ϵ was calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624nm). In the present study the values were $\epsilon_{\text{O}/455} = 25.67$; $\epsilon_{\text{B}/455} = 3.09$; $\epsilon_{\text{O}/624} = 0.86$; $\epsilon_{\text{B}/624} = 16.45\text{g/cm}$, L = path length of the cuvette (1cm in this case).

Degree of Polymerisation of the cellulose of pretreated and hydrolysed WIS

The samples (0.5g DW) were placed in beakers containing 20mL deionized water, 0.188g sodium chlorite, 63 μ L glacial acetic acid and placed in a water bath at 70°C. Three more doses of sodium chlorite (0.188g) and glacial acetic acid (63 μ L) were added after 2, 3 and 4h. After a total of 4h, the remaining solids were filtered through a sintered-glass filter and washed with deionized water (80mL hot and 200mL cold). The remaining material was treated with 5mL of 17%(w/v) NaOH, whereafter it was kept at room temperature for 2mins and then pressed with a glass rod for 3min. Thereafter, 2.5mL of 17%(w/v) NaOH was added and the suspension stirred for 5min followed by identical dosages after 10 and 15min respectively. The solution was kept at room temperature for 30min, after which 16.5mL of water was added and again kept at room temperature for 1h. The extracted α -cellulose was washed with 25mL of 8.3% (w/v) NaOH, 250mL water, 50ml 2M glacial acetic acid and 400mL water consecutively whereafter it was dried overnight. α -Cellulose viscosities were measured using the TAPPI T230 test method where 0.125g α -cellulose was placed in a test tube with 12.5mL distilled water for 1h. Thereafter, 12.5ml Cupriethylenediamine solution was added and stirred for 5min and incubated for 2h at room temperature until solubilization of cellulose was complete. The soluble cellulose pulp viscosity was measured using a capillary viscometer (Brookefield viscometer) with 0.5% cellulose solution, using 0.5 M Cupriethylenediamine as a solvent. Pulp viscosities determined as centipoise (cp) were converted to degree of polymerization (DP) according to Eq. (5):

$$DP^{0.905} = 0.75 [954 \log(X) - 325] \quad (5)$$

Where X = TAPPI viscosity in centipoises (TAPPI, 1999).

Scanning electron microscopy of solid residue from enzymatic hydrolysis

The samples of WIS and solid residue from enzymatic hydrolysis were washed with deionized water and dried at 30°C for 24h. The samples were mounted on a 10mm stub using double-coated tape. They were then coated with gold in an Edwards Pirani10S150A sputter coater and finally examined at different magnifications, accelerating voltage of 7kV and working distance of 10mm.

Analytical analysis

Glucose, xylose, arabinose, cellobiose and acetic acid present in the liquid fraction from enzymatic hydrolysis was analysed by HPLC. The samples were analysed on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) with column temperature set to 65°C with a mobile phase of 5mM sulphuric acid and a flow rate of 0.6ml/min. The concentrations of compounds were measured with a RI detector (Shodex, RI-101) operated at 5°C.

The phenolic acids and aldehydes (ferulic acid, vanillin, vanilic acid, syringic acid, syringaldehyde and p-coumaric acid) were analysed on a PhenomenexLuna C18 reversed phase column (4.6 x 150 mm, 5 µm particle size) equipped with a PhenomenexCation-H Cartridge Luna C18 precolumn. Column temperature was set to 25 °C and flow rate to 0.7ml/min. The mobile phases used for elution were 5 mM trifluoroacetic acid in water and 5mM trifluoroacetic acid in acetonitrile. The phenolic acids and aldehydes (ferulic acid, vanillin, vanilic acid, syringic acid, syringaldehyde and p-coumaric acid) peaks were detected with a Dionex Ultimate 3000 diode array detector at 280 nm.

RESULTS AND DISCUSSION

Kinetic profile of enzymatic hydrolysis

The cellulose to glucose conversion yield (%) and rate (g/L•h) gave an indication of the efficacy of the enzymatic hydrolysis as it proceeded for 72h (**Fig. 1**).

The kinetic profile clearly indicated that the rate at which glucose was being liberated from cellulose was severely reduced as hydrolysis proceeded. The data suggested that there was an initial fast phase (1) until 18h, characterized by a high cellulose conversion of approximately 61.7%. The initial phase (1) was followed by an intermediate phase (2) where a further 20% of cellulose was hydrolysed (81.7% total) until 48h. The final 24h corresponded to the recalcitrant phase (3), where only 4.3 % additional cellulose was hydrolysed (86 % total).

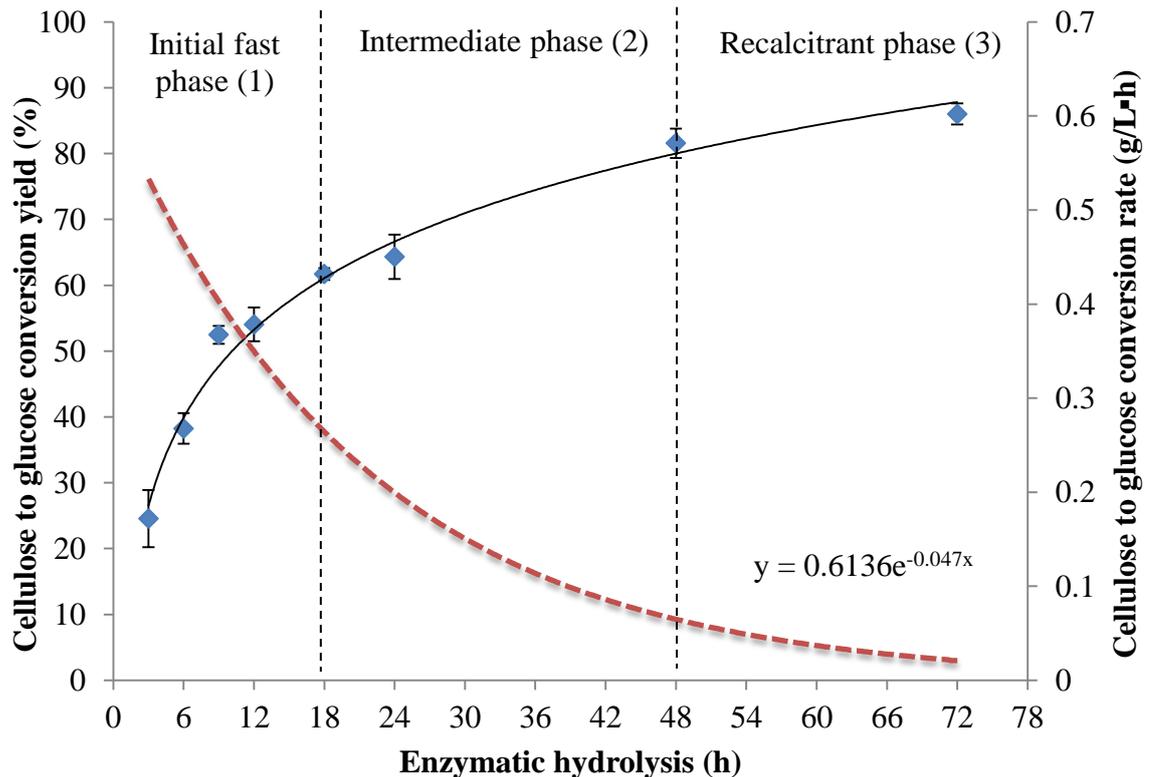


Fig 1: Kinetic profile of enzymatic hydrolysis of steam pretreated sugarcane bagasse (SB) showing the cellulose conversion to glucose %(\diamond) and cellulose conversion rate (g/L·h). Striped lines indicate the three distinctive phases of enzymatic hydrolysis. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm.

The decrease in the cellulose conversion rate (g/L·h) mirrored the trend seen in the cellulose conversion to glucose yield (%). The decreasing rate was expressed by the following function, $y = 0.6136e^{-0.047x}$, where y represents the cellulose conversion to glucose rate (g/L·h) as a function of time, denoted by x (h) (**Fig 1**). The magnitude of the negative exponent indicated the rate at which the cellulose conversion to glucose rate was decaying, with 0.6136 representing the initial rate at time 0h. The average

cellulose to glucose conversion rate for the initial phase (1) was 0.4 g/L·h decreasing to an average of 0.13 g/L·h for the intermediate phase (2) and finally 0.04 g/L·h in the recalcitrant phase (3).

The decrease in cellulose conversion yield and rate indicated that there was a definite slowdown in the liberation of sugars from the material. It is possible that the enzymes were losing activity due to thermochemical deactivation and end-product (glucose, cellobiose and xylose) inhibition. However, the assay was performed at 2% (w/v) solid loading which minimizes the effect of end-product inhibition (Cara et al., 2007; Kristensen et al., 2009). No cellobiose accumulation, and low concentrations of xylose (0.8g/L) were observed (data not shown). Additionally, glucose (15g/L) has been shown to inhibit the activity of cellulase significantly (García-Aparicio et al., 2006). However, in the present study, a maximum glucose concentration of 11g/L was only reached after 72h of enzymatic hydrolysis, at which stage the reaction had already passed into the slowdown phase, due to substrate recalcitrance. Even though the activity change was not monitored, other studies have shown the stability of the enzymes used in the present study over a period of 24h (Pribowo, Arantes, & Saddler, 2012). It could be assumed that the enzyme-related factors would have a decreased influence, compared to the substrate-related factors, on overall enzymatic hydrolysis (EH) performance at this solid loading. The results from monitoring the changes in the structure and chemical composition during EH, and its correlation with the decreased cellulose to glucose conversion rate and yield, are presented in the following sections.

Chemical composition of enzymatic hydrolysis residue

The raw industrial SB used in the present study contained 44% cellulose, 28.4% hemicellulose and 22.3% lignin (**Table 1**). This data is similar to the average chemical composition of SB found in literature (Zhao, Peng, Cheng, & Liu, 2009). The WIS was enriched in cellulose (57.7%) and lignin (29.0%), with decreased residual hemicellulose (6.0% xylose and 0.3% arabinose). The composition of the remaining solids was also monitored during the enzymatic hydrolysis kinetic. As expected, the cellulose content was reduced with the subsequent enrichment of other components such as lignin and ash during EH. The cellulose content of the final remaining residue had 3 times less cellulose (17.6%) and double lignin (61.9%) compared with the starting WIS (**Table 1**). Even though a high cellulose conversion yield was achieved (86%) (**Figure 1**), the

analysis of the residual solid indicated that there was still significant amount (8.3g/100g WIS) of cellulose remaining after 72h. This residual cellulose represents part of the sugar fraction that cannot be liberated due to the combination of biomass recalcitrance and decrease enzyme action. The increment in the lignin to cellulose ratio could contribute to the recalcitrance of the material, either as physical barrier and/ or by irreversible binding to cellulases. The adsorption pattern of the enzymes was analysed to study if the increment of lignin implied an increase in adsorption. Furthermore, structural analysis was used to investigate the specific biomass nature in recalcitrance.

Table 1: Chemical composition (polymers) of raw material, steam pretreated material and solid residue from enzymatic hydrolysis.

| Material | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Ash (%) | Cellulose (g/100g WIS) | Lignin/Cellulose ratio |
|--------------------------|----------------------|--------------------------|-------------------|----------------|-------------------------------|-------------------------------|
| Raw bagasse | 44.0±0.6 | 28.4±0.3 | 22.3±0.2 | 1.3±0.3 | n.d | 0.5 |
| Steam pretreated bagasse | 57.7±2.2 | 6.5±0.1 | 29.0±0.5 | 1.3±0.09 | 57.73 | 0.5 |
| 3h EH residue | 53.5±1.2 | 4.0±0.5 | 33.5±0.8 | 2.8±0.39 | 46.56 | 0.6 |
| 6h EH residue | 46.5±0.7 | 4.2±0.6 | 36.9±0.3 | 3.7±0.66 | 36.68 | 0.8 |
| 9h EH residue | 42.8±0.5 | 2.9±1.0 | 41.7±1.0 | 3.9±0.0 | 29.92 | 1.0 |
| 12h EH residue | 35.9±1.8 | 3.2±0.7 | 47.4±0.6 | 3.6±0.01 | 22.08 | 1.3 |
| 18h EH residue | 34.9±1.3 | 3.7±0.5 | 49.5±4.0 | 5.8±0.31 | 20.54 | 1.4 |
| 24h EH residue | 29.2±0.4 | 3.1±0.2 | 54.8±1.2 | 4.8±0.42 | 15.53 | 1.9 |
| 48h EH residue | 24.2±0.2 | 3.5±0.4 | 56.2±2.1 | 5.7±0.32 | 12.54 | 2.3 |
| 72h EH residue | 19.4±0.6 | 2.2±0.7 | 61.9±1.6 | 5.8±0.16 | 9.14 | 3.2 |

**All data represented was the average value of triplicate experiments. EH residue: solid residue from enzymatic hydrolysis.

The lignin rich residue from enzymatic hydrolysis could be used in various ways to contribute to the economic feasibility of 2nd generation bio-ethanol from SB. Nowadays, lignin is mostly burned in boilers to supply energy for the paper and pulping mills and is being investigated as a value added product used in the production of adhesives, polyolefin, flavourings, binders, activated charcoal, pyrolysis, epoxies and

phenolic resins (Satheesh Kumar, Mohanty, Erickson, & Misra, 2009; Stewart, 2008; Villegas & Gnansounou, 2008).

Additionally, the residual hemicellulose was partially hydrolysed as Spezyme contains endo-xylanase activity. It was clear from the monomer profile of the material that the hemicellulose fraction in the raw material was composed mostly of arabinoxylans (**Table 2**), which is the major component of the hemicellulose in grasses such as SB (de Vries & Visser, 2001; Grohmann et al., 1989; Wyman, 1996). Similar to lignin, hemicellulose also acts as a physical barrier inhibiting cellulase action by decreasing the accessible surface area for enzyme action (de Vries & Visser, 2001; Grohmann et al., 1989; Schell et al., 1989; Scott et al., 1986a; Wyman, 1996). Therefore, hemicellulose decreases cellulose conversion to glucose yield and rate (de Vries & Visser, 2001; Grohmann et al., 1989; Schell et al., 1989; Scott et al., 1986a; Wyman, 1996). However, steam explosion solubilises and removes most of the acetyl, arabinose and xylose from the material (**Table 2**) decreasing its inhibitory role (Alvira et al., 2010b).

Table 2: Chemical composition (monomers) of raw material, steam pretreated material and solid residue from enzymatic hydrolysis.

| Material | Glucose (%) | | Arabinose (%) | Acetyl (%) |
|------------------|-------------|----------|---------------|------------|
| Raw bagasse | 48.4±0.6 | 22.2±0.3 | 1.5±0.5 | 4.7±0.2 |
| Steam pretreated | | | | |
| bagasse | 57.7±2.2 | 6.0±0.1 | 0.3±0.0 | 1.2±0.02 |
| 3h EH residue | 53.5±1.2 | 3.3±0.5 | 0.1±0.02 | 1.3±0.75 |
| 6h EH residue | 46.5±0.7 | 3.9±0.6 | 0.2±0.04 | 0.8±0.11 |
| 9h EH residue | 42.8±0.5 | 2.5±1.0 | 0.1±0.06 | 0.7±0.12 |
| 12h EH residue | 35.9±1.8 | 2.9±0.7 | 0.1 ±0.04 | 0.7±0.16 |
| 18h EH residue | 34.9±1.3 | 3.2±0.5 | 0.2±0.03 | 0.9±0.06 |
| 24h EH residue | 29.2±0.4 | 2.7±0.2 | 0.2±0.01 | 0.7±0.07 |
| 48h EH residue | 24.2±0.2 | 3.0±0.4 | 0.2±0.03 | 0.8±0.06 |
| 72h EH residue | 19.4±0.6 | 1.8±0.7 | 0.1±0.08 | 0.6±0.21 |

**All data represented was the average value of triplicate experiments. EH residue: solid residue from enzymatic hydrolysis.

Enzyme Adsorption

The total amount of enzyme adsorbed on the substrate (adsorbed, irreversibly linked or stuck within pores) was calculated as the difference between the initial protein added (46.44 mg/g WIS corresponding to 0.93 mg/ml) and the free protein determined in the supernatant. The enzymes showed rapid adsorption to the material at the beginning of the fast hydrolysis phase, with about 35% of the initial protein adsorbed after 6h (**Fig. 2**). It is clear that after equilibrium between the enzyme in the liquid and solid phase was reached, there was no significant change in the protein concentration of the supernatant with 63% of the loaded enzyme remaining as free protein at the end of the EH.

A similar adsorption (30%) was determined for other steam exploded herbaceous biomass such as corn stover (Pribowo et al., 2012). The binding capacity of the substrate and/or lignin is determined by the feedstock, type and conditions of pretreatment and the nature of cellulases. For example, the greater lignification of softwoods and hardwoods, involves higher cellulase binding capacity than found in corn stover (Nakagame, Chandra, & Saddler, 2010). Furthermore, the lignin from steam explosion has a decreased affinity for cellulase adsorption, compared to other pretreatments, due to lower phenolic hydroxyl groups (Sewalt, Glasser, & Beauchemin, 1997). Likewise, some components of the enzyme system such as β -glucosidase exhibit lower adsorption ability to lignin (Berlin et al., 2006). Our data indicated that the amount of enzyme bound to the substrate stayed relatively constant after equilibrium had been reached, and did not increase during enrichment of the substrate lignin content as hydrolysis progressed.

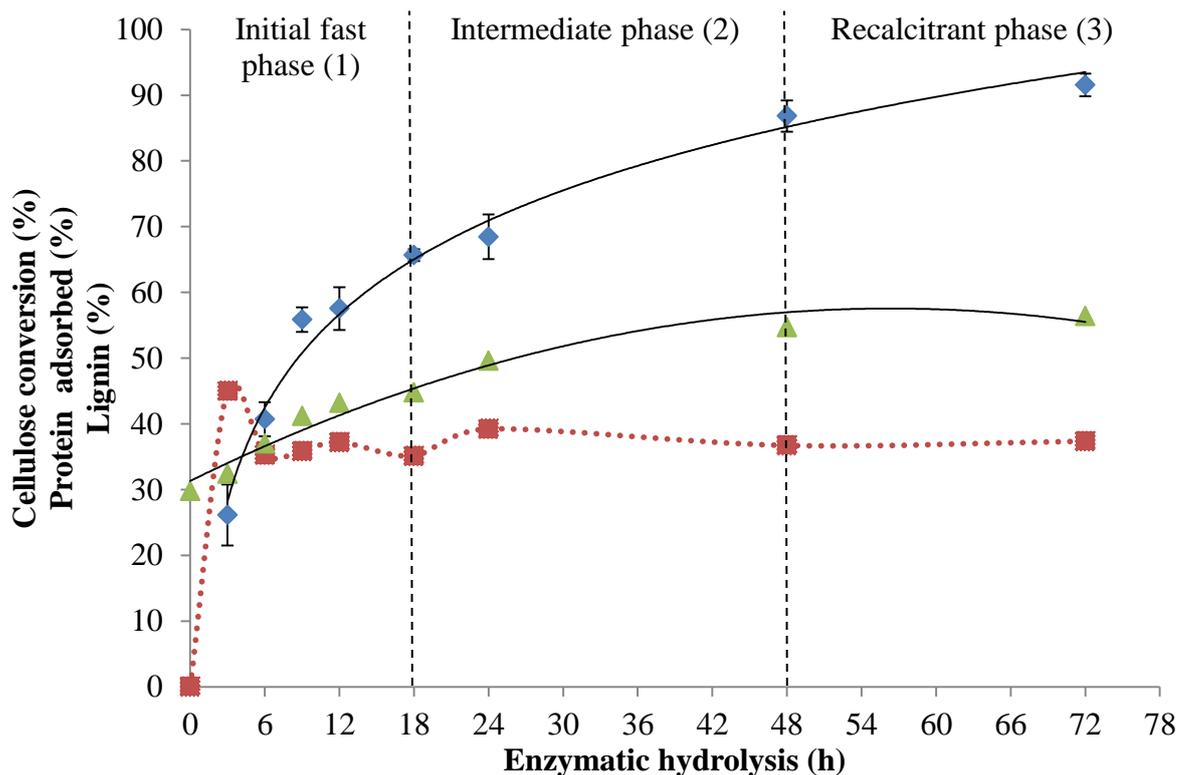


Fig. 2: Profile of enzymatic hydrolysis of steam pretreated SB showing the cellulose conversion % (◇), lignin content (Δ) and % protein adsorbed (□). Striped lines indicate the three distinctive phases of enzymatic hydrolysis. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm. The total initial protein loading was 46.44mg/g WIS corresponding to 100% protein loading.

Whether or not non-specific enzyme/lignin binding was occurring as EH progressed, enough free enzyme (65%) was available for cellulose hydrolysis. Therefore it was suggested that the major factors decreasing the cellulose to glucose conversion rate and yield were not enzyme related.

FT-IR analysis on the solid residue from enzymatic hydrolysis

The FT-IR analysis of the steam pretreated SB WIS during enzymatic hydrolysis indicated that the absorption bands at 1603, 1513, and 1453 cm^{-1} increased drastically as enzymatic hydrolysis proceeded (0–72h) (**Fig. 3A**). These bands correlate to skeletal vibrations in the aromatic C=C stretches, which could be attributed to the lignin structure (Sun, Sun, Sun, Fowler, & Baird, 2003; Zhao, Wang, & Liu, 2008). The increase in the lignin band intensity is in accordance with chemical characterization that also indicated enrichment in the lignin content as enzymatic hydrolysis proceeded.

Similarly the increase in the band at 1235 cm^{-1} as enzymatic hydrolysis proceeded, indicated an increase in the lignin content as this band represents guaiacyl ring breathing, C-O guaiacyl aromatic methoxyl linkage as well as C-O lignin stretching (Zhao et al., 2008).

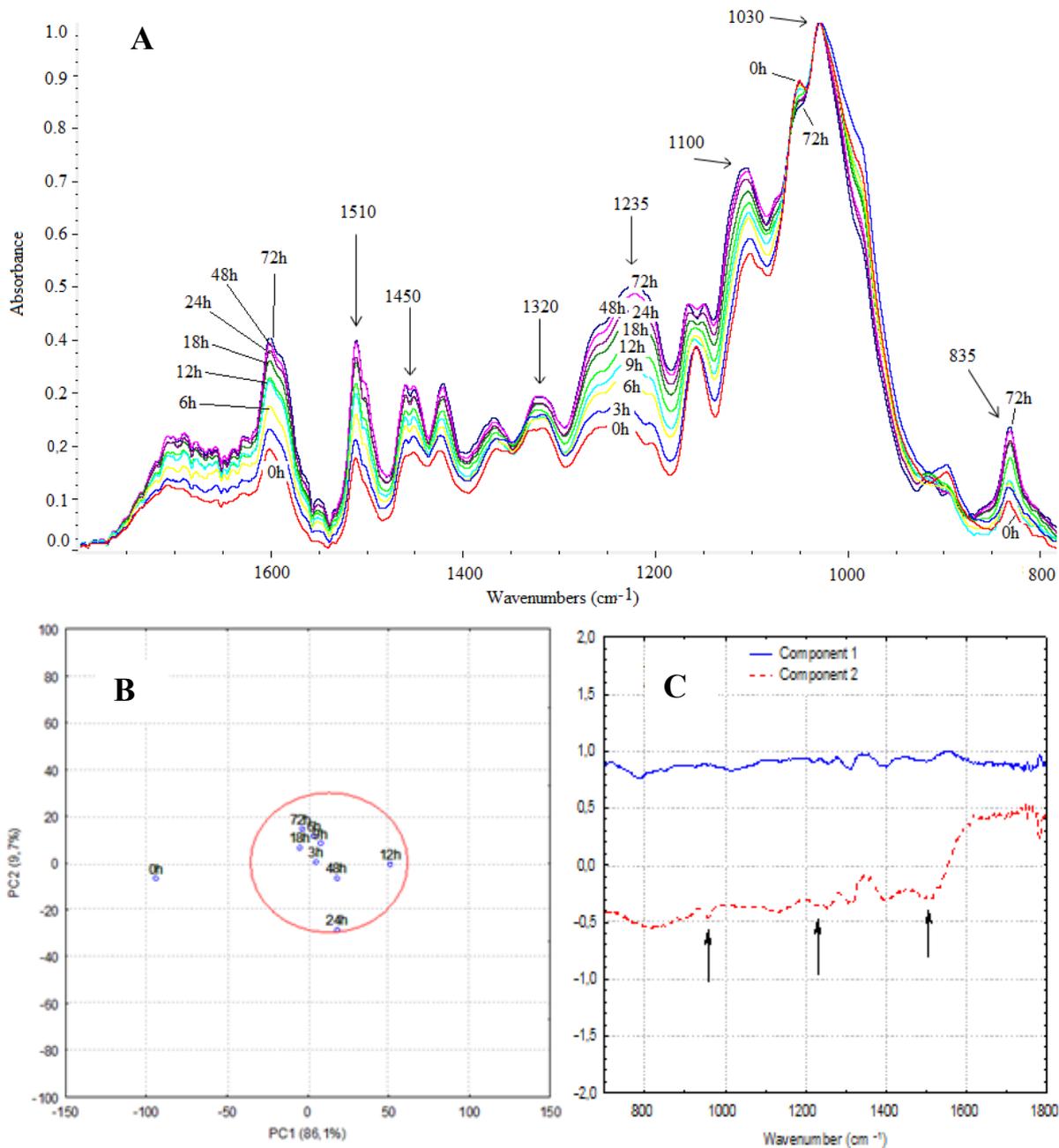


Fig. 3: FT-IR spectra(A), FT-IR score plots (B) indicating the pretreated material (0h) and the solid residue from enzymatic hydrolysis (3-72h) and FT-IR data matrix load plots (C) of the residue from steam pretreated enzymatically hydrolysed SB. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm.

Furthermore, the band at 835 cm^{-1} represents the C-H out of plane vibrations in the C2 and C6 position of the syringyl units of lignin (Zhao et al., 2008). It is clear from the spectra that this band also increased during enzymatic hydrolysis, indicating that the amount of lignin increased as enzymatic hydrolysis proceeded. Additionally, the band at 900 cm^{-1} decreased as enzymatic hydrolysis proceeded. This band represents the C-O-C stretching of β 1-4-glucosidic bonds between glucose monomers in cellulose (Zhao et al., 2008), indicating the cellulose content decreased in the material as enzymatic hydrolysis proceeded.

Principle component analysis (PCA) efficiently identifies bands and their respective functional groups as well as discriminating between samples by using transformation. The PCA score plot showed that samples obtained from the solid residue of enzymatic hydrolysis (3-72h) were distinctly different from the un-hydrolysed material (0h) (**Fig. 3B**). The discrimination between hydrolyzed (3-72h) and un-hydrolysed (0h) residues were a consequence of substrate changes during enzymatic hydrolysis. The samples from 0h and 3-72h were discriminated along PC1 that contained most of the spectral information (86.1%).

The loadings plot for PCA showed discrepancies in the region of 900, 1235 and 1513 cm^{-1} that were responsible for most of the spectral differences observed among the samples (**Fig. 3C**). According to discrepancy band identification it is possible to suggest which functional group is most relevant in the sample differentiation. Those bands are attributed respectively to glucosidic linkage (C-O-C), methoxyl linkage in the guaiacyl ring (C-O), and aromatic ring (C=C) found only in the lignin structure, indicating that both changes in cellulose (900 cm^{-1}) and lignin (1235 and 1513 cm^{-1}) were associated with the slowdown.

Phenolic content of the solid residue from enzymatic hydrolysis

The increase in the phenolic content opposed the trend of cellulose to glucose conversion rate (**Fig. 4**). It was observed that the phenolic content of the material gradually increased to a maximal value of 40.6 mg/g , after 24h of enzymatic hydrolysis, whereafter it stabilised.

Grasses such as SB contain lignified cell walls as well as phenolics and phenolic acids in non-lignified cells (Akin, 2007). It has been shown that SB contains especially high levels of phenolic acid esters such as p-coumaric and

ferulic acid within their cell walls which contribute to biomass recalcitrance (Akin, 2007; Akin, 1990; Akin & Chesson, 1989; Akin, Hartley, Morrison, & Himmelsbach, 1990). Furthermore, ferulic and p-coumaric acid also esterify to arabinose which links to xylose increasing the recalcitrance of grasses such as SB (Borneman, Ljungdahl, Hartley, & Akin, 1991).

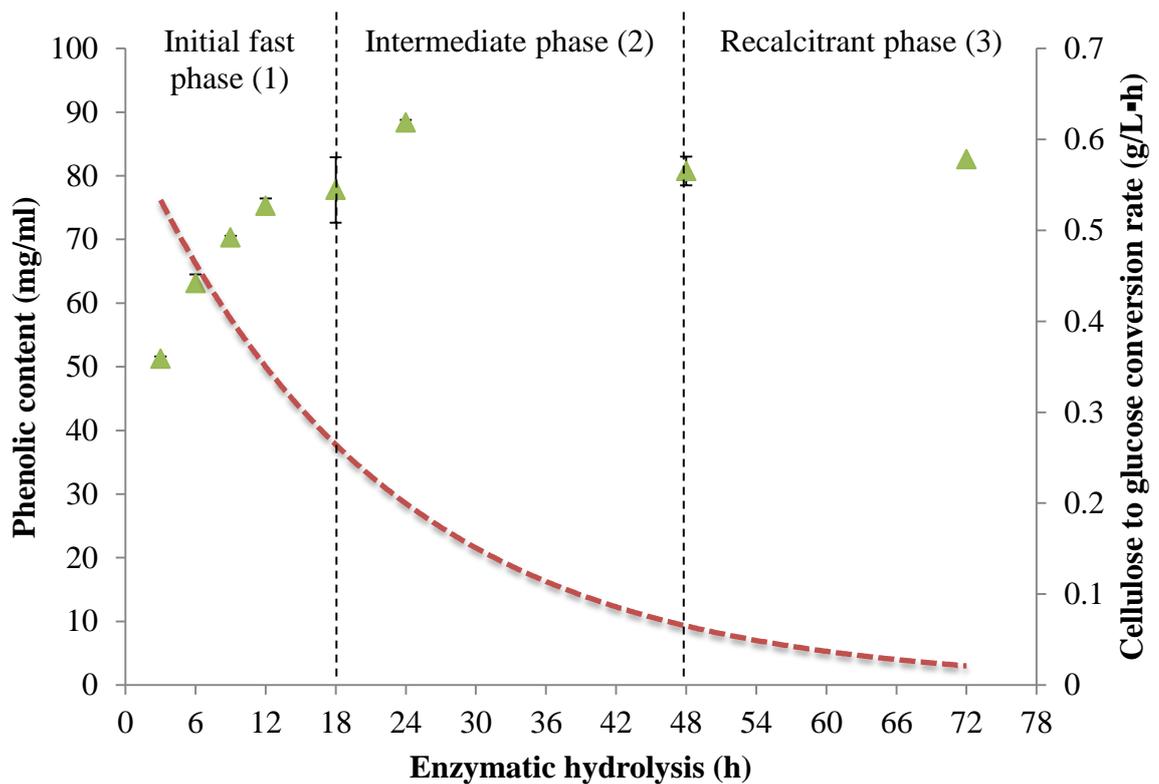


Fig. 4: Profile of enzymatic hydrolysis of steam pretreated sugarcane bagasse showing the cellulose to glucose conversion rate ($\text{g/L}\cdot\text{h}$) (Δ) and phenolic content (mg/g) (\circ) in the solid residue from enzymatic hydrolysis. Striped lines indicate the three distinctive phases of enzymatic hydrolysis. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm

Literature indicates that these phenolic compounds within SB participate in ester-ester and ester-ether bonds between polysaccharides and lignin compounds components (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Iiyama et al., 1994; Rose, 2003; Sun, 2004; Sun, 2011; Xu et al., 2005). These lignin-carbohydrate complexes (LCCs) contribute to the structural complexity of the material, due to the covalent and hydrogen bonding between the components (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Iiyama et al., 1994; Rose, 2003; Sun, 2004; Sun,

2011; Xu et al., 2005). The increase phenolic/cellulose ratio could contribute to the strength and complexity of the bonds between the remaining un-hydrolysed carbohydrate and lignin during enzymatic hydrolysis. This could be attributed to the increasing concentration of ester-linked phenolic acids, making the cellulose fraction bound to lignin harder to hydrolyse.

Substrate crystallinity and degree of polymerisation

The data indicated that there was a decrease in the crystallinity index of the pretreated WIS material (CrI) as enzymatic hydrolysis proceeded (**Fig. 5**). The initial CrI of the WIS was 55%, which decreased to 30% after 72h of enzymatic hydrolysis. Although it is accepted that the CrI of cellulose increases during EH, this effect was masked by the amorphous nature of the lignin of which the content in WIS had doubled at the end of EH. Studies using lignocellulosic material have indicated that the CrI does not increase as enzymatic hydrolysis proceeds, apparently due to cellulose (crystalline fraction of the WIS) being removed during hydrolysis (Sinitsyn, Mitkevich, Gusakov, & Klyosov, 1989).

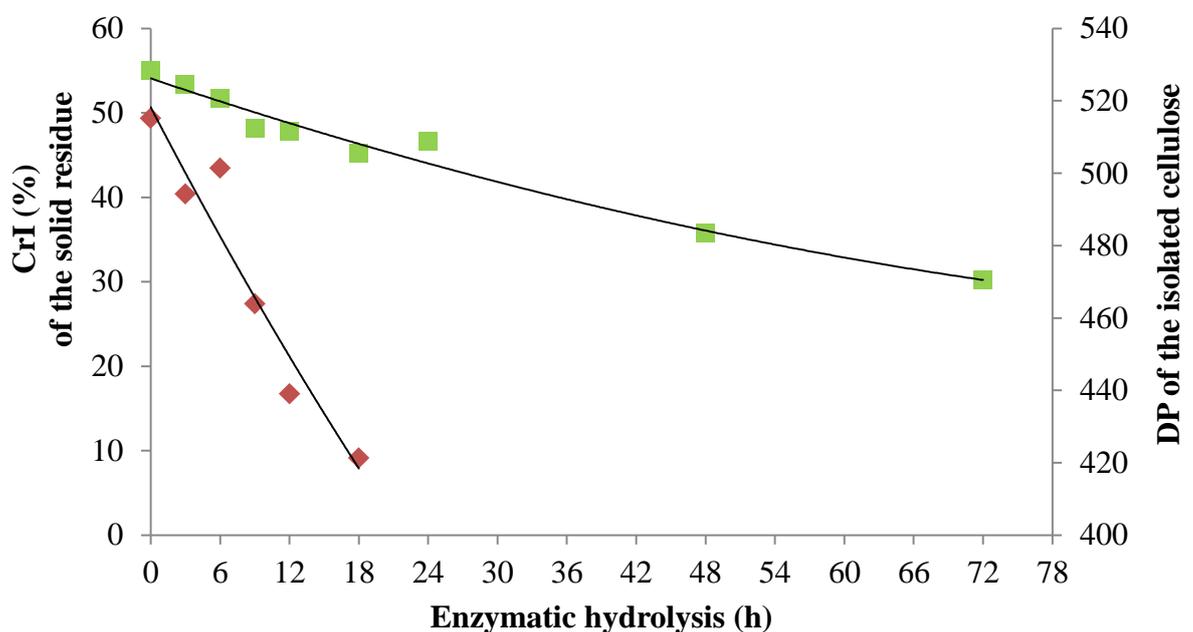


Fig. 5: Crystallinity index (□) and degree of polymerisation of extracted cellulose (◇) of the steam pretreated and solid residue from enzymatic hydrolysis. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm.

The DP of the cellulose isolated from the WIS used in the present study was 515 before EH. It was clear that the DP of the cellulose from the WIS slightly decreased

during enzymatic hydrolysis, from 515 at the start to 421 after 18h of enzymatic hydrolysis.

As the DP decreased for the 72h of enzymatic hydrolysis, it was expected that it would increase the saccharification. It has been shown that greater amounts of reducing ends supply more substrate for exoglucanase to hydrolyse (Gupta & Lee, 2009; Hallac & Ragauskas, 2011; Pan et al., 2007; Puri, 1984, Sinitsyn et al., 1989; Zhang & Lynd, 2004). Additionally, reducing the DP of cellulose shortens the glucose chain lengths (Pan, Xie, Yu, & Saddler, 2008) and shorter cellulose fragments are not as resistant to solvents as the stiff-rods of longer cellulose chains. However, during the initial fast phase of enzymatic hydrolysis the cellulose content decreased drastically, limiting the potential extra reducing ends that could be generated during hydrolysis. Therefore, at the initiation of saccharification, lower DP leads to higher rates, owing to greater amounts of free reducing ends (Hallac & Ragauskas, 2011). Similarly, after 18h it was possible that the advantageous effects of reduced DP, on the rate of hydrolysis, were insignificant or masked against the protective role of lignin.

Additionally, the high lignin to cellulose ratio after 18h previously discussed in the chemical composition (**Table 1**). caused difficulty in the solubilisation of the cellulose for the viscosity measurements. The greater lignin content may hamper chemical isolation of cellulose, which could also indicate similar problems with enzyme/cellulose interaction due to increasing substrate recalcitrance.

Interior and exterior fibre surface area

The total amount of dye adsorbed on the substrate investigated with SS increased up until 24h (289mg dye/g fiber), whereafter it gradually decreased (Fig. 6). The direct blue (DB) dye populates the smaller inner pores of the material associated with the interior fibre area, whereas the direct orange (DO) populates the external substrate surface area as it has a greater affinity for cellulose than DB (Chandra et al., 2009). It could be observed that more small pores (interior area) than large pores/surface (exterior area) characterized the material until 18h. After 18h, in the intermediate phase, the material was characterized with lower internal area than external area, which has previously been shown to be the most important factor for enzyme accessibility and cellulose conversion yield and rate (Cosgrove, 2005; Grous et

al., 1986; Hong et al., 2007; Huang et al., 2009; Luo & Zhu, 2011; Shevchenko et al., 2000; Wang et al., 2011).

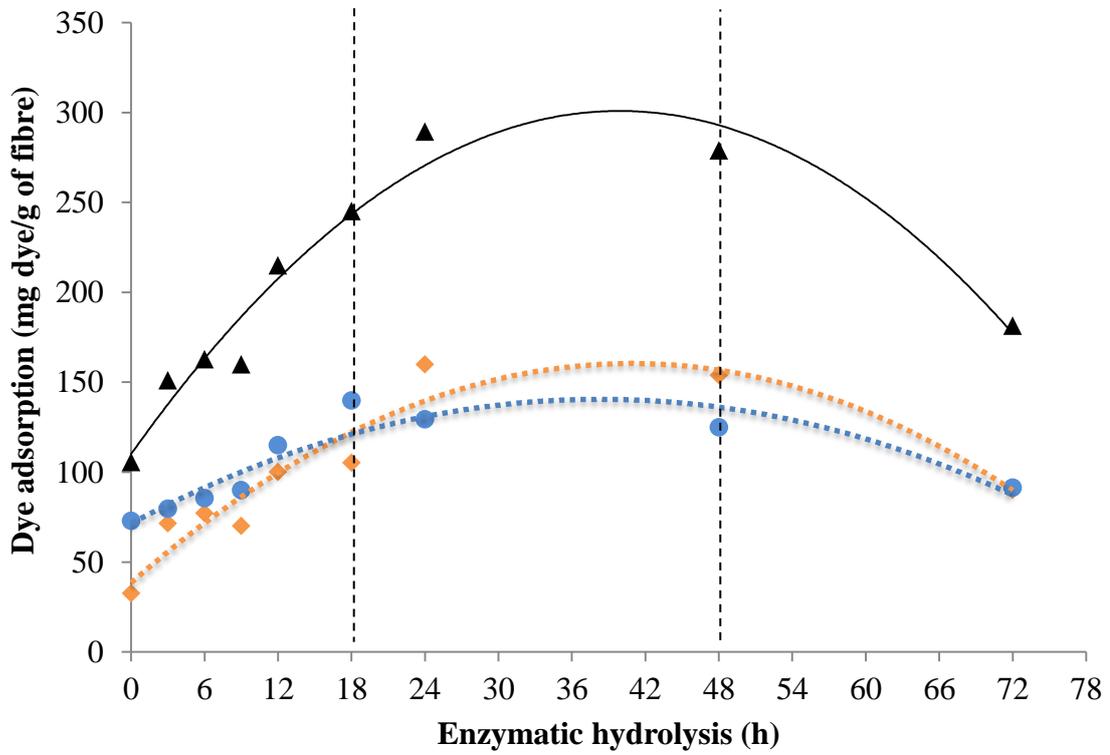


Fig. 6: Dye adsorption by the residual solid samples from the enzymatic hydrolysis of steam pretreated SB. Hydrolysis conditions: substrate 2% (w/v), enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm. Total dye (Δ), Direct blue (\circ) and Direct orange (\diamond). Dye adsorption assay conditions: Direct blue and Direct orange added in a 1:1 ratio at 70 °C and 120rpm.

This result suggests a strong modification of the material interior/exterior surface at the intermediate phase; possibly indicating that the smaller pores were opened by enzyme action. After maximal adsorption of dye on substrate after 24h, representing the maximum enzyme accessibility to the substrate, there was a decrease in the adsorption of dye. It was suggested that this decrease in adsorption could be the result of the decreasing amount of cellulose content. The DO dye has a strong affinity for cellulose and it decreased with the same trend as the total amount of dye after 24h of enzymatic hydrolysis. Thereafter, the cellulose to glucose conversion yield is not related to dye adsorption on the solid residue because there are other physicochemical characteristics that are changing, for example, the fibre reactivity. As far as we know, it

is the first time this has been reported in literature. The physicochemical environment surrounding the enzyme in the intermediate phase crosses a threshold where the enzymes could be physically blocked. This physical hindrance could possibly lead to decreased interaction between the cellulase mono-components (endoglucanase, cellobiohydrolase and β -glucosidase), decreasing their hydrolytic potential. Even though large amounts of pores are available, the fibers in the intermediate and recalcitrant phase have very high lignin/cellulose ratios (**Table 1**), which could physically impede the access of the enzymes and thereby contribute to recalcitrance.

It was clear from the SEM imaging data that significant changes on the fibre surface were occurring during enzymatic hydrolysis. As soon as enzymatic hydrolysis occurred, an increased modification/porosity in the material could be observed, and after 72h of enzymatic hydrolysis, the material exhibited a highly fractionated surface as well as a greater porosity (**Fig. 7**). In the initial fast phase, a gradual increase in the pores or fragmentation of the fibre surface could be observed (**Fig. 7B-E**). The enzyme action provoked an opening in the small pores creating holes and damaged the surface. (**Fig. 7F**) represents an image from the intermediate phase, 18h, which showed some degree of holes and damage. The same degree of damage can be observed in the images related to the recalcitrant phase, 48 and 72h (**Fig. 7G-H**), evidencing limited change in this phase.

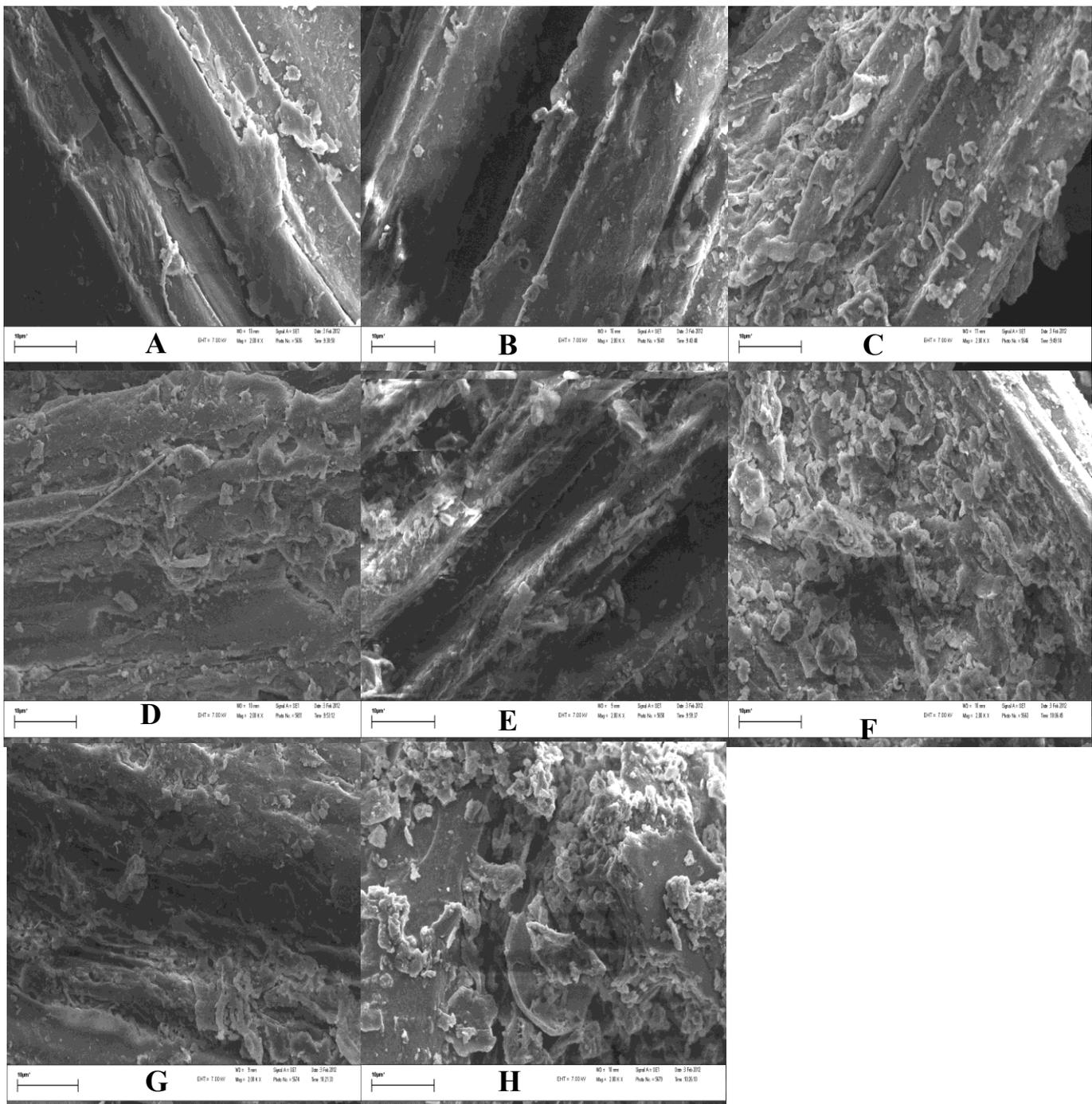


Fig. 7: SEM images of the residual solids from enzymatic hydrolysis kinetic. Figs A (0h), B (3h), C (6h), D (9h), E (12h), F (18h), G (48h) and H (72h) represent the solid residue from enzymatic hydrolysis at the indicated time at 2000x magnification.

CONCLUSIONS

Insight into the physicochemical changes that occur during enzymatic hydrolysis of SB and contribute to slowing down the EH rate of steam pretreated SB

was achieved. The kinetic study showed that after 18h of enzymatic hydrolysis the cellulose conversion rate and yield decreased significantly, and that three distinctive phases could be identified: initial fast (1), intermediate (2) and a recalcitrant phase (3).

Adsorption indicated that the amount of bound protein stayed relatively constant after equilibrium was reached, and that the protein was not non-specifically binding to lignin. Furthermore, the chemical composition, FT-IR and PCA analysis indicated that as enzymatic hydrolysis progressed, the chemical composition of the material changed, increasing the lignin/cellulose ratio due to cellulose hydrolysis.

The internal and external surface area of the solid residue from enzymatic hydrolysis indicated an increased external/internal pore surface ratio after the initial fast EH phase. The initial phase was rich in small pores, and the intermediate and recalcitrant phases were rich in large pores. The change in the pore profile was apparently due to enzymatic action that provoked fibre surface damage and pore opening. However, these pores were not accessible to enzymes because of lignin enrichment, which was physically blocking the enzyme action and reducing the fibre reactivity. Altogether, it was seen that the increasing lignin to cellulose ratio, and internal/external surface area change during enzymatic hydrolysis, had an inhibitory effect on cellulose conversion. The enzymes were less effective at hydrolyzing the increasingly recalcitrant substrate as well as being obstructed from binding to the carbohydrate fibers due to steric hindrances. These hindrances ultimately decreased the accessible surface area, explaining the characteristic slowdown of cellulose to glucose conversion rate and yield.

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See **Chapter 7**

Chapter 5: Cocktail optimization for bio-ethanol production

ABSTRACT

The applications of accessory enzymes (Cellic HTec2, Multifect xylanase, Pectinex Ultra) and surfactants (Tween 80), have shown to increase lignocellulose to glucose and xylose conversion (yields and rates), by conventional enzyme cocktails. Additionally, oxidative enzymes such as the newly classified GH61s present in new generation commercial enzyme cocktails have been implicated in increased lignocellulose conversion to glucose and xylose. These accessory enzyme components can lead to greater process economy due to increased enzymatic hydrolysis (EH) rates and yields. When using steam pretreated sugarcane bagasse (SB) we report the impact of accessory enzyme (Cellic HTec2, Multifect xylanase, Pectinex Ultra), and surfactant (Tween 80) addition on glucose and xylose release rate and yield by two commercially available (Cellic CTec2 and Optiflow) enzymes. The impact of solid loading % (w/v) on enzymatic hydrolysis as well as the effect of an optimised commercial enzyme cocktail (Cellic CTec2/HTec2) vs. a commercial standard (Spezyme/Novozym 188) cocktail on ethanol yield and rate was also reported.

Results showed that xylanase addition was the only statistically significant accessory enzyme for increased glucose and xylose release rate and yields from the pretreated SB by the (CTec2 and Optiflow) enzymes within the studied range. The xylanase addition was possibly increasing the accessible surface area for cellulases due to removal of the arabinoxylans rich hemicellulose fraction. The data also suggested that lower solid loadings (3.6% w/v) resulted in greater sugar yields and rates for all enzyme combinations. However, higher solid loadings (up to 13.2% w/v) resulted in greater concentrations of liberated sugar. The enzyme preparations, Cellic CTec2 and Cellic HTec2, were selected for statistical optimization as they outperformed the Optiflow/Multifect xylanase enzyme mixture based on glucose and xylose yields and rates. The response surface of the data indicated that 100% theoretical ethanol could be achieved if 4.8% solids (w/v) in combination with 0.15ml/g WIS CTec2 and 0.8ml/g WIS HTec2

during Simultaneous Saccharification and Fermentation (SSF). The required enzyme dosage was decreased with a steepest ascend method resulting in an optimized enzymatic cocktail containing 0.15ml/g WIS CTec2 and 0.213 ml/g WIS HTec2. The optimized cocktail, which was volumetrically equivalent to a Spezyme/Novozym 188 loading of 20FPU/g WIS, resulted in 79.2% ethanol yield after 72h of SSF, 24% higher than the Spezyme/Novozym 188 standard. This increase was attributed to the presence of higher levels of cellulases, β -glucosidase, xylanase and the new GH61 oxidative enzymes in the newer generation CTec2/HTec2 enzyme system.

Altogether, it was shown that commercially available cocktails differ depending on the presence of enzyme mono-components which lead to varying glucose, xylose and ethanol yields and rates, which would influence process economics positively. The optimized cocktail chosen (Cellic CTec2/HTec2) from Novozymes contains high levels of cellulase, β -glucosidase, endoxylanase and oxidative enzymes, leading to increased ASA when compared to other enzymes mixtures, increasing the product formation.

Keywords: Bio-ethanol, enzymatic hydrolysis, fermentation, solid loading, xylanase, cellulase

INTRODUCTION

The most abundant, low cost and renewable carbon source in the world is lignocellulose (Jagtap et al., 2012; Suwannarangsee et al., 2012). Lignocellulosic material such as SB mainly consists of cellulose (40%), hemicellulose (30%), lignin (20%), extractives and ash (10%) (Demirbaş, 2005; Pandey, 2000; Sindhu et al., 2010a). The cellulose polymer consists of linear glucose monomers, which are highly crystalline (Limayem & Ricke, 2012b). Moreover, the cellulose fraction is encrusted by lignin and hemicellulose, which together with its crystallinity, reduces their ability to be hydrolysed by enzymes (Brett & Waldron, 1996; Grabber et al., 2009; Himmel, 2008; Rose, 2003; Sun, 2004; Sun, 2011; Van Dyk & Pletschke, 2012; Xu et al., 2005).

Pretreatment methods, such as steam explosion, are used to modify the lignocellulosic structure, which increases the enzymatic hydrolysis efficiency (Zhang et al., 2009). Depending on the feedstock and pretreatment method utilized, varying enzymes

and combinations are required for the degradation of the lignocellulose. The classical view for the degradation of cellulose to glucose involves three cellulases working synergistically (Jagtap et al., 2012; Suwannarangsee et al., 2012): (1) Endoglucanase (EG, E.C.3.2.1.4) produces free cellulose fragments by acting randomly along the low crystalline cellulose chains. (2) cellobiohydrolase (CBH, E.C.3.2.1.91) degrades the cellulose further by acting as a exoglucanase releasing disaccharide cellobiose units. (3) β -glucosidase (BGL, 3.2.1.21) cleave the cellobiose units into monomeric glucose (Howard et al., 2003). The hydrolytic system for the degradation of hemicellulose involves: (1) Endo-1,4- β -D-xylan xylanohydrolase (EC.3.2.1.8) and (2) β -xylosidase (EC.3.2.1.37) which work together to release xylose from the xylan backbone.

Besides the classical enzymes, a new group of fungal derived proteins (GH61s) have recently been associated with the hydrolysis of lignocellulose by the fungal kingdom (Phillips et al., 2011). Currently GH61s can accurately be called copper-dependent polysaccharide monooxygenases (PMOs) (Beeson et al., 2012). PMOs use oxygen and reducing equivalents from cellobiose dehydrogenase (CDH) to oxidatively cleave glycosidic bonds on the cellulose surface without needing separate sugar strands (Langston et al., 2011). Oxidative cleavage occurs at the C1 and C4 position, which generates non-reduced sugars for CBHs (Beeson et al., 2012; Horn & Vaaje-Kolstad et al., 2012). Even though *T. reesei* expresses PMOs, with GH61B comprising the majority action in *T. reesei*, their activity is low. The addition of PMO (GH61B and GH61E) genes from *Thielavia terrestris* to *T. reesei* improves the hydrolytic ability of the resulting cellulase mixture by 1.6 – 2 fold, which drastically improves the economics of lignocellulose digestion (Harris et al., 2010; Phillips et al., 2011). New generations of cellulosic enzymes such as Cellic CTec2 from Novozymes contain GH61s (Cannella et al., 2012).

Furthermore, in order to encourage large scale lignocellulosic bio-ethanol, successful biochemical conversion is essential. Therefore, identification, production and mechanistic studies that extend the knowledge of optimum cocktails and conditions represent areas of interest (Ferreira et al., 2009). Various studies have shown the synergistic effect, leading to higher EH rates and yields, that could be achieved by supplementing conventional cellulase cocktail with additional factors such as xylanase, pectinase, expansin, PEG 6000, Tween 20, BSA and Tween 80 (Berlin et al., 2007; Beukes et al., 2008; Beukes & Pletschke, 2011; Beukes & Pletschke, 2010; Boisset et al.,

2001; Chen et al., 2008; Eckard et al., 2011; Kumar & Wyman, 2009a; Kumar & Wyman, 2009d; Suwannarangsee et al., 2012; Zhang & Lynd, 2004; Van Dyk & Pletschke, 2012). Even though the study of the enzymatic hydrolysis of lignocellulosic material has occurred for decades, advances in the available enzyme cocktails, accessory enzymes and surfactants occur continuously. In this context, the aim of this work was to identify which of these factors would provide the greatest contribution to the liberation of sugars from lignocellulosic material. In the present study, commercially available cellulases were supplemented with xylanases, pectinase and surfactant to study the enzymatic hydrolysis of steam pretreated SB. Factorial designs and response surface methodology was used to identify and optimize a commercial available cocktail for maximal bio-ethanol production. The chosen optimal cocktail was also compared to the industrial Spezyme/Novozymes 188 standard cocktail at equivalent volumetric dosage.

MATERIALS AND METHODS

Raw material

Industrial SB was received from TSB Sugar in Malelane Mpumalanga, South Africa. The samples were packed in zipped plastic bags and stored in a temperature and moisture controlled room (20°C and 65% humidity) until used for chemical composition, pretreatment and enzymatic hydrolysis.

Pretreatment

SB was pretreated in a batch pilot steam explosion unit equipped with a 19-L reaction vessel, a boiler capable of supplying saturated steam up to 240°C and 30 bar by injection to the vessel, and a cyclone tank to allow the abruptly decompression to atmospheric pressure. A built-in automated panel display (IAP, Germany) was used to control steam conditions, valve operation, as well as the processing temperature and pre-set residence time for each steam explosion. For each pre-treatment batch, 940g dry weight (DW) (1000g with 6 % moisture) was used. After steam pretreatment at 195°C for 10 min, the exploded material was collected in buckets, weighed and then pressed in a manual press using a hydraulic jack, to collect the liquid and solid fractions separately. Water insoluble solid (WIS), which was washed to remove inhibitors, was used as substrate in the EH and SSF experiments.

Compositional analysis of the raw and pretreated material

Carbohydrate content and lignin of extractive-free raw material and pretreated solids was determined according to the National Renewable Energy Laboratory (NREL, Golden, Colorado USA) method (Sluiter et al., 2008). Approximately 0.3g (DW) of raw material and WIS was measured into tubes and 3ml of 72% w/w Sulphuric acid was added. After mixing the tubes were submerged into a water bath maintained at 30°C, mixed every 5min for 60min and finally diluted with 84ml de-ionized water and autoclaved at 121°C for 60min. The autoclaved samples were aliquoted and analysed for monomeric sugars by a HPLC. The lignin was calculated as the sum of the acid insoluble lignin and the acid-soluble lignin. The dried samples (0.3g) were combusted in a furnace at 575°C according to the NREL method and the remaining ash was measured (Sluiter et al., 2005).

Enzymes and activities

The commercial enzyme preparations used for the study were Spezyme CP, Optiflow, Multifect Xylanase and Pectinex Ultra kindly donated by Genencor (Genencor International, Rochester, NY, USA) and Novozymes 188, Cellic CTec2 and Cellic HTec2 from Novozymes A/S (Bagsværd, Denmark). Spezyme CP is a cellulase produced by *Trichoderma reesei* with an activity of 60 FPU/ml and protein concentration of 140mg/ml. Optiflow is a cellulase produced by *Trichoderma reesei* with an activity of 130FPU/ml and protein concentration of 183mg/ml. Multifect Xylanase mixture is produced by *Trichoderma sp.* and with an activity of (12 FPU/ml cellulase.and 60FPU/ml xylanase activity) and protein concentration of 47.7mg/ml. Pectinex Ultra is an enzyme produced by *Aspergillus niger* with an activity of (176FPU/ml xylanase and 29FPU/ml mannanase activity) and protein concentration of 40mg/ml. Cellic CTec2 is a cellulase, xylanase and β -glucosidase mixture produced by genetically modified strain of *Trichoderma reesei*. Cellic HTec2 is an endoxylanase produced by genetically modified strain of *Trichoderma reesei*. Novozym 188 is a β -glucosidase produced by *Aspergillus niger* with an activity of 700IU/ml and protein concentration of 120mg/ml. The Cellulase and β -glucosidase activity of the enzyme preparations were determined with Cellulase and β -glucosidase assays as previously described by Ghose 1987 (Ghose 1987). The protein content of the enzyme preparations was determined by BCA assay (BCA-Compatible Protein Assay Kit,) using bovine serum albumin as protein standard. The activity and protein concentration of the Cellic CTec2 and Cellic HTec2 enzyme preparations were not determined.

Enzymatic hydrolysis of WIS in the 2³ factorial design

Approximately 2g (DW) of steam-pretreated biomass was transferred to a 250ml Erlenmeyer flask containing 100ml of 50mM Citrate, pH adjusted to 5. Mixing was provided by water bath set at 100rpm at 50°C. The factorial design is displayed in **Table 5.1**. The factorial design consisted of 2 different enzyme combinations. Combination 1 contained Cellic CTec2 and Cellic HTec2 and combination 2 contained Optiflow and Multifect Xylanase. Additionally, Pectinex Ultra as well as Tween 80 surfactant was used in both combination 1 and 2. Statistical analysis (Statistica 10) was used to construct Pareto charts to give an indication of the effect that commercial xylanase, pectinase and surfactant had on EH of the material.

Table 5.1: 2³ Factorial design to determine the effect that xylanase, pectinase and surfactant addition has on glucose and xylose release from WIS by cellulase. **

| Design: | Cellulase | Xylanase | Pectinase | Surfactant |
|---------------------|--------------------|--------------------|--------------------|-------------------|
| 2**(4-1) | preparation | preparation | preparation | (g/g WIS) |
| Standard Run | (ml/g WIS) | (ml/g WIS) | (ml/g WIS) | |
| 8 | 0.50 | 1.70 | 0.10 | 0.10 |
| 2 | 0.50 | 0.05 | 0.05 | 0.10 |
| 1 | 0.05 | 0.05 | 0.05 | 0.01 |
| 4 | 0.50 | 1,70 | 0.05 | 0.01 |
| 3 | 0.05 | 1.70 | 0.05 | 0.10 |
| 5 | 0.05 | 0.05 | 0.10 | 0.10 |
| 6 | 0.50 | 0.05 | 0.10 | 0.01 |
| 7 | 0.05 | 1.70 | 0.10 | 0.01 |

** All ranges were based on common dosages used within the literature with cellulase (Kumar & Wyman, 2009c), xylanase (McIntosh & Vancov, 2010; Zhang et al., 2009), pectinase (Zhang et al., 2009) and surfactant (Tu & Saddler, 2010; Ouyang et al., 2011).

Enzymatic hydrolysis of WIS in the solid loading experiment

Approximately 2 – 10% (w/v) of steam-pretreated biomass was transferred to a 250ml Erlenmeyer flask containing 50ml of 50mM Citrate, pH adjusted to 5. Mixing was provided by water bath set at 100rpm at 50°C. After a temperate period of approximately

15min, Spezyme CP cellulase was added to ensure an activity of 15FPU/g of WIS. The amount of protein in the cellulase applied corresponded to 44mg/g of WIS. Novozymes 188Cellobiase (β -glucosidase) was also added to ensure an activity of 15U/g of WIS to prevent end-product inhibition by cellobiose. The amount of Cellobiase corresponded to 2.44 mg/g.

Microorganism

The yeast strain *Saccharomyces cerevisiae* MH1000 used was kindly provided by the Department of Microbiology (Stellenbosch, South Africa). This culture was maintained on a medium culture containing yeast extract (10g/L), bacteriological peptone (20 g/L), glucose (20g/L) and agar (15g/L).

Inoculum and preconditioning culture

Pure yeast growth in culture medium previously described (Materials and methods, Microorganism) was added to 250ml Erlenmeyer flask, which contained 50ml of modified mineral medium described by Verduyn et al. (1992): glucose (20g/L), yeast extract (20g/L), $(\text{NH}_4)_2\text{SO}_4$ (7.5g/L), KH_2PO_4 (3.4g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.8g/L), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (0.05g/L), citric acid (0.5g/L) and 1ml of a trace element solution, containing: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (4.5mg/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.3mg/L) $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.5mg/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.3mg/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (3 mg/L), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.4 mg/L), H_3BO_3 (1 mg/L), KI (0.1 mg/L) and filled up with reverse osmosis treated water. The Erlenmeyer flask was incubated in a rotary shaker at 30°C and 150 rpm. After 18h, the cells from the growth culture were harvested and transferred to a preconditioning 1L Erlenmeyer flask, for a starting optical density of 0.2 at 600nm after the transfer. The 1L Erlenmeyer preconditioning flask also contained 300ml of the following medium: glucose (20g/L), yeast extract (20g/L), ammonium Sulphate $(\text{NH}_4)_2\text{SO}_4$ (7.5g/L), potassium dihydrogen phosphate (KH_2PO_4) (3.4g/L), magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (0.8g/L), trace element solution (1ml/L), calcium chloride hydrate ($\text{CaCl}_2 \cdot \text{H}_2\text{O}$) (0.05g/L), citric acid (0.5g/L), liquid hydrolysate (20 %v/v) consisting of (glucose (4g/L), xylose (20g/L), arabinose (0.8g/L), formic acid (3g/L), acetic acid (9.5g/L), Hydroxymethylfurfural (HMF) (0.2g/L) and furfural (1.66g/L) and filled up with reverse osmosis treated water. After 18h of preconditioning the cells were harvested during the exponential growth phase, centrifuged and washed with 1x phosphate buffered saline (10mM PBS), pH 7.4. The supernatant was discarded and the cells were re-suspended in 1.25ml of PBS and

transferred to 100ml Schott bottle containing the fermentation medium for final yeast loading of 5g/L wet cells.

Simultaneous saccharification and fermentation with CCD & steepest ascend

Simultaneous saccharification and fermentation was performed in 100ml Schott bottles. Each Schott bottle contained 50ml working volume containing the fermentation medium described above as well as industrial SB and enzyme concentrations depicted in **Table 5.2**. The initial yeast loading was 5 g/L as previously described (Materials and methods, Inoculum and preconditioning culture). The Schott bottles were incubated in a rotary shaker at 35°C and 120 rpm. To optimize the Cellic CTec2 and HTec2 composition for efficient SB hydrolysis and fermentation an experimental design method known as a central composite design (CCD) (Box & Wilson, 1951) was applied considering three factors: Cellic CTec2 cellulase, Cellic HTec2 and solid loading (w/v) (**Table 5.2**). This method has been effective in optimizing enzyme cocktails and their compositions (Jagtap et al., 2012; Ratnam et al., 2005; Suwannarangsee et al., 2012). The enzyme preparations were dosified in terms of ml/g WIS. All 16 experimental points lay within the design space and all experiments were run in triplicate to minimize variation. A 2³ CCD with 6-face centered star points and two (2) center points approach was chosen (**Table 5.2**). The response values, ethanol yield (%) and ethanol production rate (g/L·h), was defined as the average of triplicates.

Table 5.2: 2³ central composite design to identify a Cellic CTec2 and HTec2 enzyme cocktail for maximal ethanol production yields and rates on steam pretreated SB WIS.

| 2**(3) central composite, nc=8 ns=6 n0=2 Runs=16 + 2 centre points | | | |
|--|---------------------|------------------------|---------------------|
| Run | CTec2 (ml/g WIS) | Solid loading (w/v) | HTec2 (ml/g WIS) |
| 1 | 0.025 | 4.8 | 0.1 |
| 2 | 0.025 | 4.8 | 0.8 |
| 3 | 0.025 | 9.3 | 0.1 |
| 4 | 0.025 | 9.3 | 0.8 |
| 5 | 0.25 | 4.8 | 0.1 |
| 6 | 0.25 | 4.8 | 0.8 |
| 7 | 0.25 | 9.3 | 0.1 |
| 8 | 0.25 | 9.3 | 0.8 |
| 9 | 0.025 | 7.1 | 0.45 |
| 10 | 0.25 | 7.1 | 0.45 |

| | | | |
|---------------|--------|-----|------|
| 11 | 0.1375 | 4.8 | 0.45 |
| 12 | 0.1375 | 9.3 | 0.45 |
| 13 | 0.1375 | 7.1 | 0.1 |
| 14 | 0.1375 | 7.1 | 0.8 |
| 15 (C) | 0.1375 | 7.1 | 0.45 |
| 16 (C) | 0.1375 | 7.1 | 0.45 |

Calculations

The total released glucose and cellobiose yield after the different reaction times were used to calculate the enzymatic digestibility of the WIS. The cellulose to glucose conversion yield (%) was calculated according to Eq. (1) (Dowe & McMillan, 2001):

$$\text{Cellulose conversion (\%)} = \frac{[\text{Glucose}] + 1.053 [\text{Cellobiose}]}{1.111 f [\text{Biomass}]} \times 100\% \quad (1)$$

Where:

[Glucose] Glucose concentration (g/L) released during enzymatic hydrolysis

[Cellobiose] Cellobiose concentration (g/L) released during enzymatic hydrolysis

[Biomass] Dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L)

f Cellulose fraction in dry biomass (g/g)

1.053 Correction factor of cellobiose to equivalents of glucose.

1.111 Conversion factor of cellulose to equivalents of glucose

The ethanol yield (%) in all experiments was calculated according to Eq. (2):

$$\text{Ethanol yield (\%)} = \frac{E}{E_t} \times 100 \quad (2)$$

Where E = ethanol (g/L) from HPLC analysis.

E_t = theoretical ethanol (g/L) from consumed glucose which was 0.51g ethanol/g sugar (Jin et al., 2012)

Chromatographic analysis

Glucose, xylose, arabinose, cellobiose, ethanol, glycerol, formic acid and acetic acid present in the liquid fraction resulting after enzymatic hydrolysis and SSF was analysed by HPLC. The samples were analysed on an Aminex HPX-87H Column

equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) with column temperature set to 65 °C with a mobile phase of 5 mM sulphuric acid and a flow rate of 0.6 ml/min. The concentrations of compounds were measured with a RI detector (Shodex, RI-101) operated at 5 °C. The by-products HMF and furfural present in the liquid fraction generated by pretreatment, were analysed on an Phenomenex Luna C18(2) reversed phase column equipped with a Phenomenex Cation-H Cartridge Luna C18(2) precolumn (Separations, Johannesburg, South Africa) with column temperature set to 5 °C and a flow rate of 0.7 ml/min. The mobile phases used for elution were 5 mM trifluoroacetic acid in water (A) and 5mM trifluoroacetic acid in acetonitrile (B). Separation was carried out by gradient elution with 5% mobile phase B, increasing to 11% B over 14 minutes and then increasing to 40% B over 3 minutes. The mobile phase composition was then kept constant at 40% for 2 minutes, followed by a decrease to 5% B over 5 minutes and ending with a final step of constant composition at 5% B for 4 minutes in order to equilibrate the system. HMF and furfural concentrations were measured with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm.

Statistical software and fitting of data

The experimental data obtained was analysed with Microsoft Excel 2010 commercial software and imported into Design expert 1.6 and Statistica 10 (StatSoft. Inc., USA). The statistical software package Design-Expert (Stat-Ease Inc., Minneapolis, MN, USA) was used for regression analysis of experimental data and to plot response surfaces. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

Material Composition

It was clear from the chemical composition that steam pretreatment (195°C for 10min) enriched the cellulose (44.0 → 46.2%) and lignin (22.3 → 34.04%) content of the material and decreased the hemicellulose (25.67 → 4.78%) content in the solid WIS fraction (**Table 5.3**). This was due to hemicellulose solubilisation during the steam pretreatment and removal as pretreatment liquor (Alvira et al., 2010b; Bobleter, 1994;

Garrote et al., 1999). The solid was used for EH and SSF, while the liquid hydrolysate was used for preconditioning the yeast. The liquid hydrolysate sugar content contained glucose (4g/L), xylose (20.47g/L) and arabinose (0.83g/L). Acetic (9.55g/L) and formic acid (2.98g/L) were the greatest by-products formed during the steam pretreatment and present in the hydrolysate; although furfural (1.42g/L) and HMF (0.32g/L) were also present. Yeast preconditioning was done as a precautionary measure against possible inhibition by acetic-, formic acid, HMF and furfural formed during SSF and pretreatment degradation products present in the WIS (García-Aparicio et al., 2006; Klinke et al., 2004; Martín et al., 2007).

Table 5.3: Chemical composition of raw SB and steam pretreated SB WIS.

| Material | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|--------------------------|---------------|-------------------|------------|
| Raw SB | 44.0±0.6 | 25.67±0.5 | 22.3±0.2 |
| Steam pretreated SB WIS* | 46.2±2.2 | 4.78±0.1 | 34.04±0.5 |

*WIS recovery was 68.15%

2³ Factorial design to determine the effect that xylanase, pectinase and surfactant addition has on glucose and xylose release from WIS by cellulase

Pareto charts which highlight the most important factors among a large set of factors were constructed (**Figure 5.1 & 5.2**). Factors that crossed the red line were significant with a confidence level of 95%. Xylanase addition was the only positive significant factor increasing the glucose and xylose release by CTec2 and Optiflow enzymes within the studied range. The data also indicated that xylanase addition played a more significant role in the early stages of glucose and xylose release, by cellulases, in the range studied (**Figure 5.1 & 5.2**).

The resulting SB WIS after pretreatment had a low hemicellulose content (4.78%), which could be readily released by the xylanase enzymes (**Table 5.3**). The rapid hydrolysis of the remaining hemicellulose fraction improved the rate of cellulose EH (**Figure 5.1 & 5.2**), as it has been shown that xylanase supplementation improves EH of lignocellulose even with low xylan levels (Bura et al., 2009; García-Aparicio et al., 2007;

Kumar & Wyman, 2009a; Van Dyk & Pletschke, 2012). Additionally, endoxylanase and endoglucanase exhibits strong synergism during EH of SB (Beukes et al., 2008). The SB hemicellulose fraction in the present study is mainly composed of arabinoxylans (22.2% xylose and 1.55% arabinose) which agrees with SB hemicellulose composition found in literature (de Vries & Visser, 2001; Grohmann et al., 1989; Wyman, 1996). These arabinoxylans contain monomeric and dimeric hydroxycinnamic acid (HCA) esters directly etherified by the hydroxyls on the lignin surface resulting in ester-ether crosslinks between the CW hemicelluloses and lignin (Himmel, 2008; Iiyama et al., 1994; Rose, 2003; Saulnier, 1999). Additionally the arabinoxylans are feruloyated allowing cross-linking and covalent bridge formation between CW components (Encina, 2005; Himmel, 2008; Iiyama et al., 1994; Rose, 2003; Saulnier, 1999). Therefore, it is possible that the rapid removal of arabinoxylans by xylanase decreased the substrate recalcitrance, by increasing the access to cellulose, resulting in a rapid cellulose hydrolysis rate (**Figure 5.1 & 5.2**).

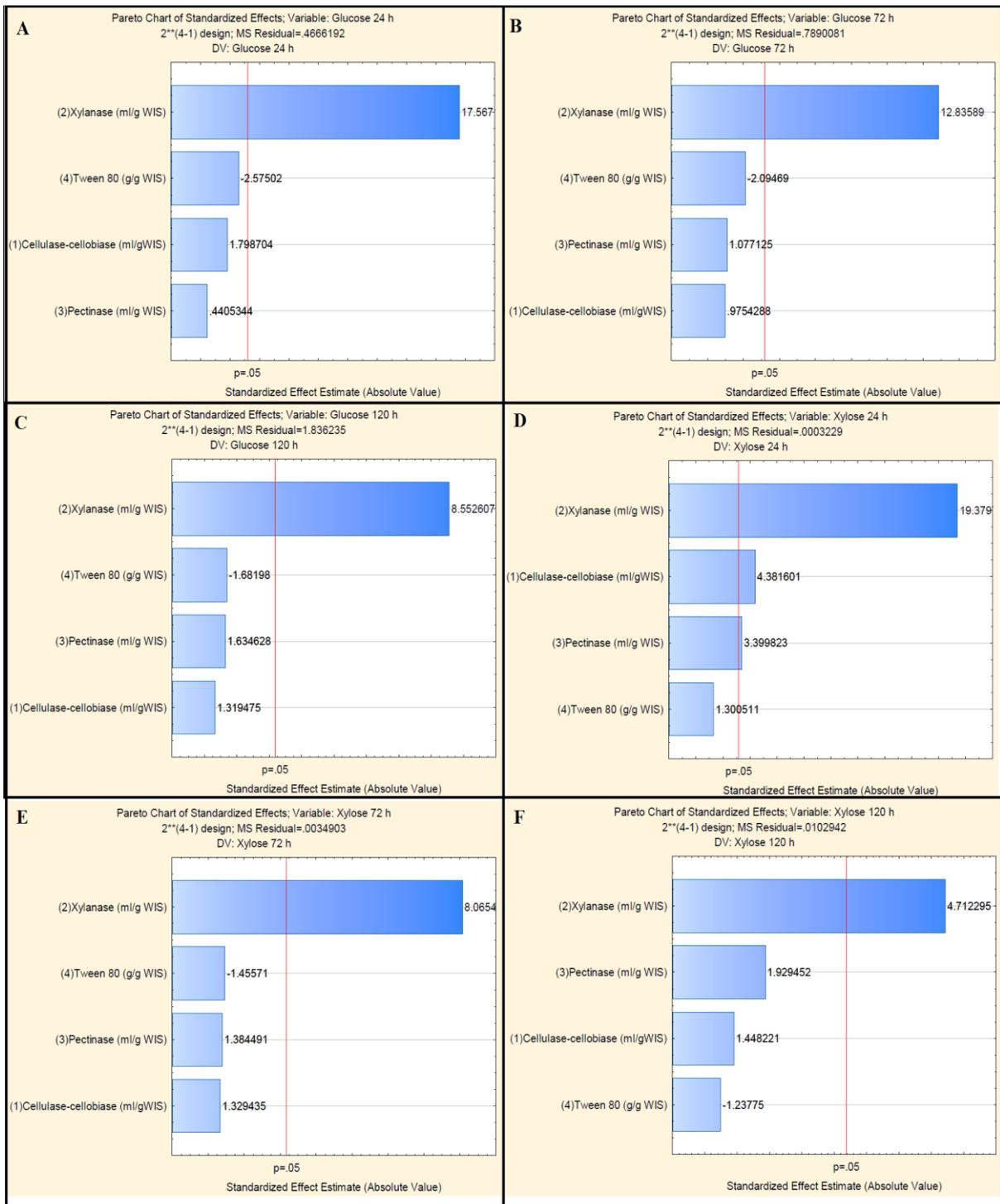


Figure 5.1: Pareto charts showing the influence of the supplementary enzymes HTec2 xylanase, Pectinex Ultra, and surfactant Tween 80 on the rate and yield of glucose released after 24h (A), 72h (B), 120h (C) and rate and yield of xylanase released after 24h (D), 72h (E) and 120h (F) of steam pretreated SB by the commercial cellulase CTec2 enzyme.

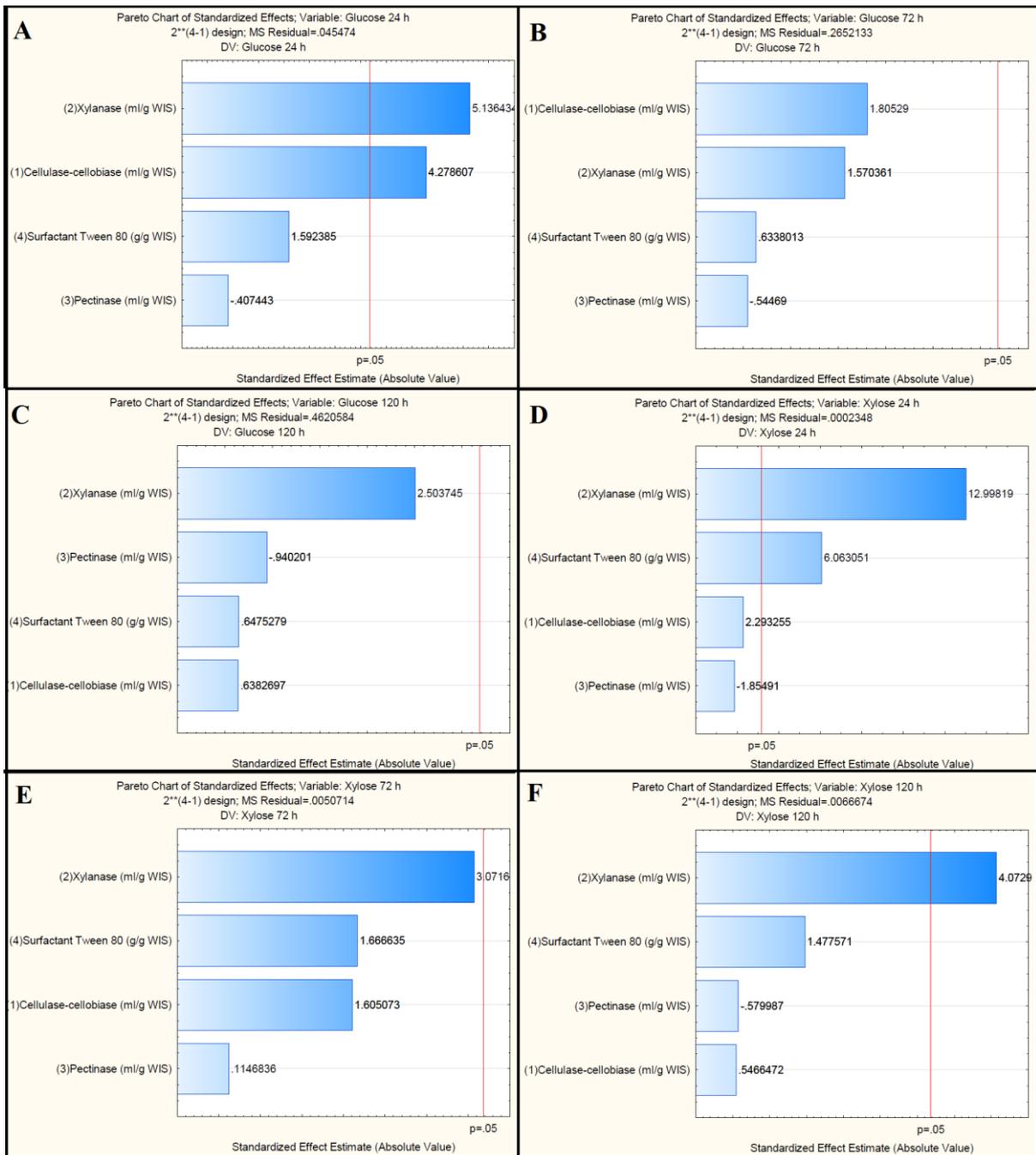


Figure 5.2: Pareto charts showing the influence of the supplementary enzymes Multifect xylanase, Pectinex Ultra, and surfactant Tween 80 on the rate and yield of glucose released after 24h (A), 72h (B), 120h (C) and rate and yield of xylose released after 24h (D), 72h (E) and 120h (F) of steam pretreated SB by the commercial cellulase Optiflow.

Additionally, even though xylanase addition was significant in improving the rate and yield of glucose and xylose release from the WIS by both commercial cellulases in the range studied, CTec2 was superior (**Figure 5.1 & 5.2**). Similar yields and rates of (SB, corn stover, switchgrass, coastal bermuda grass and wheat straw) EH by (Cellic

CTec2/HTec2) vs. conventional enzyme mixtures, such as the widely used (Celluclast/Novozym 188) and (NS50013 cellulase complex/NS50010 β -glucosidase) mixtures, have been shown (Cannella et al., 2012; Chen, et al., 2012a; Chen, et al., 2012b; Xu et al., 2011). The reported data suggested that hemicellulose hydrolysis by CTec2/HTec2 increased the ASA making the cellulose fraction easier to hydrolyse, resulting in high EH rates and yields (Cannella et al., 2012; Chen, et al., 2012a; Chen, et al., 2012b; Xu et al., 2011). Similarly, the CTec2/HTec2 mixture contains GH61s, which increase the ASA, improving the performance of the cellulase complex up to twofold (Beeson et al., 2012; Cannella et al., 2012; Harris et al., 2010; Horn & Vaaje-Kolstad et al., 2012; Phillips et al., 2011). Furthermore, there was no cellobiose accumulation in our study, indicating that sufficient levels of β -glucosidase were present in both mixtures tested. The specific yields of glucose and xylose release related to synergism was not quantified. However, the additive effect of the cellulase, β -glucosidase and xylanase as well as GH61 present in the CTec2/HTec2 combination was more effective than the Optiflow/Multifect Xylanase mixture as both have similar combined activities (**Section 2.6; Table 2.6**) (Cannella et al., 2012; McIntosh et al., 2012). Therefore, the CTec2/HTec2 mixture was used for further enzymatic hydrolysis optimization.

Effect of solid loading on cellulose to glucose conversion yield

The Pareto charts indicated that xylanase was the only significant factor in the range studied. However, the factorial design was performed at a low (2% w/v) solid loading to select the best enzyme performers. Differences are observed better at low solid loadings due to decreased end-product inhibition. However, higher solid loadings are required to make the SSF more favorable for the downstream distillation process economics (Kristensen et al., 2009; Ohgren et al., 2006; Wingren et al., 2003; Zacchi & Axelsson, 1989). On the other hand, higher solid loadings contribute to lower saccharification yields (Cara et al., 2007; Kristensen et al., 2009). Therefore, the effect of solid loading (w/v) on the cellulose conversion of steam pretreated SB by the Spezyme/Novozyme 188 control cocktail was determined (**Figure 5.3**).

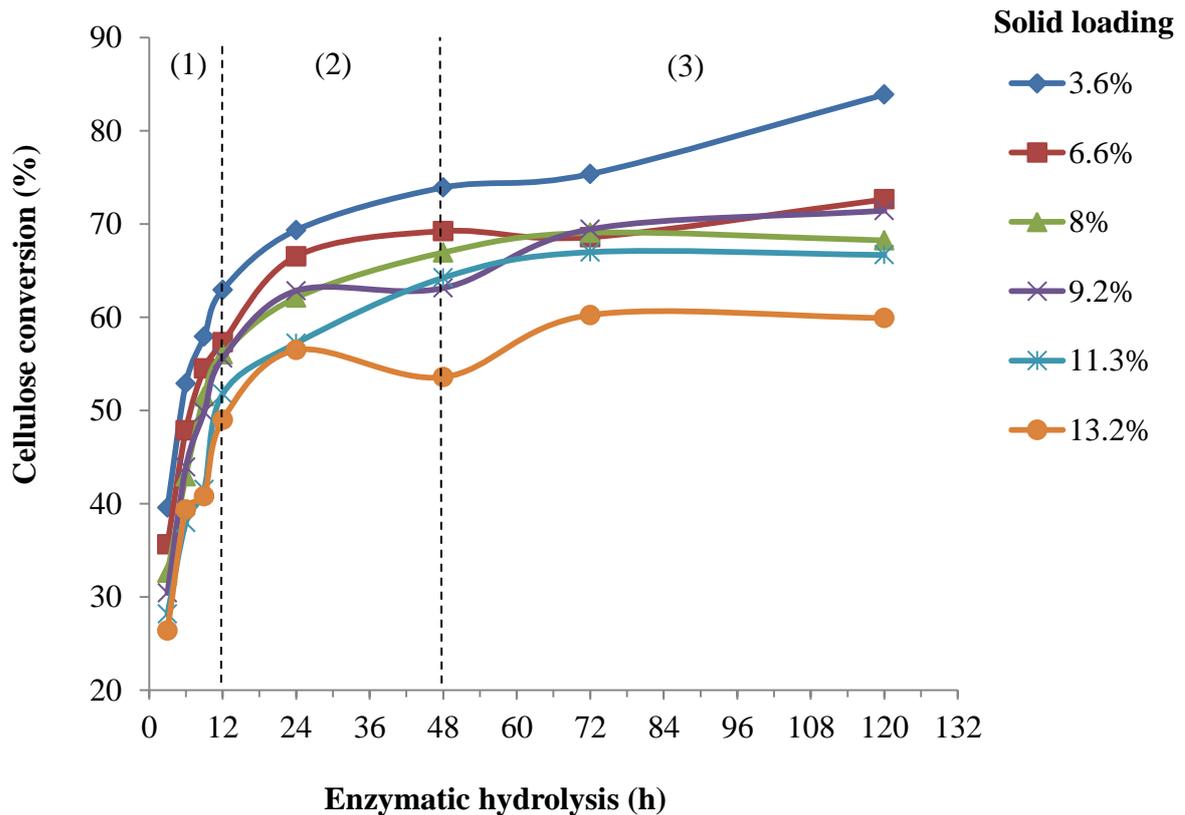


Figure 5.3: Cellulose conversion (%) with increasing solid loadings (w/v) during EH of steam pretreated SB with the Spezyme/Novozyme 188 control cocktail. Hydrolysis conditions: enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm. The three distinctive phases: (1) fast initial, (2) slower intermediate and (3) slowest recalcitrant of EH are indicated by striped lines.

Even though the solid loadings varied drastically, the same trend was seen indicating three different phases of EH, as previously shown (**Chapter 4**). However, the phases were identified at earlier times, indicating that increasing the solid loadings (w/v) is an important factor in overall EH performance, as brings about slowdown (decreased EH rate and yield) earlier, which has previously been shown (Cara et al., 2007; Kristensen et al., 2009; Lu et al., 2002). The slowdown of enzymatic hydrolysis was observed at all solid loadings tested. Even though the material was uniformly dosed at 15FPU/g WIS, increased solids (w/v) decreased the cellulose to glucose conversion yield (**Figure 5.4**).

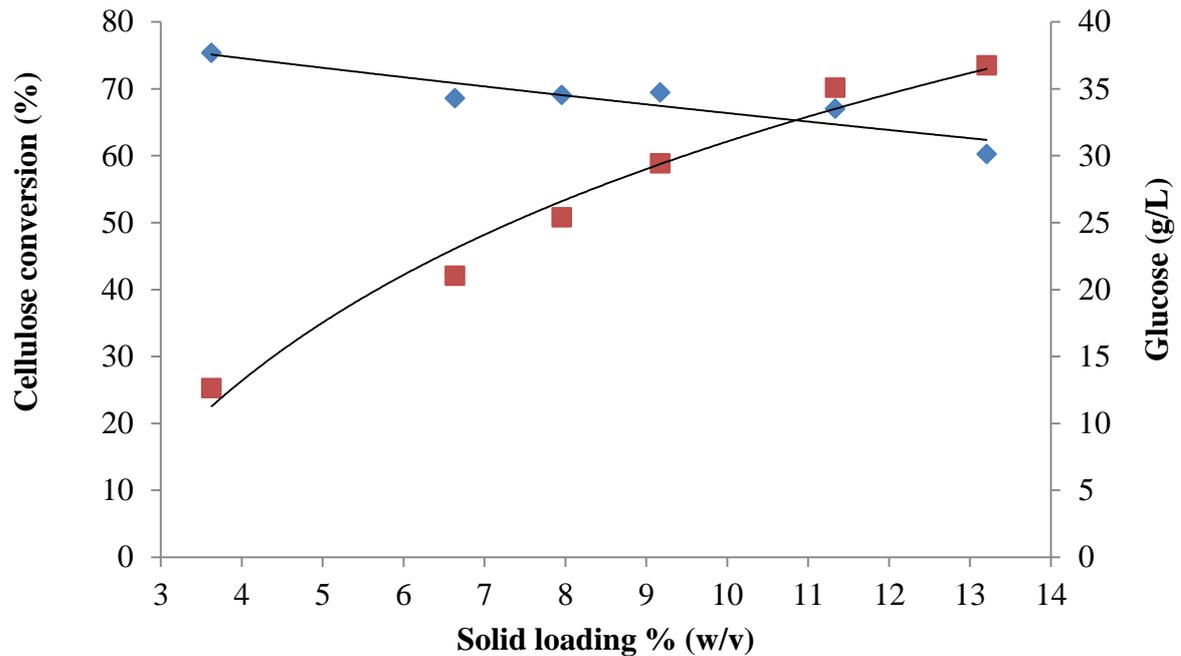


Figure 5.4: Effect of solid loading (w/v) on the cellulose conversion after 72 h (%) (◇) and glucose concentration (□) of enzymatic hydrolysis of steam pretreated SB. Hydrolysis conditions: enzyme loading 15FPU/g WIS, 50 °C, pH 5.0, 100rpm, 72h.

It was observed that after 72h of enzymatic hydrolysis the glucose concentration was 13g/L for the 3.6% (w/v) solid loading and increased to 36.7g/L at 13.2% (w/v). Although higher solid loadings resulted in higher glucose concentrations as expected, it was at the cost of cellulose to glucose conversion yield (%), which decreased as the solid loading increased. At 3.6% (w/v) solid loading, the cellulose to glucose conversion yield after 72h was 75.3%, however at the same time only 60.2% conversion yield was reached at 13.2% (w/v) solids. Therefore, it was clear that the glucose yield per unit substrate loaded was decreasing as a 3.7x increased solid loading only resulted in a 2.8x increase in glucose concentration. Similar results have shown a decrease in EH yield from 76.2% at 2% (w/v) to 66% at 10% (w/v) after 72h (Cara et al., 2007), which agrees with 75.3% at 3.6% (w/v) to 67% at 10% (w/v) in the present study (**Figure 5.4**). Furthermore, this trend has also been confirmed by various other studies (Jørgensen et al., 2007; Kristensen et al., 2009; Schwald et al., 1989; Tengborg et al., 2001; Varga et al., 2004). This pattern was observed in all samples, which could be explained by mixing problems, as higher solid loadings decrease the initial water content. The decreased water content (increased viscosity) influences the rheology of the reaction mixture, ultimately decreasing the

contact between cellulase and substrate (Jørgensen et al., 2007; Kristensen et al., 2009). Additionally, glucose, cellobiose and xylo-oligosaccharide product inhibition at higher solid loadings could influence the cellulose conversion (García-Aparicio et al., 2006; Jørgensen et al., 2007; Kadam et al., 2004; Kristensen et al., 2009; Schwald et al., 1989; Tengborg et al., 2001; Varga et al., 2004). A compromise would have to be established between achieving high enough glucose concentrations to produce ethanol during fermentation while simultaneously achieving acceptable enzymatic hydrolysis yields. Additionally, different feed strategies could be investigated to avoid high initial substrate loadings. The increased solid loading decreases the liquid volume for the enzymes to diffuse, probably decreasing its adsorption (% of the total protein). Therefore, to limit the inhibitory effects associated with higher solid loadings such as decreased enzyme adsorption and feedback inhibition, and for gaining insight into the efficiency of the enzymatic cocktail during SSF, a Central Composite Design (CCD) design was performed using *S. cerevisiae* MH1000 at solid loadings of 4.8, 7.1 and 9.3 % (w/v) as a factor. These solid loadings chosen for the CCD were within the range of solid loadings that reached greater than 69% cellulose conversion (**Figure 5.4**).

Optimization of cocktail components with a Central composite design and response surface methodology

The model was graphically represented in contour plots (**Figure 5.5 & 5.6**) with the red regions indicating the highest ethanol yields (%) and production rates (g/L•h). The response for the Cellic CTec2 design predicted that a 100% theoretical ethanol yield and rate could be reached at CTec2 loadings of 0.15ml/g WIS + 0.8ml/g WIS Htec2. Further increases in CTec2 loading resulted in a decrease in ethanol yield (%) and rate (g/L•h). This was most probably due to enzyme saturation at high cellulase dosages, as once cellulase addition increases above the saturation point, the specific activity and synergism of the enzyme mixture decreases (Kim et al. 1998). Additionally, it is possible that oxidative action by GH61s forming gluconic acid, enzyme competition, or a combination of these factors could be responsible (Cannella et al., 2012; Jagtap et al., 2012; Lee & Fan, 1982; Phillips et al., 2011). However, the design indicated that High HTec2 dosages are required (0.8ml/g WIS) (**Figure 5.5A & B**) reach the optimum. The results indicated a positive interaction between the Cellic enzymes from Novozymes resulting in maximal theoretical ethanol yields and rates. Additionally, in both scenarios, lower solid loadings (w/v) were desirable.

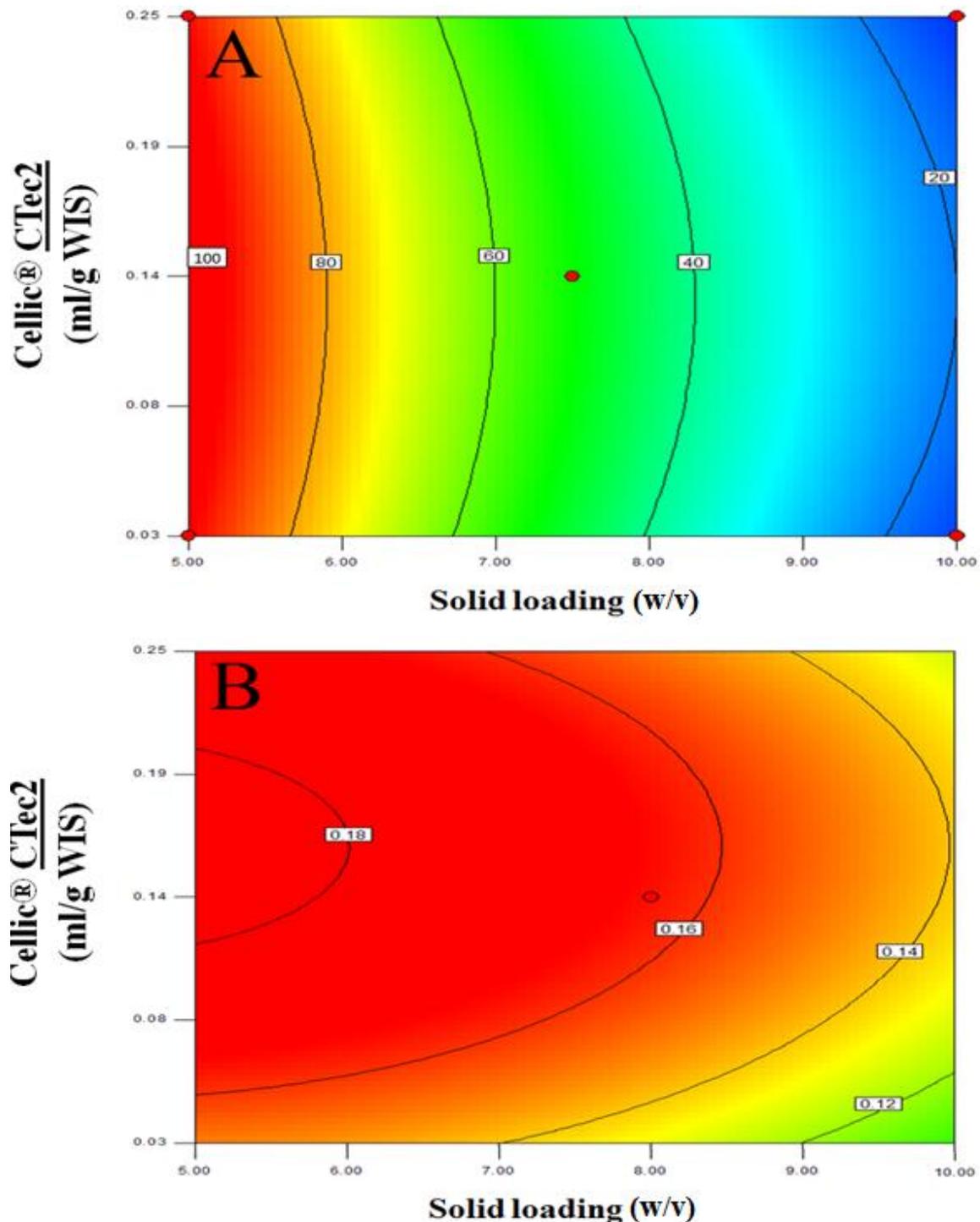


Figure 5.5: The contour plot of the 2^3 experimental design optimisation of the CTec2 enzyme complex based on maximal theoretical ethanol yield (%) (A) and maximal ethanol production rate (B). In both contour plots the CTec2 enzyme was in the presence of a high fixed dosage of Htec2 hemicellulase at 0.8ml/g WIS. Shaking was provided with orbital shaker set at 120 rpm and 35°C. The red regions indicate the highest ethanol yields (%) and production rates (g/L·h).

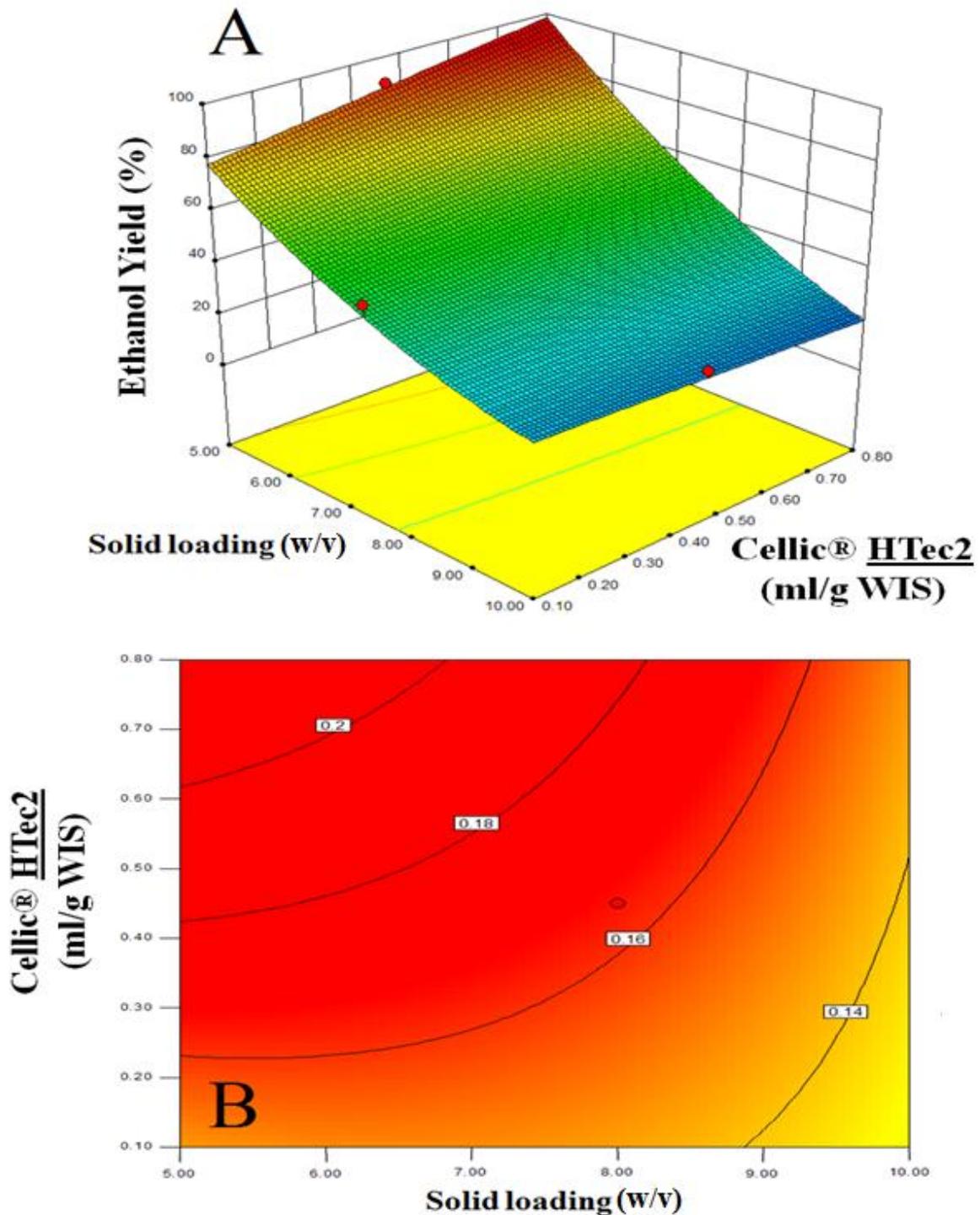


Figure 5.6: The contour plot of the 2^3 experimental design optimisation of the HTec2 enzyme complex based on maximal theoretical ethanol yield (%) (A) and maximal ethanol production rate (B). In both contour plots the HTec2 enzyme was in the presence of an optimum fixed dosage of CTec2 cellulase at 0.15ml/g WIS (A & B) previously identified (Figure 5.5A & B). Shaking was provided with orbital shaker set at 120 rpm and 35°C. The red regions indicating the highest ethanol yields (%) and production rates (g/L·h).

The contour plots indicated that high ethanol rates and yields could be achieved at low solid loadings within the enzyme range studied. However, a significant amount of HTec2 would be required. The contour plots indicated that more HTec2 would always lead to higher ethanol yields (%) (**Figure 5.6A**) and rates (**Figure 5.6B**). Even though the HTec2 optimum was out of the range studied (**Figure 5.6B**), the high dosage required to reach the optimum was not economically viable. Therefore, further enzyme screening was needed to identify the lowest HTec2 dosage required to achieve at least 80% ethanol yield. Moreover, it was interesting to observe that HTec2, had such a great influence on the process, as it was expected that CTec2 cellulase would be the determining factor in the range studied. However, previous studies have shown similar results by using Cellic enzymes at similar dosages, indicating that high yields and rates require higher HTec than CTec loadings (Xu et al., 2011).

Steepest Ascend SSF using data from CCD to decrease cocktail loading

As a result of the high HTec2 dosage indicated by the contour plots, a steepest ascend was applied to find a viable dosage for ethanol production. A fixed CTec2 cellulase loading (0.15ml/g WIS), previously identified as the statically best option if the highest ethanol yield and rate should be achieved, was chosen (**Figure 5.5**). In addition, increasing HTec2 dosages from (0 – 0.8ml/g WIS) were administered. It was decided that a volumetric dosage of 0.363ml/g WIS, which is equivalent to an industrially high-end dosage of approximately 20FPU/g WIS (Spezyme/Novozym 188), would represent the maximum economically viable dosage. The (Spezyme/Novozyme 188) mixture was used as the benchmark enzymatic cocktail as it is widely used for the saccharification of lignocellulose to produce bio-ethanol (Jin et al., 2012; Kim et al., 2008;. Qiu et al., 2012; Ximenes et al., 2011; Zhang et al., 2009; Zhang & Lynd, 2010). It was clear from the data that HTec2 xylanase addition was once again a major factor in the ethanol profile (**Figure 5.7**). After 72h of SSF, high ethanol yields could be achieved. However, this was at the cost of very high xylanase additions

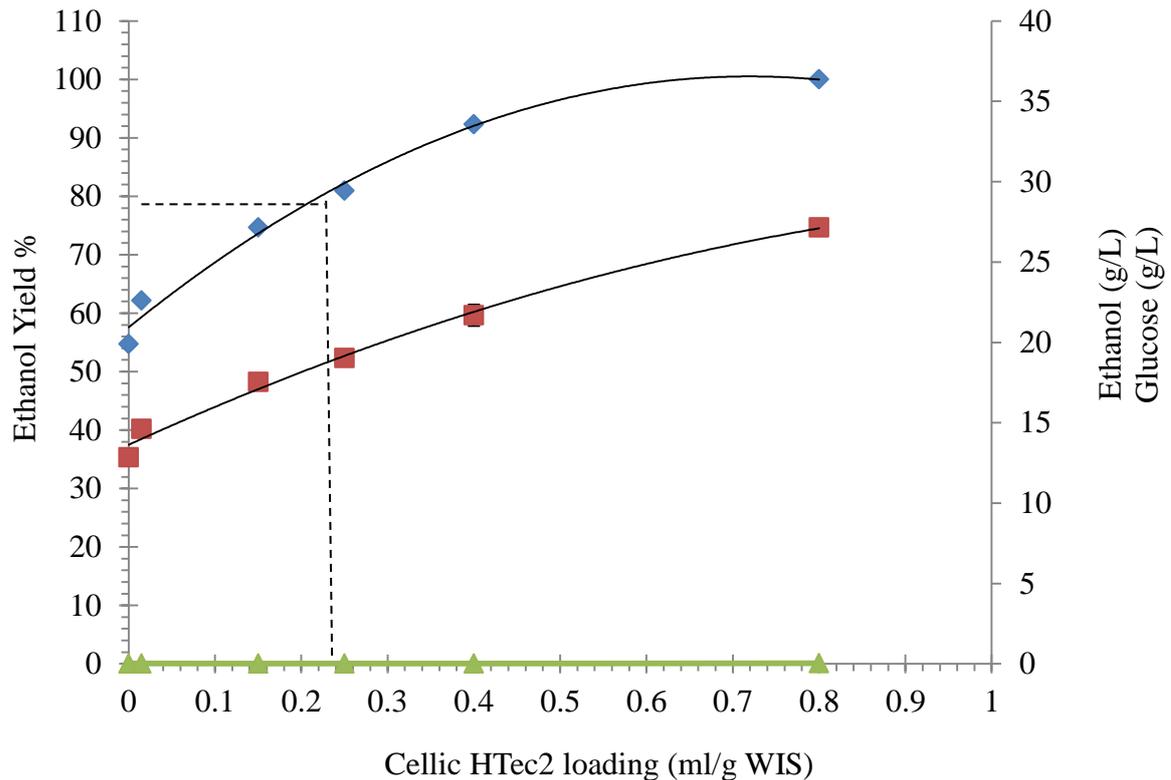


Figure 5.7: Steepest ascend showing ethanol yield (%) (◇), concentration (g/L) (□) and glucose (g/L) (△) after 72h of SSF of steam pretreated SB with fixed CTec2 cellulase loading (0.15ml/g WIS) and increasing HTec2 xylanase additions at 9.3% solids (w/v). Shaking was provided with orbital shaker set at 120 rpm and 35°C. All data points represent the average of triplicate values with standard deviation less than 1% for all samples. Striped lines indicate the 0.213 ml/g HTec2 maximal dosage cut-off with the corresponding ethanol yield (%).

Theoretical ethanol yields of 100% were achieved for all samples after 72h of SSF, however 0.8ml/g WIS HTec2 endoxyylanase addition was required. In the samples where no HTec2 xylanase was added, ethanol yields of 54% were reached after 72h of SSF. It was clear that HTec2 xylanase addition was required for effective ethanol yields during SSF, however determining the balance between dosage and yield was a priority. It has been described that 70% digestibility represents a good balance between dosage and yield (Arantes & Saddler, 2011). Therefore, a target of 70% or higher theoretical ethanol yield was set, as long as the enzyme dosage required fell within a suitable range. The highest dosage acceptable for our enzyme cocktail would be equivalent to 20FPU/g WIS of the widely used Spezyme CP/Novozym 188 cocktail. Using our data and a 20FPU/g WIS Spezyme CP/Novozym 188 equivalent cut-off for the dosage a cocktail containing

(0.15ml/g WIS CTec2 cellulase + 0.213ml/g WIS HTec2 endoxylanase) was chosen. This cocktail resulted in 79.2% ethanol yield (**Figure 5.7**) similar to results previously shown for a range of Cellic CTec2/HTec2 combinations on corn stover (Chen, et al., 2012a; Chen, et al., 2012b).

Optimized cocktail vs. Spezyme/Novozyme 188 control cocktail

The chosen optimized 0.363ml/g WIS cocktail, which consisted of 0.15ml/g WIS CTec2 and 0.213 ml/g HTec2, was compared to an equivalent volumetric dosage of conventional enzymes (Spezyme/Novozyme 188 industrial standard) used in literature. The data indicated that the chosen cocktail was definitely more efficient for the production of ethanol during SSF than the Spezyme/Novozyme 188 standard at volumetric equivalent dosages (**Figure 5.8**). After 72h of SSF the optimized (CTec2/HTec2) cocktail achieved 79% vs. the standard (Spezyme/Novozyme 188) 55% ethanol yield. Moreover the results from the experiment was within 1% of the previous ethanol yields (%) achieved for the cocktail (**Figure 5.7 & 5.8.**). In brief, a 24% greater ethanol yield (%) was achieved with the optimized cocktail.

The increased performance of the optimized cocktail vs. the older industrial standard cocktail is most likely due to the greater activity and presence of cellulases, xylanase, β -glucosidase and the newly classified GH61 oxidative enzymes, within Cellic CTec2/HTec2 (Cannella et al., 2012; Phillips et al., 2011). It has previously been shown that supplementation of GH61 oxidative enzymes, which have recently been included in next generation commercial enzyme cocktails such as Cellic CTec2 (Sims, 2011), increases the synergistic action of cellulases (Harris et al., 2010; Vaaje-Kolstad et al., 2010). Previous work has shown a 25% increase in cellulose conversion by CTec2 vs. Celluclast/Novozymes 188 cocktail at equal dosages of 7.5FPU/g dry material. GH61 enzymes oxidize the endoglycosidic bond in the cellulose chain, creating more strands for the cellulase complex to hydrolyse (Beeson et al., 2012; Cannella et al., 2012; Horn & Vaaje-Kolstad et al., 2012; Langston et al., 2011).

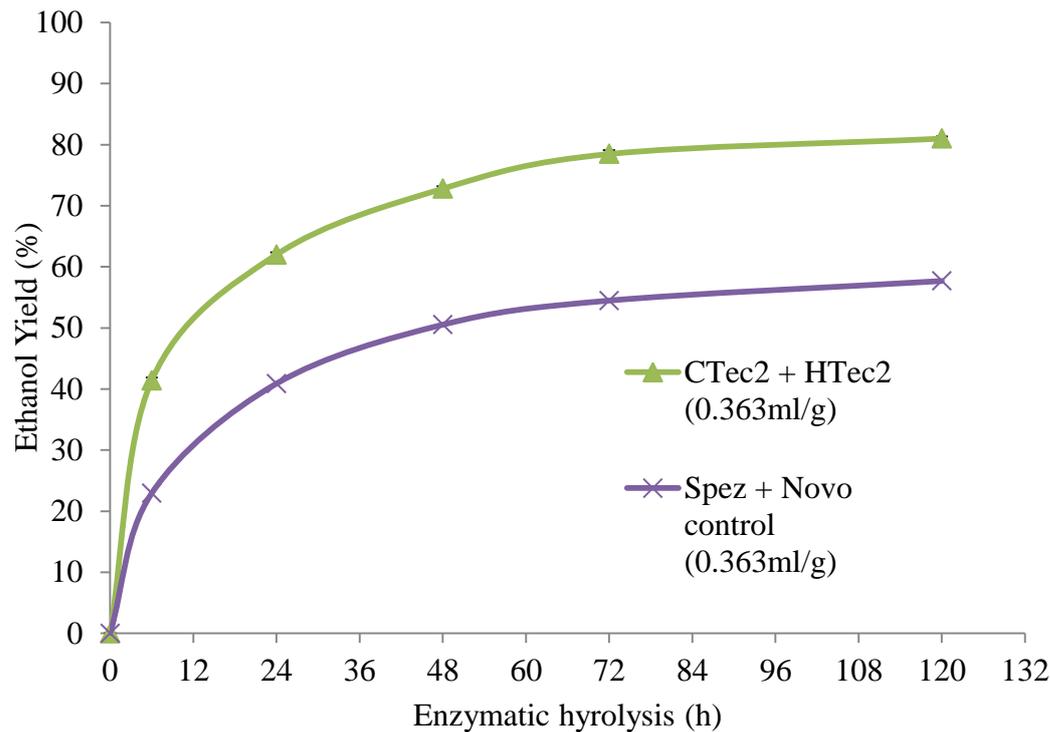


Figure 5.8: Time course showing the ethanol yield (%) of the optimized 0.363ml/g WIS CTec2+HTec2 cocktail vs 0.363ml/g WIS Spezyme/Novozyme 188 cocktail at 10% solids (w/v). Shaking was provided with orbital shaker set at 120 rpm and 35°C.

CONCLUSIONS

Xylanase addition was the only significant factor in the studied range, when compared with pectinase and Tween 80, for improving the EH rate and yield of steam pretreated SB WIS. The higher yields achieved with xylanase addition was due to synergism between the cellulase and xylanase enzymes, as well as increased ASA for cellulose degradation, achieved by enzymatic arabinoxylan removal.

A compromise could be established between sugar concentration and cellulose conversion as a function of the solid loading. Lower solid loadings had greater cellulose conversion yields (%) although the sugar concentration (g/L) decreased for all enzymes studied. Furthermore, the (Cellic CTec2/HTec2) cocktail outperformed the (Optiflow/Multifect xylanase) cocktail within the studied range, possibly due to better synergism between the enzyme ratios in the Cellic mixture. Similarly, the presence of

oxidative GH61 enzymes in the Cellic cocktail could be enhancing the cellulases, possibly by increasing the ASA.

The selected cocktail (Cellic CTec2/HTec2) was statistically optimized to contain 0.15ml/g WIS CTec2 and 0.213ml/g HTec2 which represents an volumetric dosage equivalent of 20FPU/g WIS of the industrial standard Spezyme/Novozyme 188 cocktail. The optimized cocktail resulted in a 24% increased ethanol yield (%) vs. the control, even though equivalent volumetric dosages were administered. This increase was probably due to the presence of a greater amount of cellulase, xylanase, β -glucosidase and oxidative enzymes (GH61s) in the Cellic mixture, that have shown to increase the activity of conventional cellulase cocktails. As a result, an optimal commercial enzyme cocktail was identified (0.15ml/g CTec2 + 0.213ml/g HTec2) which reached the 80% ethanol yield target. Altogether, it was seen that the use of newer generation enzymes could increase bio-ethanol production from lignocellulose, an important factor in the economic viability of the process.

REFERENCES

See **Chapter 7**.

Chapter 6: General conclusions and remarks

The economics involved in the conversion of lignocellulose to bio-ethanol still represents the major factor impeding the commercial growth of the bio-ethanol industry. The recalcitrant nature of lignocellulosic plant material is a significant barrier for efficient enzymatic hydrolysis as it slows down the conversion of biomass to fermentable sugars and decreases the ultimate yield. This decrease in overall EH performance (rate and yield) in the industry, calls for the addition of different enzymes that work synergistically to alleviate slow conversion rates and low final yields. However, great amounts of enzyme are still required. This has a negative effect on the viability of the process, as enzyme addition is the one of the most cost intensive part of the production of bio-ethanol from biomass. Therefore, research has tried to elucidate the factors responsible for slowing down the rate and decreasing yields of enzymatic conversion of biomass to fermentable sugars. However, the exact causes of slowdown remains debated.

In the present study, steam-pretreated industrial SB was enzymatically hydrolysed and the kinetics studied. Three distinctive phases: (1) an initial fast, (2) slower intermediate and (3) slowest recalcitrant phase of enzymatic hydrolysis was observed as the reaction progressed. Chemical analysis of the residual biomass after each individual step of EH revealed a drastic increase in the lignin/cellulose ratio with time. Molecular studies confirmed the increasing lignin:cellulose ratio. Similarly, the phenolic content of the material increased as the reaction progressed. The increase in lignin to cellulose was causing spherical hindrances and decreasing the Accessible Surface Area (ASA), blocking future enzyme action, resulting in decreased EH rates. Furthermore, the increasing phenolic/cellulose nature of the remaining material as EH progressed, increased the cellulose conversion difficulty, as these phenolics participate in ester-ester and ester-ether bonds between arabinose, xylose, cellulose and lignin, increasing the substrate recalcitrance.

Modifications in the internal and external surface area of the material as enzymatic hydrolysis progressed indicated changes in the ASA, which is the most important factor for efficient hydrolysis. Changes in the substrate morphology were correlated with visual observations made by scanning electron microscopy. The modifications in the material

during EH indicated that the ratio of smaller/bigger pores, decreased as EH progressed. Moreover, the smaller pores were more prevalent in the initial fast hydrolysis phase. Previous work suggested that the amount of small pores available is more important for fast EH rates and high yields than the larger pores. The three distinctive phases of EH rate and yield correlated to the modification of pore size from greater amount of small pores in the rapid phase, to a greater amount of big pores in the recalcitrant phase. It was clear that substrate factors were an important factor in the distinctive slowdown of steam pretreated SB EH conversion rate to fermentable sugars as time progressed.

Optimization of EH for the fermentation of biomass sugars to bio-ethanol, has the potential to positively influence the economic viability of biochemical biomass conversion. In conjunction with understanding the important biomass substrate characteristics, an understanding of the biological catalysts that break down the substrate is of equal importance to overcome the decreasing biomass EH rate and yield. Furthermore, a variety of new commercial enzyme mixtures, process conditions and supplementary chemicals are available to improve the enzymatic action. Therefore selecting the correct enzymatic cocktail and conditions for the feedstock used in a biomass to bio-ethanol production facility are of crucial importance.

In the present study, steam pretreated industrial SB was enzymatically hydrolysed by different commercially available enzyme mixtures. Their performances as well as the influence of supplementary enzymes, surfactant and varying solid loadings, were tested. Xylanase (Cellic HTec2) and (Multifect Xylanase) was the only significant additive improving the glucose and xylose EH rate and yield by the cellulase mixtures (Cellic CTec2) and (Optiflow). Xylanase addition acted synergistically with the cellulase mixtures, as well as increasing the ASA due to hemicellulose (mainly arabinoxylan) removal, thereby increasing the overall enzyme performance. Two Novozymes products: Cellic CTec2 cellulase and Cellic HTec2 endoxylanase were selected for optimization as higher EH yields and rates were achieved vs. the Optiflow/Multifect Xylanase cocktail. The superior performance with the Cellic cocktail was probably due to the presence of the newly classified GH61 oxidative enzymes within these enzymes, as the cellulase and xylanase activities of the cocktails were similar. Even though Cellic CTec2 had more β -glucosidase than Optiflow, no cellobiose accumulation was observed with either enzyme. The oxidative action of GH61s, present in the Cellic cocktail, are able to increase the

efficiency of cellulases two fold as GH61s increase the ASA by generating more ends for the cellulases to hydrolyse.

An optimal Cellic cocktail dosage was identified, consisting of 0.15ml/g WIS Cellic CTec2 + 0.213 ml/g HTec2 that was volumetrically equivalent to 20FPU/g WIS Spezyme + Novozym 188 mixture. During SSF reactions with the ethanol producing *S. cerevisiae* strain MH1000, 79.2% theoretical ethanol conversion from biomass was achieved with the optimized cocktail after 72h at 5% solids. In comparison, only 55% ethanol yield was achieved with the Spezyme/Novozymes standard cocktail at the same conditions. The increased ethanol yield and rate with the optimised cocktail (0.15ml/g WIS Cellic CTec2 + 0.213 ml/g HTec2) was due to the presence of greater cellulase, xylanase and β -glucosidase activities as well as GH61s proteins that have been shown to significantly improve the efficiency of newer generation enzymes. It is evident that there are definite differences between commercially available hydrolytic enzymes used for the degradation of biomass and those cocktails that contain higher levels of lignocellulose degradation enzymes perform better. The optimized Cellic CTec2/HTec2 cocktail resulted in a 24.2 higher ethanol yield (%) compared to the industrial Spezyme/Novozyme 188 standard. Therefore, it is recommended to continuously evaluate the enzyme cocktails used within a process to incorporate the best enzyme cocktails available for optimal EH product yield, rates and economics.

Altogether, it was possible that the selected optimum cocktail (CTec2 +HTec2) was less susceptible to the recalcitrance of steam pretreated SB (**Chapter 4**) during EH than the older industrial standard dosage of Spezyme/Novozymes 188. The greater cellulase, xylanase, β -glucosidase and GH61 activity within the optimum cocktail was possibly increasing the ASA for the cellulase complex. The increased ASA was achieved by hemicellulose (arabinoxylans) degradation, which also decreased the ester and ether bonds between CW components, decreasing substrate recalcitrance. Additionally, oxidative cleavage of the cellulose fraction by GH61s present in the Cellic cocktail supplied more ends for the cellulases enzymes. It is possible that the synergistic action of the enzymes present in the newer generation cocktail partially negated the negative effect of the decreased ASA caused by the increasing lignin/cellulose ratio as EH progressed, resulting in higher yields and rates vs. the older standard cocktail. Therefore a delicate relationship between biomass substrate properties and mechanism of enzyme cocktails

exist influencing the EH yield and rate. The substrate properties influence the digestibility of the biomass by creating a baseline barrier to EH, with the nature of the barrier heavily dependent on the feedstock and pretreatment utilized. However, once enzymes are administered, it is important that the selected cocktail contain high levels of cellulases, xylanases, β -glucosidases and GH61s, with the exact composition varying with the feedstock, to negate the substrate barrier and achieve high EH yields and rates.

Performing physicochemical analysis on SB varieties that were modified for 2nd generation bio-ethanol production, and comparing them to the industrial SB used in the present study, should still be performed. This would give interesting insights into the nature of recalcitrance between varieties. Lastly, physicochemical studies should be used to compare the slowdown in cellulose conversion rate and yield when commercial and optimized cocktails are used. A study can be conducted by washing material as it enters the slowdown phase of EH. The residue can be subjected to subsequent EH steps with a range of control and next generation enzyme cocktails to investigate the exact action that improved cocktails have on substrate recalcitrance. An enzyme cocktail that is less susceptible to substrate recalcitrance can be identified which could lead to lower enzymes dosages, improving the biomass to bio-ethanol process economy.

Chapter 7: References

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