Study of Enzymatic Hydrolysis to Improve Sugar Production from Steam-Pretreated Sweet Sorghum Bagasse and Triticale Straw

by

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DECLARATION

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Summary

The production of ethanol from lignocellulosic biomass (second generation biofuels) is currently being investigated worldwide for the development of biofuels that can be used as an alternative to fossil fuels. This follows the growing concerns over potential land protection, conflicts with food suppliers as well as arguments involving the reduction of greenhouse gas emission. Sweet sorghum bagasse and triticale straw are especially promising as these crops can produce high yields under a wide variety of conditions. They also have no food value and lower greenhouse gas emission compared to other feedstocks, making them attractive for second generation bio-ethanol production.

Among the numerous different possibilities, enzyme-based processes are one of the most promising for the production of biofuel. This is mainly due to its specificity, higher yields, generation of lower amounts of inhibitory compounds formed during hydrolysis and fermentation as well as the high potential of improvement through biotechnology. At present, the enzymatic hydrolysis of lignocellulosic materials is a bottleneck in the process of bio-ethanol production and the associated production costs are still too high, thereby preventing the commercialisation of this process. The selection of feedstock varieties (selected based on their response to pretreatment and agronomic data) and optimisation of enzymatic hydrolysis for specific feedstock and pretreatment conditions is thus of key importance for obtaining an efficient sugar yield and therefore, ethanol yield.

A need also exists for developing realistic and quick methods to evaluate feedstock digestibility. Increasing research is being directed towards the development of high-throughput systems based on distributing milled pretreated material into micro-well plates to evaluate multiple variables during enzymatic hydrolysis. Since the milling can be considered as an additional treatment, the application of other methods with minimum impact on lignocellulose structure is preferred. Such a method is based on handsheets as described in the TAPPI standard methodology.

In this context, the main aim of this thesis was to improve enzymatic hydrolysis by identifying optimum enzyme combinations that are specific for feedstock varieties and steam-explosion pretreatment conditions of sweet sorghum bagasse and triticale straw. The

specific goal was to obtain an optimum combination of enzyme preparations at minimum dosage that provide 80 % of cellulose conversion. Based on preliminary results, two enzyme preparations characterised as cellulase and xylanase were selected for optimisation by central composite design and subsequent response surface plots with the use of Design Expert® software. A second aim of the thesis was to develop a micro-assay method that incorporates an additional homogenisation step prior to making the handsheets. The effects of different homogenisation treatments on fiber length and digestibility were evaluated and compared with lab-scale results. These enzymatic hydrolysis studies were conducted using pretreated bagasse and straw and the enzyme combinations selected in the optimisation study.

Results showed that the optimum combination of cellulase and xylanase proved to be more effective during enzymatic hydrolysis compared to a conventional enzyme mixture. These optimised cocktails consisted of 0.15 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.32 $mL.g^{-1}$ WIS Cellic® HTec2 for sorghum and 0.10 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.20 $mL.g^{-1}$ WIS Cellic® HTec2 for triticale. This improvement is, however, dependent on the feedstock. Sorghum required double the enzyme dosage used for triticale to reach a cellulose conversion of 80 %. This was possibly due to the higher cellulose content thereof and because the material was less digestible. With regards to the micro-assay, a homogenisation step showed to slightly improve the digestibility of the pretreated materials depending on the feedstock and enzyme combination applied. However, this method revealed the ability of the micro-assay to distinguish between the two different feedstocks as well as the two enzyme preparations.

In conclusion, optimisation of enzymatic hydrolysis integrated with pretreatment can significantly improve the overall sugar yield. For sorghum, the optimised cocktail yielded 401.0 kg $sugar.ton^{-1}$ bagasse compared to the 328.2 kg $sugar.ton^{-1}$ bagasse obtained with the control enzyme combination. The optimised cocktail for triticale yielded 320.7 kg $sugar.ton^{-1}$ straw and the control cocktail 275.5 kg $sugar.ton^{-1}$ straw. Data confirmed that although feedstocks were similar in terms of biomass type and chemical composition, their different raw material properties and pretreatment conditions required them to have different optimum enzyme loadings for hydrolysis. The higher yields obtained with the optimised cocktails were also confirmed when enzymatic hydrolysis was performed at micro-scale. This is a useful screening method, but the differences observed should be kept in mind when high-throughput systems are applied.

Opsomming

Die vervaardiging van etanol vanaf lignosellulosiese biomassa (tweede generasie bio-brandstowwe) word tans wêreldwyd ondersoek vir die ontwikkeling van bio-brandstowwe wat as alternatief teenoor fossiel-brandstowwe gebruik kan word. Dit is die gevolg van toenemende besorgdheid oor potensiële landbewaring, konflik met voedselverskaffers asook argumente rondom die afname in die vrystelling van kweekhuisgasse. Soet sorghum bagasse en triticale strooi is veral twee belowende plantaardige grondstowwe, aangesien hul hoë opbrengste verskaf onder ‘n verskeidenheid kondisies. Hul het ook geen waarde as voedselbron nie en lei tot ‘n netto laer vrystelling van kweekhuisgasse, wat hul baie gewild maak vir die vervaardiging van tweede generasie bio-etanol.

Onder die verskeie moontlikhede wat bestaan, is ensiem-gebaseerde prosesse die mees belowende vir die vervaardiging van bio-brandstowwe. Dit is grotendeels te danke aan die spesifisiteit daarvan, die hoër opbrengste, die generering van laer hoeveelhede inhiberende komponente tydens hidrolise en fermentasie sowel as die hoë potensiaal daarvan om te verbeter deur tegnologie. Tot op hede vorm die ensiematiese hidrolise van lignosellulosiese materiaal ‘n bottelnek in die proses van bio-etanol vervaardiging. Die produksiekostes wat daarmee geassosieer word, is ook steeds baie hoog en verhoed dus die kommersialisering van hierdie proses. Die seleksie van grondstof variëteite (geselekteer op grond van hul reaksie op vooraf-behandelings- en agronomiese data) en optimisering van die ensiematiese hidrolise stap vir spesifieke grondstof en vooraf-behandelingstoestande is dus van uiterste belang om ‘n voldoende suiker opbrengs, en gevolglik etanol opbrengs, te verkry.

Daar is ook ‘n aanvraag om realistiese en vinnige metodes te ontwikkel vir die evaluering van grondstof verteerbaarheid. Toenemende navorsing word dus nou gerig op die ontwikkeling van hoë deurvoer sisteme wat gebaseer is op die verspreiding van gemaalde vooraf-behandelde materiaal in mikro-titer plate om verskeie veranderlike faktore tydens ensiematiese hidrolise te evalueer. Aangesien die maling van materiaal as ‘n addisionele behandeling beskou kan word, word die toepassing van ander metodes wat minimum impak op die lignosellulosiese struktuur het, verkies. So ‘n metode word gebaseer op *handsheets* soos beskryf in die TAPPI-standaard metode.

Met hierdie as agtergrond, was die hoof doel van die tesis om ensiematiese hidrolise te verbeter deur optimum ensiem kombinasies saam te stel wat spesifiek is vir variëteite en stoom-voorafbehandelde toestande van sorghum bagasse en triticale strooi. Die spesifieke doel was om 'n optimum kombinasie van ensiem-bereidings teen 'n minimum dosis te verkry wat 'n omskakeling van 80 % van die sellulose na suikers, verskaf. Gebaseer op voorafgaande resultate is twee ensiem-bereidings wat as sellulase en xylanase gekarakteriseerd is, geselekteer vir optimisering deur 'n sentrale saamgestelde ontwerp en die daaropvolgende respons-vlak grafieke met die hulp van Design® Expert sagteware. Die tweede doel van die tesis was om 'n mikro-toets metode te ontwikkel wat 'n addisionele homogeniserings-stap insluit voor die maak van die *handsheets*. Die effek van die verskillende homogeniserende behandelings op die vesellengtes en vertering was geëvalueer en vergelyk met laboratorium-skaal resultate. Hierdie ensiematiese hidrolise studies was uitgevoer met behulp van voorafbehandelde bagasse en strooi en deur gebruik te maak van die ensiem kombinasies wat geselekteer is in die optimiserings-studies.

Resultate het getoon dat die optimum kombinasies van sellulase en xylanase meer effektief was gedurende ensiematiese hidrolise in vergelyking met 'n konvensionele ensiem-mengsel. Die geoptimiseerde kombinasies het bestaan uit 0.15 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.32 $mL.g^{-1}$ WIS Cellic® HTec2 vir sorghum en 0.10 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.20 $mL.g^{-1}$ WIS Cellic® HTec2. Hierdie verbetering is egter afhanklik van die plantaardige grondstof. Sorghum het dubbel die hoeveelheid ensiem benodig as wat deur triticale gebruik is om 'n 80 % sellulose omskakeling te behaal. Dit was moontlik as gevolg van die hoër sellulose fraksie daarvan en omdat die materiaal minder verteerbaar is in vergelyking met triticale. Met betrekking tot die mikro-toets het 'n homogeniserings-stap aangedui dat dit die verteerbaarheid van die vooraf-behandelde materiale tot 'n mate kon verbeter, afhangend van die grondstof en ensiem kombinasies wat gebruik is. Hierdie metode het egter die vermoë van die mikro-toets om tussen twee verskillende plantaardige grondstowwe te onderskei, sowel as tussen die twee ensiem bereidings, onthul.

Ten slotte, die optimisering van ensiematiese hidrolise wat geïntegreer is met voorafbehandeling kan die algehele suiker opbrengs merkwaardig verbeter. Vir sorghum het die geoptimiseerde ensiem kombinasie 401.0 kg $suiker.ton^{-1}$ bagasse gelewer in vergelyking met die 328.2 kg $suiker.ton^{-1}$ bagasse wat verkry is met die kontrole ensiem kombinasie. Die geoptimiseerde ensiem kombinasie vir triticale het 320.7 kg $suiker.ton^{-1}$ strooi gelewer en

die kontrole kombinasie 275.5 kg suiker.ton^{-1} strooi. Data het bevestig dat, alhoewel die grondstowwe eenders was in terme van die tipe biomassa en chemiese komposisie, hul verskillende rou materiaal eienskappe en vooraf-behandelingstoestande daartoe gelei het dat hulle verskillende optimum ensiem kombinasies tydens hidrolise vereis het. Die hoër opbrengste verkry met die geoptimiseerde kombinasies was ook bevestig met die uitvoer van ensiematiese hidrolise op mikro-skaal. Dit is ‘n bruikbare siftingsmetode, maar die verskille wat opgemerk is, moet in gedagte gehou word wanneer hoë deurvoer sisteme toegepas word.

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List of Abbreviations

Abbreviation	Description
1G	First Generation
2G	Second Generation
3G	Third Generation
5-HMF	5-hydroxymethyl-2-furaldehyde
ADB	Algae-Derived Biofuels
AFEX	Ammonia Fiber Explosion
ANOVA	Analysis Of Variance
BCA	Bicinchoninic Acid
BG	β-glucosidase
BIS	Biofuels Industry Strategy
BX	β-xylosidase
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolases
CBP	Consolidated Bioprocessing
CCD	Central Composite Design
CD	Catalytic Domain
CDH	Cellobiose-dehydrogenase
CMC	Carboxy-Methyl Cellulose
DMC	Direct Microbial Conversion
DNS	Dinitrosalicylic acid
DP	Degree of Polymerisation
DS	Degree of Synergism
EG	Endo-glucanases
EH	Enzymatic Hydrolysis
EX	Endo-xylanase
FPA	Filter Paper Activity
FPU	Filter Paper Units
GH61	Glycoside Hydrolases Family 61
GHG	Greenhouse Gas
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric Acid
HPLC	High-Performance Liquid Chromatography
IU	International Unit
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
KOH	Potassium Hydroxide
LAPs	Laboratory Analytical Procedures
LHW	Liquid Hot Water
NREL	National Renewable Energy Laboratory
PCA	Perchloric Acid
PEF	Pulsed Electrical Field

PEG	Polyethylene Glycol
RI	Refractive Index
RSM	Response Surface Methodology
SE	Steam-Explosion
SHF	Separate Hydrolysis and Fermentation
SNG	Synthetic Natural Gas
SO_2	Sulphur Dioxide
SSB	Sweet Sorghum bagasse
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TIA	Technology Innovation Agency
TS	Triticale straw
UV	Ultra-Violet
WIS	Water-Insoluble Solids
WSS	Water-Soluble Solids
XOS	Xylo-oligomers

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1. Introduction

The depletion of conventional fossil fuel reserves (petrol) coupled with the escalation in the consumption of energy have led to the search for renewable sources of energy such as biomass that are also sustainable [1], [2]. The use of renewable energy could help to increase national energy security, by reducing dependence on imports of fossil-fuel, whose prices are high and unstable (political instability in oil-producing regions). The second largest energy carrier in SA (after coal) is imported crude oil that is used for the supply of liquid fuels in the transport sector [3]. These fuels meet about 20 % of the total energy demand of South Africa, but represent up to 75 % of the total energy costs [4]. Biofuels from biomass represent one of a few alternatives for short-term diversification to enhance the security of supply in the transport sector, which depends almost totally (98 %) on fossil-based fuels [5].

The interest in biofuels is also driven by the increasing greenhouse gas (GHG) emissions, mainly CO_2, and health and safety concerns. The transportation sector is responsible for 58 % of CO_2 emissions in South Africa, which is an important factor contributing to global warming [6]. Biofuels, which are obtained from vegetal biomass, are renewable products that can reduce GHG emissions. The CO_2 generated when the biomass is combusted is used together with water by vegetal biomass in order to produce the carbohydrates constituents and O_2 through photosynthesis [7]. It can therefore be argued that the cycle is closed as long as the biomass is planted (Figure 1).

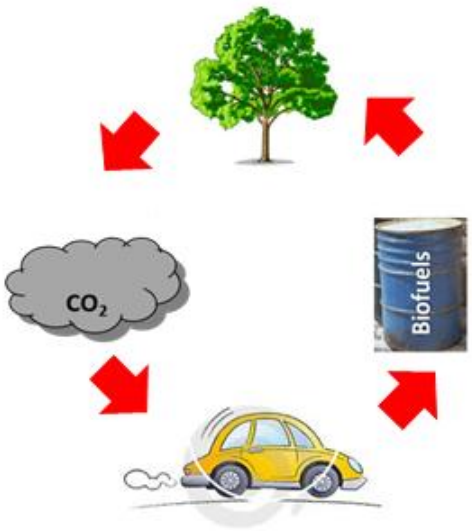

Figure 1: Carbon-cycle for biofuels. The plants transform CO_2 and water into biomass using solar power. The biomass is then transformed into biofuels and the combusted CO_2 is released back into the atmosphere.

As a result, South Africa has joined the worldwide trend in developing measures to promote both the production and use of biofuels. The Department of Minerals and Energy prepared the Biofuels Industry Strategy (BIS) in 2007 to fix a five-year target for blending biofuels of 2 % (corresponding to approximately 400 million litres per year) by 2013 [8]. The proposed feedstocks included first generation (1G) biomass (i.e. soya, canola and sunflower for biodiesel; sugarcane and sugar beet for bio-ethanol) [8]. However, only recently (August 2012) a mandated blending of E8 and B2 for bio-ethanol and biodiesel, respectively, has been established in order to support commercial production of these biofuels [8], [9]. These refer to blends of transportation fuels (of an oil origin); E8 to a blend of 8 % bio-ethanol with petrol and B2 to a 2 % blend of biodiesel with diesel.

Ethanol is currently produced by fermentation of glucose contained in crops rich in sugar or starch. The ethanol produced from crops designed for the food market and established technology is referred to as 1G. Most of the glucose can be extracted directly from sugar-rich plants, which entails solubilisation with warm water, followed by pressing [10]. The glucose from the starch is obtained by available technology based on the enzymatic hydrolysis (EH) of the starch by means of amylases [10]. However, the increasing demand for bio-ethanol will not only require the search for alternative feedstocks, but also the integral use of the whole plant, including the lignocellulose part. In this context, the development and use of novel dedicated energy crops with improved agronomic traits, such as sweet

sorghum (*Sorghum bicolor*) and triticale (*x Triticosecale*), has been suggested as a strategy to produce biofuels without impacting food security or the environment [11]. The use of these crops generates lignocellulosic residues (bagasse and straw of sorghum and triticale, respectively) that provide a supplement to the sugar and grain-based ethanol. The incorporation of modules for 2nd generation ethanol production in current facilities of 1st generation ethanol could facilitate its gradual introduction in the (bio)fuel-mix, given its already established capacities and logistics system [12].

In spite of the advantages of second generation (2G) ethanol, the technology of conversion needs improvements in order to be competitive with oil-derived fuels. Among the conversion technologies, those based on enzymatic hydrolysis are considered promising given its specificity and the potential of improvement by means of biotechnology [13]. Cellulose and hemicellulose can be hydrolysed enzymatically into sugars that are fermented into ethanol, which is recovered by distillation. The lignocellulose, however, needs to be subjected to a pretreatment step to alter its recalcitrant structure and make the polysaccharide fraction more susceptible to enzymatic attack [13].

One of the steps that contribute extensively to the cost of cellulosic ethanol production is the enzymatic hydrolysis [13]. The enzyme requirements rely on feedstock, pretreatment applied and nature of the enzyme preparations [13]. In this context, this thesis is focused on the improvement of the enzymatic hydrolysis of pretreated bagasse and straw from selected varieties of sorghum and triticale, respectively. At the same time, a method for rapid evaluation of both digestibility of different lignocellulosic materials and hydrolytic potential of enzyme preparations was developed.

1.1. Outline of the Thesis

The thesis is divided into eight chapters. Chapter 2 corresponds with the literature review, which puts into perspective 2G ethanol within biomass as renewable energy. The different sections of the literature review describe the chemical composition and structure of lignocellulose, the main stages of ethanol production (pretreatment, enzymatic hydrolysis and fermentation) and different ethanol production schemes from lignocellulosic biomass based on enzymatic hydrolysis. The aspects influencing the enzymatic hydrolysis step were covered in depth in order to centre the aims and interest of the study discussed in Chapter

3. The materials and methods applied are summarised in Chapter 4. In Chapter 5 the characterisation of the commercial enzyme preparations used during this study is described. Chapter 6 and Chapter 7 comprise two manuscripts corresponding to enzymatic hydrolysis optimisation and development of a micro-assay for evaluation of digestibility and hydrolytic potential of enzyme combinations, respectively. Both of these manuscripts are also followed by an addendum which encompasses corresponding preliminary studies. In Chapter 8 some concluding remarks are presented and Chapter 9 lists recommendations for future work.

1.2. References

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2. Literature Review

2.1 Biomass as Energy Source

Biomass is defined as organic, non-fossil material of a biological origin that creates an energy source that is renewable [1]. Biomass can also constitute a source of energy that can replace conventional fuels by generating heat, heat and power combined, electricity as well as transport fuels for various sectors [2]. Biomass offers several advantages as it provides a clean, sustainable source of energy, can compensate for decreasing worldwide petroleum reserves, is able to alleviate the dependence that exists on foreign oil and also provides an economic boost to rural communities [3]–[6]. Liquid biofuels, in particular, obtained from biomass, are one of the few alternatives that can be used for short-term diversification within the transportation sector to replace petrol/diesel/jet fuels partially or totally, given that they can be certainly incorporated in the existing supply and refuelling systems [7], [8].

Biomass can be divided into different groups according to the composition thereof; sugar-rich and oil-rich crops that include sugar beet and oil-seed, starch-rich crops and lignocellulosic biomass [9]. At the same time, biofuels (solid, liquid, gas) have been grouped into three generations based on the feedstock nature, processing technology and development stage (Table 1) [7], [10]. 1G biofuels are produced with technologies that are commercially well-established and which make use of traditional food and feed crops, such as ethanol production from starch- or sugar-rich feedstocks [7]. Maize grain, for example, is diverted from the conventional use thereof to become a feedstock that can be made into fuel ethanol [7]. Sugarcane is similarly grown for the extraction of its sugars, where after the sugar is then used for the production of ethanol for fuel [7]. It thus involves the enzymatic conversion of the accessible glucose from sources such as corn starch or the direct fermentation of sucrose such as sugarcane [11]. Alternatively, oil-rich crops and wet biomass are used in the production of biodiesel and biogas, respectively [9]. 2G biofuel systems employ crops that are grown specifically for the production of lignocellulosic biomass (cellulose and hemicellulose of agricultural residues) that will be converted into ethanol or other biofuel forms [7], [11]. Lastly, third generation (3G) biofuels are those biofuels produced by algae [10].

Table 1: Classification of biofuels and the conversion technology involved (Table adapted from [12]).

Biofuel type	Biomass feedstock	Production process
First generation (conventional) biofuels		
Bio-ethanol	Sugar beets, cereal grains	Fermentation
Biodiesel	Oil crops (i.e. grape seeds)	Cold pressing/extraction & transesterification
	Waste/cooking/frying oil	Transesterification
Biogas	(Wet) biomass	Digestion
Second generation biofuels		
Bio-ethanol	**Lignocellulosic material**	**Advanced hydrolysis & fermentation**
Synthetic biofuels		Gasification & synthesis
Biodiesel (hybrid between 1G and 2G)	Vegetable oils and animal fat	Hydrogenation (refining)
Biogas – Synthetic Natural Gas (SNG)	Lignocellulosic material	Gasification & synthesis
Bio-hydrogen		Gasification & synthesis or biological production
Third generation biofuels		
Algae-derived biofuels (ADB)	Macro-algae Micro-algae	Extraction and transesterification Biochemical Thermochemical

Lignocellulose, being a type of biomass, includes any plant material that is produced by the action of photosynthesis [1]. Lignocellulosic biomass is abundant and can be produced quickly and at a lower cost than food crops, making it reasonably advantageous [13]. Bio-ethanol production from lignocellulosic materials also utilises the non-edible portion of the plant, thereby reducing the overall waste [13].

Current concerns with 1G biofuels are causing research and development to be catapulted towards 2G biofuels, which represents a more sustainable option for ethanol production [13], [14]. Bio-ethanol that is produced from lignocellulosic biomass fermentation (2G) has several advantages when compared to sugar- or starch-derived bio-ethanol (1G), from both an energetic and environmental point of view [15]. One advantage entails that 2G biofuels that are generated from forest and agricultural residues and by-products present a promising alternative to the current problem of resource competition with the food production from agriculture [16]. The hope exists that it would be possible to produce these biofuels in large scale, without it having any effect on food production. This will, however, not be a problem as lignocellulosic feedstocks are mainly agro-forestry wastes that are cellulose-based [17]–[19].

Apart from land, biofuel feedstocks are in competition with other productive resources such as fertilizer, pesticides and water, which will eventually lead to the degradation of land and water [16]. More pressure is being placed on the environment as the production of biofuels expands [16]. The pressure will increase the loss of biodiversity as well as GHG emissions, specifically in areas with a high organic matter content and biodiversity [16], [20], [21]. One significant advantage of the use of bio-ethanol is, however, the decrease in GHG emission which will be larger due to the lower overall fossil fuel input that is required during the process [15]. This was one of the main reasons for the development of biofuels [2]. When using ethanol that is produced from cellulosic material, the projected GHG emission savings can approach 90 % compared to gasoline [20], [22]. Similarly, a reduction in GHG emissions of 88 % is obtained using cellulosic ethanol when compared to ethanol produced by corn, which can only reduce it by about 13 % [20], [23]. The production of biofuel as transportation fuel is also able to help reduce the build-up of CO_2 in two ways: by recycling the CO_2 which is released by the combustion thereof as fuel and by displacing the use of the fossil fuels [5]. Some of the main benefits of biofuels are given in Table 2.

Table 2: The major benefits that biofuels provide (Table adapted from Balat *et al.* 2011 [5]).

Economic Impacts	Sustainability Fuel diversity Increased number of rural manufacturing jobs Increased income taxes Increased investments in plant and equipment Agricultural development International competitiveness Reducing the dependency on imported petroleum
Environmental Impacts	GHG reductions Reducing of air pollution Biodegradability Higher combustion efficiency Improved land and water use Carbon sequestration
Energy Security	Domestic targets Supply reliability Reducing use of fossil fuels Ready availability Domestic distribution Renewability

2.2 Lignocellulosic Biomass: Composition and Structure

The lignocellulosic materials which can be used in the production of bio-ethanol can be classified into six groups: agricultural residues, hardwoods (angiosperms), softwoods (gymnosperms), cellulose wastes (e.g. recycled paper sludge, waste office paper) and municipal solid wastes [24], [25].

Lignocellulose, a complex carbohydrate polymer consisting of fibrous material, forms the "architecture" of plant cell walls [13], [17]. The plant cell walls' three dimensional structure, along with the complex interactions among different components, are all responsible for the complexity in the degradation of lignocellulose via enzymes [13], [26]. Three main structural components make up lignocellulose: cellulose, hemicellulose and lignin, where cellulose and hemicellulose are the main components for bio-ethanol production [24], [27]. Minor components that make up the remaining fraction within lignocellulosic biomass include proteins, pectins, lipids, minerals, soluble sugars, extractives and ash (inorganic material), which all have an influence on downstream processing [18], [24]. The proportion of the main structural compounds varies depending on the type of feedstock, as it can be observed in Table 3.

Table 3: The composition of different lignocellulosic raw materials (% dry weight).

Raw Material	Glucan	Mannan	Galactan	Xylan	Arabinan	Lignin	Reference
Agricultural residues							
Corn stover	34.4	0.6	1.4	22.8	4.2	17.2	[28]
Herbaceous crops							
Switchgrass	35.2	0.2	0.9	21.7	2.8	27.4	[29]
Sugarcane bagasse	41.3	0.3	0.5	21.8	1.8	23.4	[29]
Wheat straw	23.3	-	-	19.0	3.0	-	[30]
Sorghum straw	22.0	-	-	16.9	3.7	-	[30]
Arund donax	17.7	-	-	23.3	2.2	-	[30]
Sugarcane tops	22.3	-	-	18.7	3.1	-	[30]
Sweet Sorghum bagasse	35.1	0.9	1.5	19.4	1.4	18.6	[31]–[33]
Sweet Sorghum bagasse (MSJH16)	37.9			18.6	1.0	18.3	Chapters 6 and 7
Triticale straw	32.2	0.4	1.1	19.3	2.3	15.0	[36]
Triticale straw (EliteM13)	35.6			17.0	1.7	17.4	Chapters 6 and 7
Hardwoods							
Oil mallee	10.6	-	-	3.4	5.0	-	[30]
Eucalyptus	8.9	-	-	14.6	0.3	-	[30]
Hybrid poplar	43.8	3.9	1.0	14.9	0.6	29.1	[28]
Yellow poplar	44.7	1.8	0.4	17.1	0.3	23.9	[37]
Red maple	41.9	1.8	0.6	6.2	0.3	30.2	[37]
Softwoods							
Spruce	45.2	12.1	2.0	5.4	0.7	27.9	[38]
Pine	10.6	-	-	19.7	1.5		[30]
Douglas-fir	44.7	12.9	2.8	4.9	1.6	28.4	[31]

Cellulose comprises the largest fraction of the sugars present in lignocellulose, where glucose $(C_6H_{10}O_5)_n$ is the preferred carbon source for many micro-organisms [5], [39]–[41]. It

is a linear bio-polymer of cellobiose containing 30 - 60 % of the total amount of feedstock dry matter and consists of two D-glucose monomers that are linked to each other by β-1,4 bonds [13], [39], [42], [43]. Cellulose provides rigidity and strength to the cell wall due to the orientation of the linkages and the presence of additional hydrogen bonds [39], [42]. This contributes to the difficulty of breaking the structure thereof [5], [39].

Cellulose is usually in close contact with hemicellulose and lignin [44]. It mainly consists as a bundle of fibrillar units in a supramolecular structure of amorphous and crystalline regions, where in the latter nearly all the water is excluded [43]–[45]. Crystalline regions are typically several sheets of cellulose chains that are linked in a tight manner by intra- and intermolecular hydrogen bonds [44]. Amorphous cellulose is degraded rapidly to cellobiose in contrast to the saccharification of the crystalline cellulose which is slower [44]. The degradation rate, however, depends on cellulose crystallinity as well as the degree of polymerisation (DP), as more crystalline cellulose tends to be less degradable by enzymes and have deceased solubility [39], [44].

Hemicelluloses, in contrast, are non-cellulosic polymers of hexoses (glucose, mannose, galactose), pentoses (xylose, arabinose) and acetylated sugars [18], [27], [39], [46]. This component comprises 20 - 40 % of the total lignocellulose [5], [13]. Unlike cellulose, they have shorter chains and main chain molecules that are highly branched [39]. The composition and frequency of the branches depends on the specific xylan source [46], [47]. All of these features make the structure thereof more easily hydrolysable than that of cellulose [39]. Biomass obtained from herbaceous materials has similar components of hemicellulose to that of hardwoods, but only with the presence of lower amounts of acetyl groups [39].

Hemicellulose consists largely of aldopentoses (xylose, mannose and arabinose) and has glycans that are cross-linked [43], [48], [49]. They are able to coat the microfibrils, but can also span the distance between these microfibrils, linking them together in order to form a network [43]. Herbaceous biomass is high in hemicellulose, such as 5-carbon (5C) hemicellulosic xylose/arabinose sugars [50]. The hemicelluloses from hardwoods, grasses as well as agro-industrial by-products, are mainly composed of xylans (β-(1-4)-linked D-xylopyranoside monomer units) which form the majority of hemicellulose chains [11], [40], [46], [47]. Hardwoods may also contain glucomannans [40]. Contrary to these,

softwood hemicelluloses consist primarily of glucans such as arabinogalactans and xyloglucans and xylans such as glucomannans and arabinoglucuronoxylans [40]. Figure 2 illustrates the chemical structure of the xylans present in grasses, softwoods and hardwoods [11].

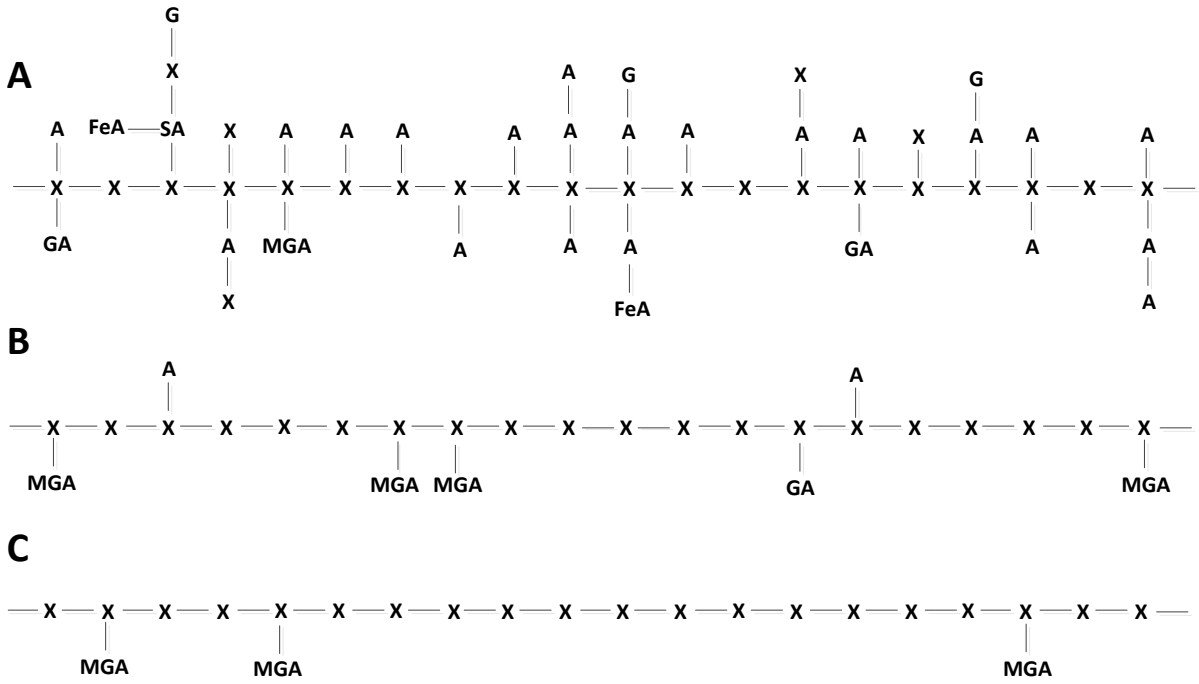


Figure 2: A representation of the chemical structure of different xylans, reflecting the major side-chains and linkages: A) grasses and cereals, B) softwood, and C) hardwood. (X - xylose, G - galactose, A - arabinose, GA – glucuronic acid, MGA – 4-*O*-methyl-glucuronic acid, FeA – ferulic acid) (Figure adapted from Deutshmann *et al.* 2012 [11]).

Pectins are hydrophilic polysaccharides that make out part of the intracellular network and convey elasticity to the cell by means of its association with water. These pectins vary highly in nature and have galacturonic acid as their main component [26].

Lignin is a very complex, phenolic, aromatic polymer that consists of phenylpropanoid units such as sinapyl alcohols that form the main component within the lignin network [13], [42], [43], [51]. It also provides rigidity to the structure of the cell wall and accounts for 15 - 25 % of the total amount of lignocellulose that is present [13], [42]. The basic units of lignin are closely bound to cellulose and hemicellulose by various linkages which forms part of a very complex matrix [39]. An impermeable barrier thus exists which prevents enzyme activity and thereby the enzymatic degradation of the cellulose and hemicellulose fractions [5], [39].

Generally, softwoods have the highest content of lignin and herbaceous crops, such as grasses, have the lowest lignin content (Table 3) [52]. Lignin is the most abundant non-carbohydrate constituent within the lignocellulosic material [43]. This, however, presents a major problem during biomass conversion as the physical structure of natural lignocellulose is largely resistant to attacks by enzymes, particularly cellulose, which is even further protected by the surrounding matrix of hemicellulose, lignin and pectin [43], [53], [54]. Lignin cannot be used for biochemical conversion into ethanol, but can still be used afterwards to supply the required energy for the process (combustion to generate electricity and process streams) [2].

The proportion and composition of the structural compounds will vary between feedstocks, tissues, etc. [2]. Generally the plant cell wall is divided in a middle lamella, primary wall and secondary wall [2]. In plants, cellulose and hemicellulose are closely packed in the primary cell wall and are linked to lignin which is situated in the secondary cell wall [55]. The hemicellulose and lignin fractions act as physical barriers for the cellulolytic enzymes that are employed during enzymatic hydrolysis and thus prevent the access of hydrolytic agents to cellulose due to the partly covalent association between lignin and hemicellulose [25], [55].

Figure 3 illustrates how these three main components fit into the framework of lignocellulosic biomass.

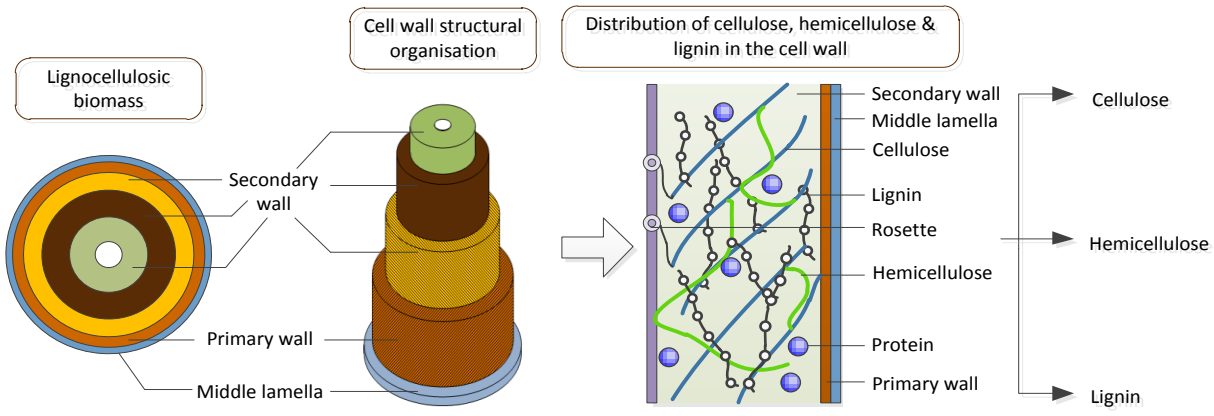


Figure 3: A diagrammatic representation of the framework of lignocellulosic biomass (Figure adapted from Sarkar *et al.* 2012 [2]).

The choice of raw material used depends mostly on biomass availability, economic issues as well as environmental issues [56]. The ethanol yield, however, varies depending on the origin of the specific biomass, the pretreatment method that is applied as well as the fermentative organism that is used [3].

The two feedstocks that will be focussed on during this study are the herbaceous energy crops triticale straw (TS) and sweet sorghum bagasse (SSB) due to their ability to provide a chance for reduced fossil fuel dependence [7]. The compositional analysis of triticale and sorghum lignocellulosic residues indicate that they contain between 60 and 72 % polysaccharides and have a 15 - 25 % lignin content, which is relatively low and makes them good candidates for the production of cellulosic ethanol through appropriate pretreatment, hydrolysis and subsequent fermentation [57], [58]. The interesting properties of these two feedstocks are described next.

2.2.1 Triticale straw

Triticale is a cereal hybrid that is produced by the crossing of wheat (*Triticum*) and rye (*Secale*) [58]. The use of it as feedstock is advantageous as it only has a 2 - 5 % lower starch content than wheat, which is considered the main raw material for the production of fuel alcohol in Canada [58], [59]. The price of triticale is significantly lower than wheat, due to reduced input costs and higher yields per hectare and thus holds the advantage of remaining a competitive feedstock [58], [60], [61]. About 0.51 ton triticale straw is produced per ton of triticale grain [62]. The interest in the straw is therefore to use the whole plant (not just the starch grain) for the production of ethanol through 2G technology.

Triticale presents additional advantages such as a high grain yield in unsuitable conditions, their resistance to soil-climatic conditions, tolerance to dryness, more acid soils and their low requirement of nutrient substances [60]. It also has no need for as much fertilizer as required by other types and varieties that provide the same yield [60], [61]. Triticale has a low susceptibility to diseases as well as pests that attack wheat and rye, resulting in a reduced necessity of chemical protection against any agents that can be seen as harmful [60], [63].

Replacing wheat with cheaper crops such as triticale for the production of fuel alcohols, provides an attractive alternative while wheat prices increase and thereby also provide good economic opportunities [58].

2.2.2 Sweet sorghum bagasse

Sorghum is a tropical grass that is primarily grown in the drier, semiarid parts of the world, especially those areas that are too dry to grow corn [27]. Sweet sorghum (*Sorghum bicolor* variety *saccharatum*) is a large crop specie which belongs to the C4 family (which represents a specific group of plants that photosynthesise via the phosphoenol pyruvate carboxylase pathway). This also entails having a high resistance to drought and therefore covers both winter and summer cropping cycles [57], [64]. Sorghum is considered a multi-purpose crop which can be cultivated under a variety of environmental conditions [65]. Sorghum also has reasonable potential in terms of production costs and productivity [13].

Often compared to sugarcane, sorghum is an attractive specie and is attracting great interest due to the versatility thereof, its tolerance to abiotic stresses such as water logging, salinity, alkalinity and drought, as well as its ability to grow on land that is unsuitable for most feedstocks [7], [32]. Sorghum is specifically more suited to warmer climates and can produce equal amounts, if not more, biomass than maize while using up to 33 % less water [7], [66]. Sorghum can also be grown on a variety of soils, including all soils that range from light sands to clay, preferably with a pH above 5.8 [7], [67], [68].

The drawbacks of sorghum are the fast deterioration thereof after harvest, difficult sugar crystallisation, difficulty with transportation, the short harvest season thereof and the fact that it has lower sugar content than sugarcane [7], [13].

However, because of its high biomass yield, high amounts of fermentable sugar per unit and low input requirements, sweet sorghum is one of the most promising herbaceous energy crops that exists as they provide opportunities for reducing fossil fuel dependence [7], [65]. Sorghum bagasse is a by-product from sugar extraction for 1G ethanol production. This bagasse can be used for the production of 2G bio-ethanol. Approximately 0.46 ton sorghum bagasse is produced per ton of harvested sorghum grain [69]. There is thus a tremendous interest to use the whole plant for ethanol production through 2G technology, rather than only the starch grain. Sorghum bagasse is therefore an excellent feedstock for the production of ethanol while also being perfectly suited for use in a bio-processing plant concept [64]. With the continuous development of biomass energy, herbaceous energy crops hold the potential of becoming a primary component of the global energy mix [7].

2.3 Cellulosic Ethanol

The biochemical conversion of biomass, which will be focussed on during this study, is advantageous as it is able to preserve the original carbohydrate structures in monomeric sugar form [24]. In contrast, thermochemical conversion damages the structure of the carbohydrates [24]. Enzyme technology is also generally accepted as the viable and ecological technology for saccharification [24]. The transformation of lignocellulose into bio-ethanol in an enzyme-based process requires several steps [13]:

1. Collection of lignocellulosic biomass
2. Pretreatment (to disrupt the lignocellulosic structure)
3. Hydrolysis (for obtaining sugars from cellulose and hemicellulose)
4. Fermentation (for the conversion of sugars into bio-ethanol)
5. Distillation and ethanol purification

These main process steps are illustrated in Figure 4.

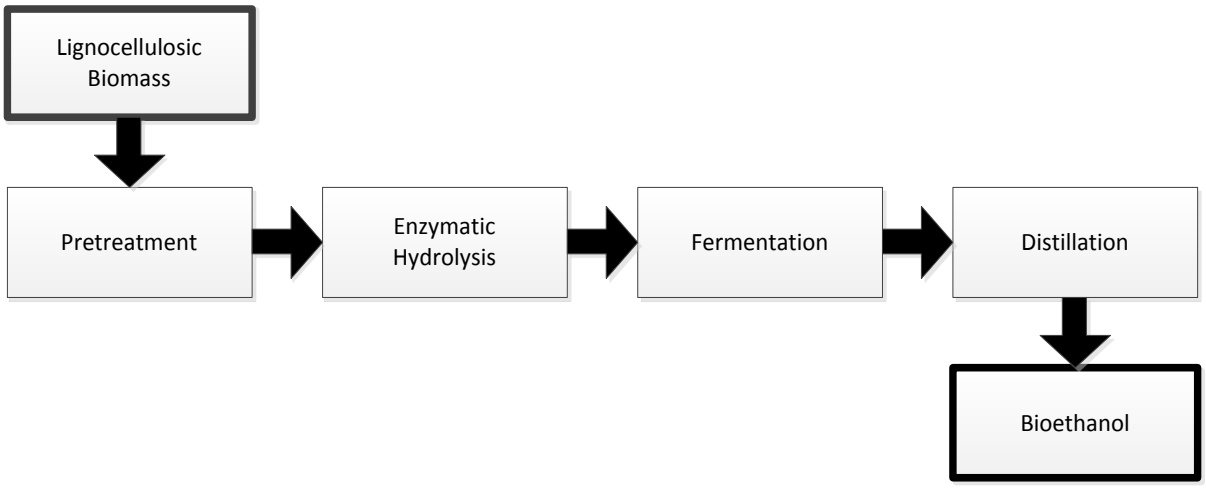


Figure 4: A simplified flow sheet for the production of ethanol from lignocellulosic biomass. During the pretreatment step, the material is prepared for the second step; enzymatic hydrolysis. Subsequent to hydrolysis, the sugars are fermented into ethanol. Lastly, the ethanol that is generated has to be isolated through the process of distillation (Figure adapted from Waldron et al.2010 [41]).

When obtaining an appropriate lignocellulosic biomass, the material is firstly subjected to a pretreatment step, as will be discussed in more detail in section 2.4. The main function of the pretreatment process is to disrupt the crystalline cellulose structure and to break down the structure of lignin [70]. Maximal fractionation of the different structural

components of the feedstock makes the cellulose more amenable to the enzymes and leads to higher sugar yields [4], [70]. Figure 5 illustrates how pretreatment and hydrolysis of the lignocellulosic materials should first be performed in order to obtain monomeric sugars that will be transformed to ethanol during the subsequent fermentation step.

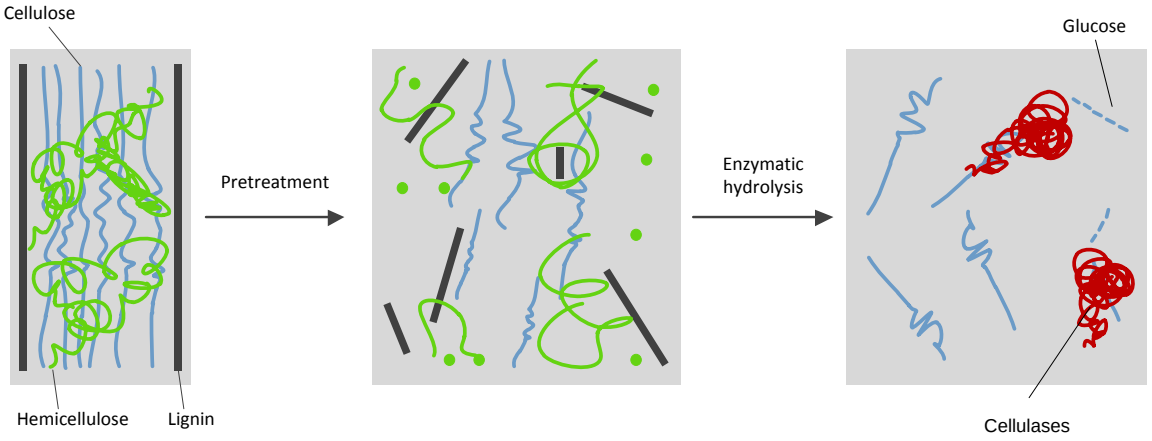


Figure 5: A general enzyme-based ethanol production scheme representing the goals of pretreatment and enzymatic hydrolysis on lignocellulosic material. Hemicellulose and lignin are solubilised while the decomposition of the plant cell wall's structural network takes place (Figure adapted from Corredor *et al.* 2008 [55]).

Each pretreatment method implies a different effect on the respective cellulose, hemicellulose and lignin fractions [70]. Different pretreatment conditions (impregnation, temperature, time) and methods should thus be selected based on the specific process configurations that will be applied for the hydrolysis and fermentation steps that follow [70]. These are also discussed in more detail in the pretreatment section 2.4. The most important factors to consider during pretreatment are as follow [17]:

1. The production of sugars (sugar yield)
2. Avoiding the loss/degradation of sugars once the polymers are hydrolysed
3. Limiting the formation/release of inhibitory compounds that could influence downstream processing
4. Reducing the energy requirement (heat and power)
5. Minimising the costs that are involved

Hydrolysis represents the key step responsible for converting the carbohydrate polymers within the lignocellulosic biomass into simple monosaccharide sugars after pretreatment [5]. Efficient enzymatic hydrolysis increases the effectiveness of the process

and radically reduces the cost of enzymes [14]. Cellulose is typically hydrolysed by a group of enzymes termed cellulases [5]. Although most of hemicelluloses are solubilised by the majority of pretreatments, the application of hemicellulases could contribute to remove residual hemicellulose and therefore enhance the cellulose conversion.

The hydrolysis reaction for cellulose conversion into sugar polymers is represented in reaction 1:

$$(C_6H_{10}O_5) + nH_2O \rightarrow nC_6H_{12}O_6 \quad (1)$$

Hydrolysis of cellulose involves the synergistic action of three types of cellulases: endo-glucanases (EG), exoglucanases and β-glucosidases (BG) [5]. These interactions are represented in Figure 6. Endo-glucanases cuts the cellulose chains at random positions where after the exoglucanases (CBH - cellobiohydrolases) cleave off cellobiose units from the new ends that were made available [71]. The β-glucosidase enzymes then hydrolyse the released cellobiose units into glucose [71]. These cellulolytic enzymes act in three steps: adsorption to the cellulose surface area, biodegradation of the material and desorption [14].

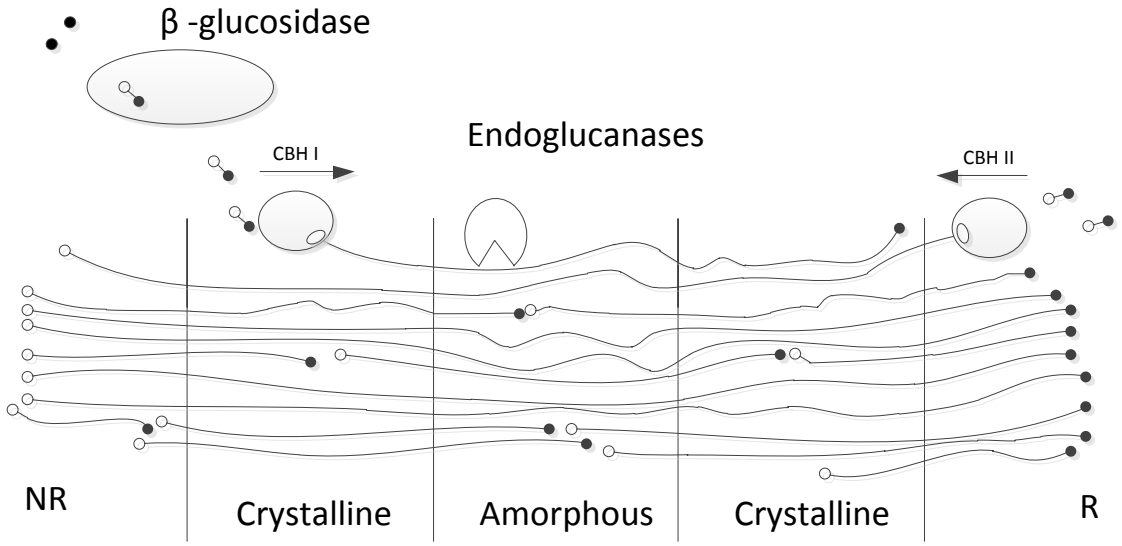


Figure 6: The main enzymes involved in the degradation of cellulose (Figure adapted from Jeon *et al.* 2010 [72]).

Hydrolysis generally yields hydrolysates that contain both hexoses (six-carbons) and pentoses (five-carbons) sugars [5]. These sugars are then subjected to the next step, being

fermentation. During fermentation, the micro-organism that is used, ideally has to fulfil certain requirements [4], [17]:

1. Utilisation of a broad range of substrates, including pentose sugars
2. High productivity and subsequent ethanol yield
3. The ability to withstand high ethanol concentrations as well as high temperatures
4. High tolerance to inhibitors and/or inhibitory products that are present within the hydrolysate

Saccharomyces cerevisiae represents the most commonly used micro-organism (yeast) that is used for fermentation as it has proved to be robust and well suited for fermenting lignocellulosic hydrolysates [5]. The bacterium *Zymomonas mobilis* has also been used for ethanol production from hexoses [14]. These organisms are both able to produce high ethanol yields (90 – 97 % of the theoretical yield) and tolerate high ethanol concentrations (10 % $v.v^{-1}$) [14]. Since there is a significant amount of hemicellulose, the fermentation of hemicellulose-derived sugars, mainly xylose, would contribute to increase the final ethanol concentration. Therefore, challenges in the fermentation step involve the co-fermentation of glucose and xylose along with the use of recombinant microbial strains [17]. Each of the aforementioned steps will be described in more detail in the sections that follow.

2.4 Pretreatment

The recalcitrance of the lignocellulosic biomass requires the application of a pretreatment step that breaks its recalcitrant structure and thereby enhances the enzymatic hydrolysis of the cellulose fraction [4], [15]. An overall consensus exists that a successful pretreatment method should [4], [13], [25], [41]:

- Maximise the surface area of the biomass
- Increase the material's porosity
- Disrupt cellulose crystallinity and the lignin barrier in order to ease enzymatic attack of the cellulose chains
- Increases the amorphous fraction of the cellulose (which is the preferred form for enzymatic attack)
- Maximise enzymatic conversion

- Minimise sugar degradation from cellulose and hemicellulose
- Maximise valuable by-products production, e.g. lignin
- Minimise the use of chemicals, energy and capital equipment
- Limit the production of toxic inhibiting products that interfere with enzyme function or fermentative micro-organisms

Pretreatment makes changes in the macroscopic and microscopic structure and size of the specific biomass and also alters the sub-microscopic structure and chemical composition [17]. It generally increases enzymatic digestibility by breaking down the macroscopic rigidity of the lignocellulosic biomass as well as decreasing the physical barriers that exist by solubilising and depolymerising the hemicelluloses to monosaccharides and oligosaccharides [73], disrupting the cellulosic crystal structure [74] or by breaking the lignin seal [45], [75]. However, if pretreatment is too harsh, the sugars can be degraded to compounds that are enzyme- and yeast- inhibiting [25]. This would then lead to a lower overall yield and productivity [15]. Alternatively, if the pretreatment conditions are too weak, it will result in low enzyme accessibility [15]. This will then eventually lead to a low overall yield and slow hydrolysis process [15]. A compromise thus exists between enhancing sugar yields from both the carbohydrate/polysaccharides while minimising the formation of inhibitors by restricting the severity of the pretreatment [15].

The availability of accessible surface area is another very important factor during pretreatment as it affects the efficiency of the subsequent enzymatic cellulose degradation [15]. Pretreatment increases available surface area in more than one way: by the formation of fragments and cracks, hydrolysis of the hemicellulose fraction to diminish shielding effects, structural changes in lignin and delignification of the wood [15]. Shielding of microfibrils and blocking of pores by lignin can thus be removed. Substrate crystallinity and the DP are also factors that are believed to have an influence on digestibility during the enzymatic and fermentation steps [15].

The choice of an appropriate pretreatment method and its conditions will differ for specific biomass feedstocks and remain to be a compromise between minimising cellulose and hemicellulose component degradation, while simultaneously maximising the enzymatic hydrolysis of cellulosic substrates [15], [46]. A variety of pretreatment methods are available, e.g. physical (e.g. grinding or milling), chemical (e.g. dilute acid or alkali), physico-

chemical (e.g. steam-pretreatment/autohydrolysis), biological or combinations of all of the above [13], [15]. In Table 4 the advantages and disadvantages of some of these pretreatment methods are indicated.

Table 4: Advantages, disadvantages and limitations of a variety of pretreatment processes used for lignocellulosic biomass (Table adapted from Balat *et al.* 2011 [5]).

Pretreatment Process	Advantages	Limitations and Disadvantages
Mechanical Comminution	- Reduction in the crystallinity of the cellulose	- Higher power consumption than the inherent biomass energy
Steam-Explosion (SE)	- Hemicellulose solubilisation; - Lignin transformation; - Cost-effective	- Destructs a portion of the xylan fraction; - Incomplete disruption of the lignin-carbohydrate matrix - Not effective in softwoods unless a catalyst is added
Ammonia Fiber-Explosion (AFEX)	- Increases the accessible surface area; - Removes lignin and hemicellulose to an extent; - Produces no inhibitors for down-stream processes	- Not suitable for biomass with a high lignin fraction
CO_2 Explosion	- Increases the accessible surface area; - Cost-effective; - No formation of inhibitory compounds	- No modification of hemicelluloses or lignin
Ozonolysis	- Reduces the lignin content; - Produces no toxic residues	- Requires a large amount of ozone; - Expensive
Acid Hydrolysis	- Hydrolysis of hemicellulose to xylose and other sugars; - Alters the lignin structure	- High cost; - Corrosion of equipment; - Formation of toxic substances
Alkaline Hydrolysis	- Removes hemicelluloses and lignin; - Increases the accessible surface area	- Requires long residence times; - Formation of irrecoverable salts which is incorporated into biomass
Organosolv	- Hydrolyses hemicelluloses and lignin	- Solvents need to be drained from the reactor, evaporated, condensed as well as recycled; - High cost
Pulsed Electrical Field (PEF)	- Ambient conditions; - Disrupts plant cells	- More research needed to make process efficient
Biological	- Simple equipment degrades hemicellulose and lignin fractions; - Requires only low energy inputs	- Very low rate of hydrolysis

Physical methods include chipping, grinding or milling of the biomass [13]. The surface area is thereby increased and decrystallisation of the cellulose is improved [13], leading to an overall increase in cellulose digestibility [71].

Chemical pretreatment involves soaking the material in concentrated or dilute acid and then heating it to high temperatures for several minutes [13]. Generally, acids such as dilute

sulphuric acid (H_2SO_4) and dilute hydrochloric acid (HCl) are used during pretreatment [2], [76]. As dilute H_2SO_4 is inexpensive and very effective, this acid have been studied the most during pretreatment and is commercially used in a wide variety of biomass types [2], [13]. This method is similar to SE in terms of chemical changes in the lignocellulose [77].

Micro-organisms such as white-, brown- and soft-rot fungi are used during biological pretreatment to solubilise hemicellulose and degrade lignin [5]. Although the hydrolysis rate during the process is slow, this method of pretreatment has only mild environmental conditions and low energy requirements [5].

Treatment with an alkali (e.g. ammonia) requires lower pressure, temperature and residence time compared to acids [13]. The material is soaked in an alkaline solution and then heated. This leads to swelling of the pores in the material which increases the internal surface area while decreasing the degree of crystallinity and polymerisation. This type of pretreatment breaks the C-O-C bonds that exist between lignin and carbohydrates along with other additional ether and ester bonds in the lignin-carbohydrate complex [1], [13]. The lignin structure is thereby disrupted and the majority of the lignin removed while the accessibility of the material to the enzymes increase [13], [64]. Alkaline pretreatment is effective in agricultural residues and herbaceous crops as they contain less lignin in general [13]. The cost of alkali pretreatment is, however, so high that this type of pretreatment is not competitive for use in large-scale production plants [25].

Pretreatment with liquid hot water (LHW) is classified under physico-chemical pretreatment [46]. An advantage of this type of hydrothermal pretreatment is that, except for water, no chemicals are added, thus making the whole process environmentally friendly [46]. Another advantage is the conversion of hemicelluloses into hemicellulosic sugars with high yields and low formation of by-products [46].

In the conversion of biomass to fermentable sugars, pretreatment of biomass has been labelled as one of the most expensive processing steps [5]. Pretreatment is therefore seen as possibly the most crucial step during the biological conversion to ethanol, having a large effect on all the other process steps that follow [13], [70]. Dilute acid at small scale was used in the project for screening and selection of the varieties for further pretreatment optimisation at pilot scale. Lignocellulose from selected varieties of triticale and sorghum were steam-pretreated under optimum conditions to generate the substrate used for

enzymatic hydrolysis during this study. These pretreatment methods are discussed in the sections below.

2.4.1 Dilute acid pretreatment

Among chemical pretreatments, dilute acid hydrolysis is one of the most widely applied methods, allowing the recovery of a high portion of hemicellulose sugars [78], [79]. Dilute acid pretreatments using either sulphur dioxide (SO_2) or H_2SO_4 are the most investigated [15]. These methods have already been applied in pilot plants and are therefore very close to commercialisation [15]. Other types of acid include nitric acid, hydrochloric acid and phosphoric acid [17], [25].

The acid medium mainly attacks the hemicelluloses (polysaccharides), which are easier to hydrolyse than the celluloses [25]. The cellulose and lignin fractions are thus kept fairly constant in the solid fraction which can be processed further [25]. The resulting liquid fraction of the hydrolysate usually consists of sugars (both hexoses and pentoses), hemicellulose decomposition products (oligomers) and monosaccharide decomposition products (furfural and 5-hydroxymethyl-2-furaldehyde (5-HMF)) [25].

Advantages of acid catalysed treatments, in particular dilute H_2SO_4, include the improvement of hemicellulose removal and partial hydrolysis of cellulose [13], [15]. Another advantage or this type of pretreatment is the successful recovery of hemicellulose-derived sugars [25].

A problem that is often associated with dilute acid hydrolysis is the toxicity of the hydrolysis for fermentative micro-organisms [18]. Disadvantages of this pretreatment also include relatively low sugar concentrations because of the low solids loading in the reactor and the degradation of sugars into furans [25].

2.4.2 Steam-explosion pretreatment

Hydrothermal pretreatment, which includes Steam-explosion (SE), have been extensively studied as it satisfies most of the requirements that are needed from an efficient pretreatment process [80].

During SE, high-pressure (20 – 50 bar) steam heats up the lignocellulosic material to temperatures between 160 – 290 ˚C [17], [71]. This could take from several seconds up to 15 minutes [71]. The steam condenses into water at high pressure and thereby impregnates

the material [17], [66]. The steam thus acts as a catalyst that releases other organic acids (mainly acetic acid) that together catalyse the hydrolysis of hemicellulose [17], [66]. After the residence time, a drastic change in pressure to atmospheric pressure transforms the water back into steam which exhibits a mechanical effect on the fibers, breaking open its structure [17], [66]. SE is thus a thermochemical-physical pretreatment.

This pretreatment combines mechanical forces with a chemical effect as a result of the hydrolysis of acetyl groups within the hemicellulose fraction [39]. Promotion of organic acid formation from the acetyl groups takes place because of the high temperatures [71]. This causes autohydrolysis [71]. The hemicelluloses also hydrolyse and solubilise partially due to the sudden change in the pressure [71]. This exposes the surface of the cellulose and increases the accessibility of the cellulose microfibrils to the enzymes [41]. During pretreatment, lignin is only removed from the material to a limited extent as it is rather disrupted and redistributed on the surface of the fiber due to depolymerisation/re-polymerisation and melting reactions [41], [71], [81]. This aids in facilitating enzyme access to cellulose fibers [81].

High temperatures may also result in the degradation or increased removal of hemicelluloses, while solubilising and transforming the compounds that are lignin-related to chemicals which may inhibit downstream processes [71], [81]. Improved cellulose digestibility and promotion of sugar degradation also takes place [71]. During steam-pretreatment, it is thus important to obtain a balance during pretreatment severity in a way that solubilisation of the hemicellulose component takes place, but minimising degradation of any sugars along with it [28], similar to other pretreatments such as dilute acid [77]. Advantages of steam-pretreatment include [28]:

- short reaction times
- the limited use of chemicals
- low consumption of energy
- reduction in the formation of sugar degradation products, such as furfurals
- higher particle sizes required (which reduces further reduction on the pre-processing of the raw material)

Steam-explosion, without the addition of a catalyst, is a promising method of pretreatment, yielding xylitol, levulinic acid as well as alcohols [17], [82], [83]. This pretreated method is also made more economically attractive by the recovery of xylose (45 - 65 %) [17], [83], [84].

Even though SE has been successfully used for the pretreatment of agricultural residues and hardwoods, it is not very effective in softwoods [71]. Because of the formation of by-products, the conditions that promote digestibility also do not give a high recovery of hemicellulose-derived sugars [71]. SO_2-catalyzed steam-pretreatment, in contrast, was found to be effective for use on softwoods, hardwoods as well as agricultural residues [46], [78]. Hemicellulose recovery and a reduction in the formation of sugar degradation products could be improved as impregnation of SO_2 prior to pretreatment resulted in lower temperatures during pretreatment along with shorter reaction times [46], [85].

With the addition of an acid, such as SO_2 as catalyst during SE, hydrolysis can be improved as acid-catalysed steam-pretreatments have been shown to generate high sugar yields [15]. Increased accessibility of enzymes to cellulose, increased hemicellulose hydrolysis and decreased production of degradation products from the sugars are some of the advantages when using an acid catalyst [18], [71]. In general, lignocellulosic materials can be impregnated with SO_2 or H_2SO_4 before being subjected to SE [71]. The use of SO_2 is preferred as this gas can penetrate the wet material faster and more easily and does not result in significant equipment corrosion problems, compared to the use of H_2SO_4. The use of SO_2 is also advantageous in the hydrolysis of softwood as it results in sugars with a hydrolysate that is easy to ferment [71].

A pretreatment method such as SO_2-catalysed steam-pretreatment that hydrolyses a high percentage of the hemicellulose fraction to their monomeric sugars is important due to their potential utilisation in fuel production [46]. A major drawback when using this catalyst is the high toxicity involved as it poses both a health and safety risk [5]. Today, however, SO_2 is used in various industrial processes by means of well-established techniques [5].

A two-step method of steam-pretreatment has also been suggested as a way of increasing sugar recovery [5], [39]. The first step injects steam at a low temperature in order to solubilise the fraction of hemicelluloses [5]. The second step is then performed by subjecting the remaining cellulosic fraction to SE at a higher temperature (> 210 ^{o}C) [5]. With

this pretreatment method higher yields are obtained, lower doses of enzymes are required during enzymatic hydrolysis and better use of the raw materials is applied [5].

The pretreatment efficiency is evaluated in terms of overall sugar yield (recovery during pretreatment and enzymatic hydrolysis) and inhibitors formation. Table 5 presents examples of conditions and sugar yields obtained when applying water- or SO_2-impregnated SE on sorghum bagasse. For comparison, this table also contain the pretreatment conditions, sugar yield and digestibility obtained for sorghum bagasse and triticale straw in this study.

Table 5: Sugar yields of water- and SO_2-impregnated Sorghum bagasse and Triticale straw in g.100 g^{-1} dry weight ($w.w^{-1}$).

	Temperature (˚C)	Time (min)	SO_2-impregnation (% $w.w^{-1}$)	Glucan (% $w.w^{-1}$)	Xylan (% $w.w^{-1}$)	Reference
Sorghum bagasse	190	5	0	54.0	14.9	[31], [57], [86]
	200	5	2	55.7	13.2	
	205	10	2	69.6	2.3	
	210	5	0	61.8	5.3	
Sorghum bagasse (MSJH16)	200	5	0	52.4	9.4	Chapters 6 and 7
Triticale (EliteM13)	190	5	0	46.6	14.5	Chapters 6 and 7

2.5 Inhibitor Formation

Inhibitory compounds that are formed during acid-based, uncatalysed SE and LHW pretreatments impact on downstream processes, which include enzymatic hydrolysis and fermentation [87]. These compounds are generally formed by hemicellulose, lignin and sugar degradation [87], due to high temperatures and acid treatment of lignocellulosic substrates [71]. These inhibitors can be grouped, based on their chemical structure, into furans, phenolic compounds and organic acids [15], [81]. Organic acid [88], sugar products [89], [90], ash [91] and phenols [92] have shown to inhibit the activity of cellulase to a certain extent [4]. Xylo-oligomers (XOS) are also strong inhibitors of the activity of cellulase [4]. The

removal of these inhibitory compounds is therefore of major importance in order to improve the performance of the enzymes and the fermentation [4].

The harshness of the pretreatment process determines the specific inhibitors that are present and their concentration [71]. Primary inhibitors include furfural, 5-HMF and acetic acid [71]. An increase in the harshness of pretreatment can also promote the formation of additional inhibitors such as levulinic and formic acid which are termed secondary inhibitors [71]. The formation of these inhibitors is illustrated in Figure 7.

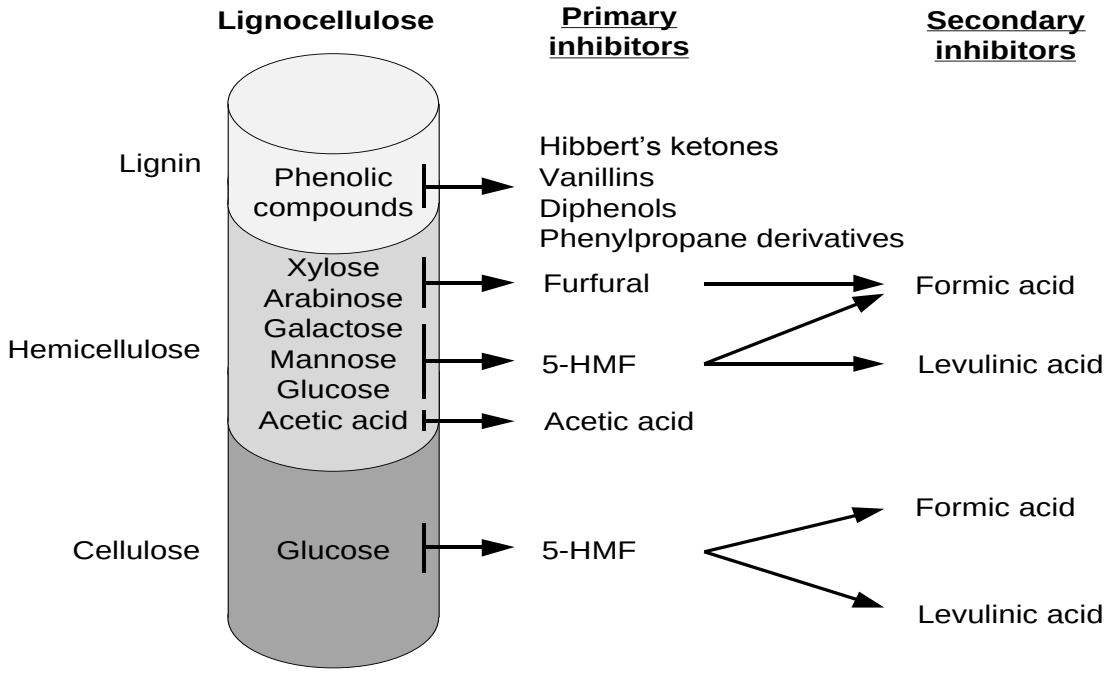


Figure 7: The generation of inhibitory compounds during hydrolysis (Figure adapted from McIntosh et al. 2010 [71]).

The mechanism by which inhibition takes place mainly depends on the inhibitors and vary between enzymes and micro-organisms [71]. It is thus difficult to predict the strength and effect of inhibition as it depends on the specific raw material, pretreatment method, hydrolysis process and the micro-organism that is used [15], [71]. Moreover, the use of high solid loadings during subsequent hydrolysis fermentation, required to reach ethanol concentrations of at least 4 % ($v.v^{-1}$), imply higher concentration of these inhibitors in downstream processes. When selecting a pretreatment method that is appropriate for use in a given feedstock, these parameters should be taken into consideration, together with the

enzyme tolerance and robustness of the fermentative micro-organism [87]. Table 6 shows the concentration of inhibition and XOS [90] of the liquid fraction obtained after SE pretreatment of sweet sorghum bagasse and triticale straw used in this study.

Table 6: Summary of the inhibitors (monomeric form) present within the liquid fraction of pretreated sweet Sorghum bagasse and Triticale straw in this study.

	By-products ($g.L^{-1}$)				XOS* ($g.L^{-1}$)
	Acetic acid	**Formic acid**	**Furfural**	**5-HMF**	
Inhibitory concentration	> 0.5 - 9.0	> 0.5 - 2.7	> 3.0	> 2.0	1.7
Sweet Sorghum bagasse	5.29	1.40	0.59	0.19	39.36
Triticale straw	1.31	0.31	0.12	0.04	23.88

* The concentration of the XOS is a rough estimation based on post-hydrolysis of the liquid fraction
Standard deviation less than 5 %

Two of the most common furans (furaldehydes), furfural (2-furaldehyde) and 5-HMF, are formed from pentoses and hexoses, respectively [15], [81]. 5-HMF is the main degradation product of glucose whereas furfural is the main product of the thermal degradation of xylose during hydrolysis [45], [71]. Degradation products such as furfural can react with the lignin fraction of the material and form new C-C bonds, which leads to the formation of pseudo-lignin (condensation products) [45]. With an increased amount of condensed lignin within the solid fraction, the enzymatic digestibility can also be reduced by adsorbing proteins and obstructing hydrolysis [45], [93]. Moreover, at concentrations higher than 1 $g.L^{-1}$, furfural causes a decrease in fermentation (and thus ethanol production), the total viable cell number in *S. cerevisiae* as well as in cell multiplication [71]. 5-HMF is closely related to furfural and concentrations higher than 2 $g.L^{-1}$ are able to decrease biomass yield by 23 % as well as the protein content in *S. cerevisiae* [71].

Numerous variants of phenolic compounds can also be found, depending on the type of lignin [15]. Phenolic (aromatic) compounds are present in diverse forms and originate from lignin degradation [71]. The concentrations thereof increase along with the harshness of hydrolysis, but then seems to level off [71]. These compounds, even at low concentrations, have shown to inhibit cell growth without having an effect on the ethanol yield in *S. cerevisiae* [71].

Organic (weak) acids, also known as low molecular weight acids, mostly include compounds such as acetic, formic and levulinic acid [15], [81]. Acetic acid is released from acetyl groups within the hemicellulose fraction during the course of hydrolysis whereas formic and levulinic acid is the result of the further degradation of furfural and 5-HMF [39], [71], [81]. *S. cerevisiae* is inhibited by concentrations of formic acid > 0.5 - 2.7 $g.L^{-1}$ and acetic acid > 0.5 – 9.0 $g.L^{-1}$ [1], [94]. At these concentrations the above mentioned inhibitors interfere with the functions involved in cell maintenance [1], [94].

Besides the products originated from pretreatment, the products formed during enzymatic hydrolysis (oligomers, glucose, cellobiose, xylose, and acetic acid from residual hemicellulose) and fermentation (ethanol, glycerol, lactic acid) act as inhibitors of the enzymes and micro-organisms [71]. The end-product inhibition can be overcome by applying a simultaneous saccharification and co-fermentation (SSCF). Different enzymes and micro-organisms have different tolerances to these inhibitors. For example, an enzyme preparation with enough beta-glucosidase and xylanase activity would be less susceptible to cellobiose and XOS inhibition. Similarly, eukaryotes such as *S. cerevisiae* are generally able to tolerate greater ethanol levels than prokaryotes such as *Escherichia coli*. High concentrations of ethanol in *S. cerevisiae*, however, reduce cell viability and inhibit the growth rate [71].

2.6 Saccharification

The lignocellulose feedstocks are subjected to hydrolysis after pretreatment to further degrade the cellulose and hemicellulose into monomeric sugars, mainly glucose and xylose. Enzymes or acid can be used to hydrolyse cellulosic materials [45]. The hydrolysis process must, however, be performed in an economically feasible manner and with the use of environmentally friendly technologies [45], [95]. The National Renewable Energy Laboratory (NREL) has estimated that a reduction in the costs associated with the enzyme process could be reduced four times more than for the dilute acid process [45], [96]. The two routes are described below.

2.6.1 Chemical saccharification

The industrial digestion of cellulose has historically been performed with acid hydrolysis, which is currently being studied for the potential disassembly of the cell wall [13], [15]. Although possible in practical terms, this process is not efficient enough for allowing

commercial ethanol production [13]. The process entails the hydrolysis of material with concentrated acid at low temperatures or dilute acids at higher temperatures. The strong acid attacks the glycosidic linkages among monosaccharide residues found in a polysaccharide [13]. Sulphuric, hydrochloric or trifluoroacetic acid are usually the applied acids during acid hydrolysis [13]. For hydrochloric and sulphuric acids, little discrimination exists among different glycosidic linkages that attack celluloses and hemicelluloses in a similar way [13]. Trifluoroacetic acid, however, preferentially breaks the weakest linkages existing of alpha linkages that are present in the branches of hemicelluloses [13].

A problem with this process is related to the need for neutralising the hydrolysed solution in order for fermentation to be carried out [13]. Limestone (calcium hydroxide) is generally used for this purpose, but results in the conversion of sulphuric acid to calcium sulphate, which cannot be recycled [13]. This is also the main factor contributing to the high cost of the technique [13]. To develop acceptable commercialisation levels, the reduction in costs associated with the consumption and re-use of acid, as well as improvement in efficiency and productivity in conversion of biomass, will be necessary [13].

The solid-liquid nature of the acid-hydrolysis of lignocellulose involves diverse chemical reactions that result not only in monomeric sugars, but also degradation compounds [13]. The hydrolysates produced from acid hydrolysis are toxic to fermenting micro-organisms and glucose yields only reach a maximum of 60 % [15]. The formation of undesired by-products such as 5-HMF and levulinic acid are also a result of acid-catalysed degradation of sugars [13]. The corrosive nature of concentrated acids that are used is, however, the main obstacle when making the process economically achievable [71].

2.6.2 Enzymatic saccharification

Due to advantages such as the minimised loss of monomers, decreased production of by-products during hydrolysis and higher conversion efficiencies, enzyme-based processes are preferred over chemical treatments such as acid or alkaline hydrolysis [45], [98]. This follows as enzyme-based treatments make use of low corrosive and moderate operating conditions and have low process requirements [45], [97], [98]. The disadvantage thereof, however, is that the process is fairly slower [25].

Successful SE pretreatment removes hemicellulose to a large extent, which leaves the cellulose fraction more accessible to cellulases [15]. The cellulose and residual hemicellulose,

however, need to be hydrolysed into fermentable sugar monomers to be transformed to ethanol producing micro-organisms [15]. The enzymatic hydrolysis step is influenced by several factors, including the type of substrate and substrate concentration, enzyme combination and its dosage and process conditions (temperature, pH, residence time, etc.).

The intrinsic recalcitrance of lignocellulose hampers the effective enzymatic conversion of the polymers of cellulose and hemicellulose [97]. Cellulases have a low specific activity compared to other enzymes (i.e. amylases) [55]. Moreover, the cellulose conversion rate falls sharply as hydrolysis proceed [55]. These factors make enzyme production and its use during hydrolysis a bottleneck for ethanol production [97].

Overall, the success of the conversion of lignocellulose into fermentable sugars relies in the feedback properties, pretreatment type and conditions, and the appropriate enzymes combination. The enzymes involved in lignocellulose degradation, their mechanism of action as well as factors that influence the enzymatic hydrolysis step are described in the next subsections.

2.6.2.1 Enzymes Involved in the Degradation of Lignocellulose

In nature, the degradation of lignocelluloses takes place through a battery of oxidative and hydrolytic enzymes that are produced by several bacteria and fungi [45], [99]. There are numerous enzymes with different catalytic activities involved in lignocellulose degradation. Glycosides hydrolases represent a wide variety of enzymes that have the function of hydrolysing the glucosidic bonds, which form between a non-carbohydrate and carbohydrate moiety or between two or more carbohydrates [83]. Besides the enzymes involved in the hydrolysis of polysaccharides, there are other enzymes involved in lignin degradation or hydrolysis of the carbohydrates-lignin complex.

The International Union of Biochemistry and Molecular Biology (IUBMB) classified the enzymes according to a code, EC, of four numbers. This EC number is based on the reaction catalysed and substrate specificity. In the case of glycosides hydrolases this number corresponds with EC 3.2.1.X, where the first digit refers to the enzyme class (hydrolase), the second digit refers to the sub-class and its capacity to hydrolyse the glycoside link, the third number corresponds to the functional group donor (water) and the fourth one refers to the acceptor molecule, that is substrate specific. Another recent system of classification is based on structural (three-dimensional folds) and amino acid sequence similarities [100]–[102] that

classified the enzymes into families and clans. This system has catalogued up to 131 families of glycosides hydrolases to date [103]. These classifications can be seen in the CAZY database, which describes the families to which the catalytic and carbohydrate-binding modules belong [100]. These modules (functional domains) are those of enzymes that modify, degrade or create glycosidic bonds [100]. Examples of these enzymes are cellulases and hemicellulases.

The core enzymes involved in lignocellulose degradation are the so-called cellulases. These enzymes are produced by superior plants, some invertebrates and mainly by micro-organisms. There are several types of micro-organisms e.g. aerobic actinomycetes, aerobic filamentous fungi, anaerobic fungi and anaerobic hyperthermophilic bacteria that can produce cellulase systems [15]. The majority of enzymes that have been developed and tested are, however, from fungi [3]. Currently, the enzymes used for biomass deconstruction are derived from fungi such as *Trichoderma* and *Aspergillus* [3]. For enhanced cellulose production, *Trichoderma reesei* has gone through multiple rounds of strain improvement since 1950 [3], [15]. This organism is currently the predominant industrial cellulolytic enzyme producer that secretes enzyme systems that are able to degrade crystalline cellulose, consisting of endo-glucanases, cellobiohydrolasas as well as β-glucosidases [15], [38], [104]. The main enzymes involved in lignocellulose degradation are listed in Table 7.

Table 7: A list of some of the main enzymes that are involved in lignocellulose degradation (Table adapted from Van Dyk *et al.*2012 [26]).

Component	Enzymes
Cellulose	Cellobiohydrolase, endo-glucanase, β-glucosidase
Hemicellulose	Endo-xylanase (EX), acetyl xylan esterase, β-xylosidase (BX), endo-mannanase, β-mannosidase, α-glucuronidase, α-L-arabinofuranosidase, ferulic acid esterase, α-galactosidase, p-coumaric acid esterase
Lignin	Laccase, Manganese peroxidase, Lignin peroxidase
Pectin	Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase

Cellulases are enzymes that specialise in breaking up the β-1,4-glycosidic bonds of glucan [3], [15]. These cellulolytic enzymes comprise 3 main enzymes: endo-1,4-β-d-glucanases, exo-1,4-β-d-glucanases or cellobiohydrolases and 1,4-β-D-glucosidases [3], [13], [15], [52]. In the most well characterised cellulase producer, *T. reesei*, five endo-glucanases, two cellobiohydrolases and two β-glucosidases have been characterised [52]. The role of endo-glucanases is to reduce the DP of the substrate significantly by randomly hydrolysing internal β-1,4-glucosidic bonds in the cellulose chain, mainly focussing on the amorphous

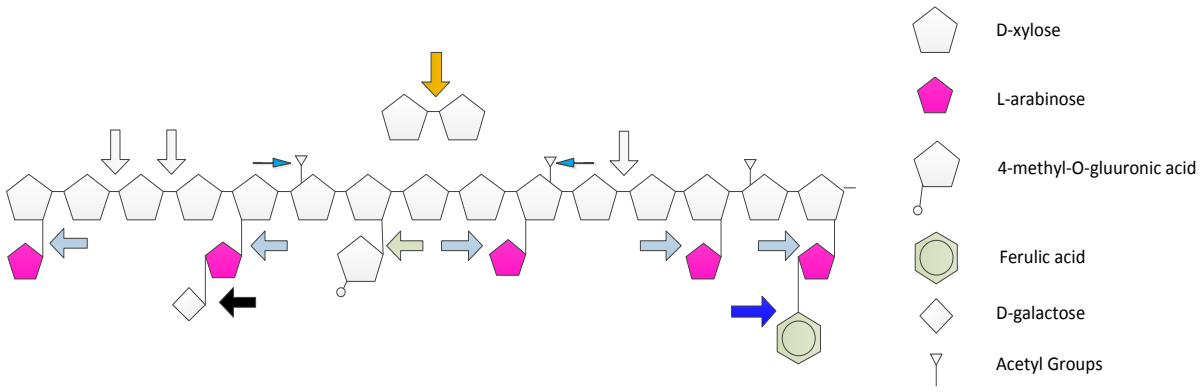
regions within cellulose [13], [15], [52], [104]. Two of these enzymes are secreted into the medium, namely EG I and EG II [104]. Exoglucanases (CBH) shorten glucan molecules by binding to the ends thereof and mainly cleaving off the cellobiose units from both reducing and non-reducing ends of the chain [13], [15], [52]. Two of these enzymes have also been identified in *T. reesei,* each occurring in several iso-enzymatic forms as CBH I and CBH II [104]. At high concentrations, cellobiose can inhibit the activity of CBH [45]. The activity of β-glucosidases is therefore required to hydrolyse the cellobiose units to glucose while cleaving off glucose units from cello-oligosaccharides [13], [15], [52]. As most production systems struggle with recovering β-glucosidases, extra supplementation is required to prevent the cellobiose from accumulating in the hydrolysis media [45]. Any end-product inhibition that may occur is thereby reduced [45]. β-glucosidases therefore also function in the control of the accumulation of cellulose inducers [104]. All three of these enzymes work together in synergism to saccharify cellulose by creating new available regions for each other, removing any obstacles and alleviating end-product inhibition that may occur [52].

Endo-glucanases and cellobiohydrolases form part of a two-domain structure [71]. Cellobiohydrolases have a small C-terminal glycopeptide (cellulose binding domain (CBD)) that is able to bind to cellulose as well as a larger core protein (catalytic domain (CD)), which contains the active site [71]. These two domains within the enzymes are normally linked by residues of 22 - 34 amino acids [71]. The CBD is positioned at the N- or C-terminal end of the protein [71]. Primarily, the function of the CBD is to increase contact time by bringing the catalytic core into close contact with the surface of the celluloses [71].

Cellulases with an exoglucanase mode of action have a “tunnel”-shaped structure in which the active site is positioned [44], [71]. Endo-glucanases that act more randomly generally have a CD which is more “cleft”-shaped, thereby exposing the active site to the exterior part of the enzyme [44], [71].

Hemicelluloses are heterogeneous and have various side groups. The hemicellulolytic system is therefore more complex [41], but it is also comprised of endo-enzymes and exo-enzymes. Endo-enzymes include endo-1,4-β-D-xylanases, which hydrolyse the bonds within the xylan chain and endo-1,4-β-D-mannanases, which cleave the internal bonds in mannan. Examples of exo-enzymes are 1,4-β-D-xylosidases, which attack xylo-oligosaccharides from the non-reducing end and also liberates xylose and 1,4-β-D-mannosidases, which cleave

manno-oligosaccharides to mannose [41]. Besides the previous enzymes, the hemicellulolytic system also requires auxiliary enzymes such as esterases that hydrolyse the bonds between hemicelluloses and lignin. A scheme of the enzyme system required to hydrolyse the hemicellulose of herbaceous material is represented in Figure 8.



Symbol	Enzyme	EC number	Link hydrolysed
	Endo-1,4-β-xylanase	3.2.1.8	β-1,4
	β-D-xylosidase	3.2.1.37	β-1,4
	α -L-arabinofuranosidase Arabinoxylan arabinofuranohydrolases	3.2.1.55	α-L-1,2; α-L-1,3; α-1,5
	α -D-glucuronosidase	3.2.1	α-1,2
	feruloyl esterase	3.2.1	ester bond
	α -D-galactosidase	3.2.1.22	α-1,6
	asetylxylan esterase	3.1.1.6	ester bond

Figure 8: A scheme of the hemicellulolytic systems specific for arabinoxylan. The number of EC of each enzyme and the link they hydrolyse is indicated in the table (Figure adapted from Aro *et. al* 2005 [105]).

A core enzyme set consists of endo-glucanase, cellobiohydrolase, β-glucosidase, endo-xylanase and also β-xylosidase [3]. Some accessory enzymes that can also be present include proteases, esterases, non-hydrolytic proteins and glycosyl hydrolases, which cleave less frequent chemical linkages that are found in the cell walls of plants [3]. Enzymes that act indirectly on the covalent bonds in plant cell wall polysaccharides, in addition to those acting directly, might be important in the breakdown of lignocellulose [3]. These potentially auxiliary enzymes firstly include non-enzymatic proteins (e.g. expansins from plants, fungi and bacteria) that contribute to wall loosening [3]. Second on the list are enzymes that

degrade non-glycosidic wall components (e.g. lignin and proteins) and thereby facilitate access of glycosyl hydrolases [3]. The third group includes enzymes that degrade small molecules, released during pretreatment, which inhibits the downstream fermentation steps or the core degradative enzymes [3]. In the future, customised enzyme mixtures will possibly differ from current mixtures in terms of the accessory enzymes acting on linkages that are scarce in the plant cell walls, rather than in the core xylanases or cellulases [3].

Due to recent studies of fungal proteins that are currently classified as glycoside hydrolases family 61 (GH61), the classical endo/exo scheme have shown to be oversimplified [24]. The discovery of new fungal proteins has led to a new model to explain the mechanism of action of cellulases [100]. Although these enzymes were originally categorised within the glycoside hydrolase family, they are not true glycosides [100]. In fact, GH61s are copper-dependent polysaccharide mono-oxygenases [24]. The GH61 can still be accessed in the CAZY database, since they promote cellulose hydrolysis when used together with cellulases [100].

These GH61 proteins contain flat substrate-binding surfaces and cleave polysaccharide chains in their crystalline form with the use of an oxidising mechanism, which is dependent on the presence of an electron donor as well as divalent metal ions [24]. Oxidation by these enzymes takes place at several carbons within the ring-structure, especially C1, C4 and possibly also the C6 carbons [24], [106]–[109].

The mechanism of action of the GH61 proteins is illustrated in Figure 9. There, a C1 and C4 oxidising GH61 can be seen generating optimal ends for CBH1 and CBH2 (the oxidised sugars are indicated in red) by cleaving of the end-glycosidic bonds within cellulose. The cellobiose-dehydrogenase (CDH) provide GH61 with electrons [24].

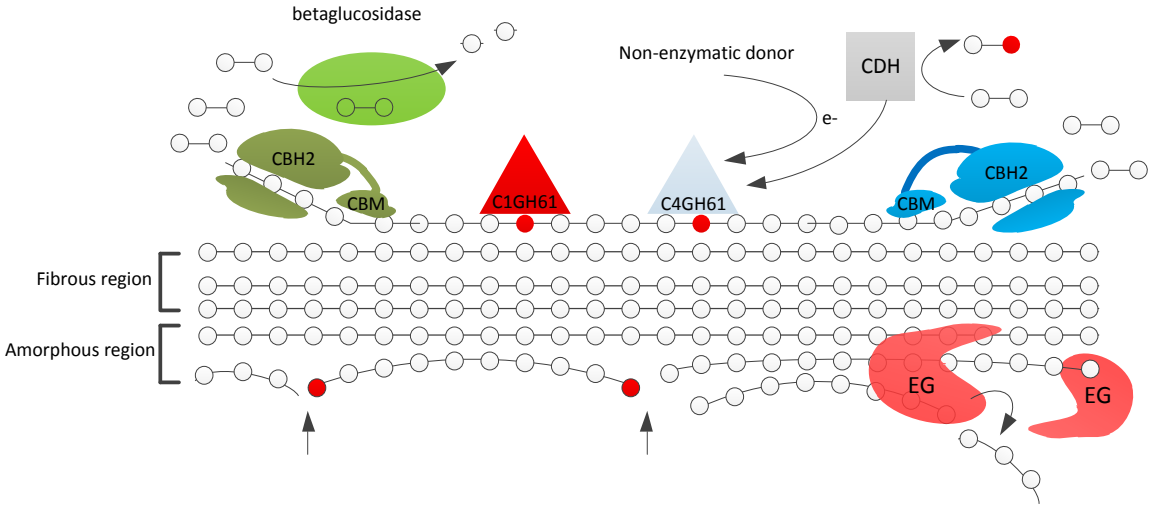


Figure 9: An illustration of the fungal enzymatic degradation of cellulose as it is currently viewed. CBM – carbohydrate-binding module, CBH - cellobiohydrolase, CDH – cellobiose-dehydrogenase, EG – endo-glucanase, GH61 – Glycoside Hydrolases family 61 (Figure adapted from Cardona *et al.* 2010 [24]).

The degradation of cellulose by cellulases and oxidative enzymes produce not only glucose, but also monomeric and dimeric oxidised sugars such as gluconic- and cellobionic acid with respect to C1 carbons [24]. As the main activity of GH61 enzymes is to oxidatively cleave the endo-glycosidic bonds within cellulose, the amount of gluconic acid is expected to be at least equal to the total number of newly formed entry sites created in the cellulose chain as a result thereof [106]. These products can induce cellulase inhibition, where gluconic acid is a stronger inhibitor than glucose, while cellobionic acid has been proven to inhibit β-glucosidase [24]. In enzymatic hydrolysis studies performed with an enzyme preparation with GH61, approximately 4 % of the glucose released during hydrolysis, were seen to be oxidised into gluconic acid [106]. Additionally, the produced gluconic acid also inhibited β-glucosidase with subsequent cellobiose accumulation [106]. β-glucosidase was, however, able to hydrolyse the cellobionic acid at a 10-fold lower rate than for cellobiose [106]. The cellulose conversion yields, however, where still 25 % higher compared to the use of conventional enzymes [106]. Interestingly, it was also noted that 35 % less gluconic acid was produced when hydrolysis was performed at 50 ˚C compared to those performed at 33 ˚C [106]. This might be due to the activity of the GH61 enzymes that do no decrease as much as the activity of the endo-glucanases and cellobiohydrolases when the temperature is reduced. This follows as the rate of glucose production is proportional to the rate of the formation of gluconic acid throughout hydrolysis. This fact highlights the necessity to adapt

process conditions and configurations not only to feedstock and pretreatment, but also to the enzyme combination used.

Micro-organisms that convert biomass present genes of GH61 [24]. The inclusion of GH61 enzymes in the new generations of cellulase preparations has a significant impact on the performance of hydrolysis [106]. Cellic® CTec2, which is produced by Novozymes, contains additional GH61s [24]. These contribute significantly to the improved performance of this product, compared to other [24]. The application of these enzymes would, however, require adaptation of the process configuration applied in order to minimise gluconic and cellobionic acid formation, since they cannot be fermented into ethanol by conventional yeast such as *S. cerevisiae*.

2.6.2.2 Factors that Influence Enzymatic Saccharification

Commercial application of enzymatic saccharification of lignocellulose substrates has been hampered not only by the high cost of cellulases and its slow conversion rate, but also by the insoluble and heterogeneous nature of lignocellulose. High enzyme loadings and/or prolonged hydrolysis times are normally required to attain near-complete cellulose conversion and can therefore also be seen as factors that limit enzymatic hydrolysis [110]. Techno-economic modelling has, however, shown that the long hydrolysis time applied for improving cellulose conversion adds significantly to the operating costs associated with the saccharification step and subsequently also to the overall bioconversion process [110].

The reaction catalysed by cellulases is a solid-liquid system comprising insoluble substrate (pretreated lignocellulose) and a soluble catalyst (cellulolytic enzymes). Both the structural characteristics of the lignocellulose and the mechanism of action of the enzymes combination will impact on the rate of the conversion of cellulose. Nevertheless, the cellulose conversion into glucose normally follows three different stages characterised by a fast, moderate and low hydrolysis rate. Figure 10 illustrates a typical progress curve for glucose release during enzymatic hydrolysis of pretreated lignocellulose with three different enzyme preparations. Hydrolysis generally starts with the quick absorption of cellulase onto the available cellulose, where after a rapid, initial hydrolysis phase follows [44]–[46], [110]. The reaction, however, quickly reaches an intermediate phase that is characterised by the moderate hydrolysis of the substrate [110]. More or less 50 – 70 % of the original substrate is hydrolysed at that point, depending on substrate (type, concentration) and enzyme

(nature, loading) [110]. The last, very slow phase is then entered, where the steady decrease of the reaction rate takes place [110].

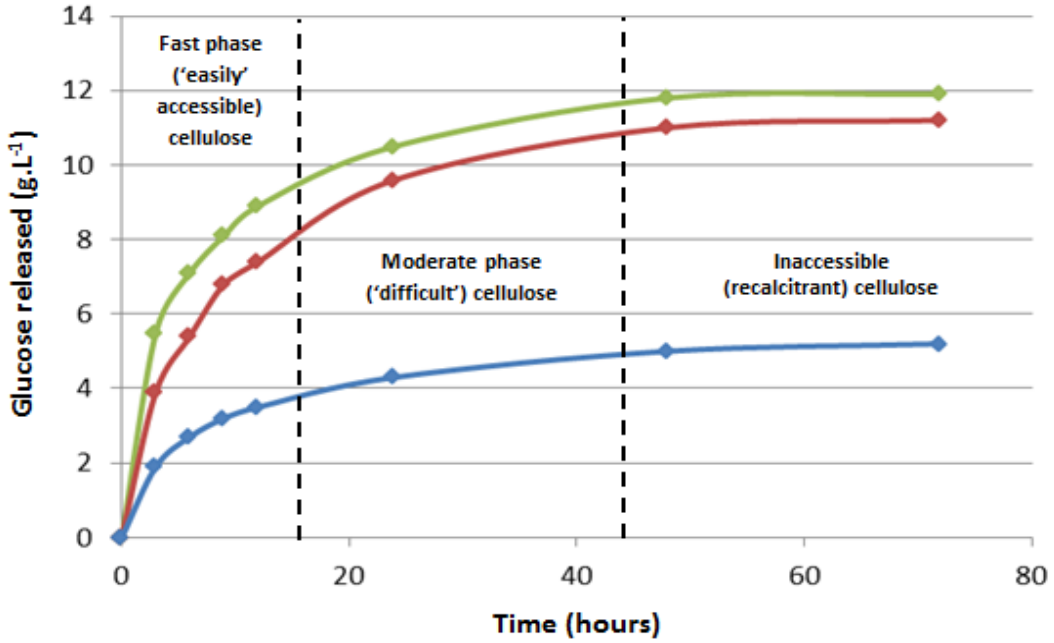


Figure 10: Typical time course for glucose release during enzymatic hydrolysis of pretreated lignocellulose. Conditions: 2 % (dw.v^{-1}) solids loading, pH 5.0, enzyme loading of 0.25 mL.g^{-1} substrate of different enzymes preparations: the Alternafuel CMAX preparation from Dyadic (blue graph), the control enzyme combination of Spezyme CP and Novozyme 188 from Genencor and Novozymes, respectively (red graph), and Cellic® CTec2 from Novozymes (green graph).

The enzymes that are needed for the conversion of plant biomass materials into sugars that are fermentable are extremely expensive as they are thermodynamically unstable and produced by living systems [3]. The real cost contribution of cellulases can be reduced by a number of possibilities [111]:

- Improvements in cellulase expression [112]
- Improvements in the cellulase machinery through protein engineering [73] and
- Improvements in cellulose hydrolysis rates by optimising reaction conditions via process engineering [113]

The outcome of these three strategies strongly relies on identification and quantification of crucial properties of substrate and enzymes that govern reaction rates [111].

There are also several other factors involved in the observed reduction of the cellulose conversion by enzymes. These factors can be divided into substrate- and enzyme-related

factors where the latter includes enzyme properties, synergism between enzymes, etc. Depending on the type of biomass, pretreatment and enzymes applied, enzyme- or substrate-related factors could have the potential to have a bigger influence than the other [46]. Hypotheses explaining this observation include (as seen in Figure 11) [45]:

- Inactivation of the enzymes because of thermal effects such as deactivation and instability [114], [115]
- Inhibition by the products formed during hydrolysis [115]–[118]
- Unproductive binding of enzymes to lignin [119]–[123]
- Transformation of substrate into a form that is less digestible [124] and/or
- The heterogeneous structure of the substrate [124], [125]

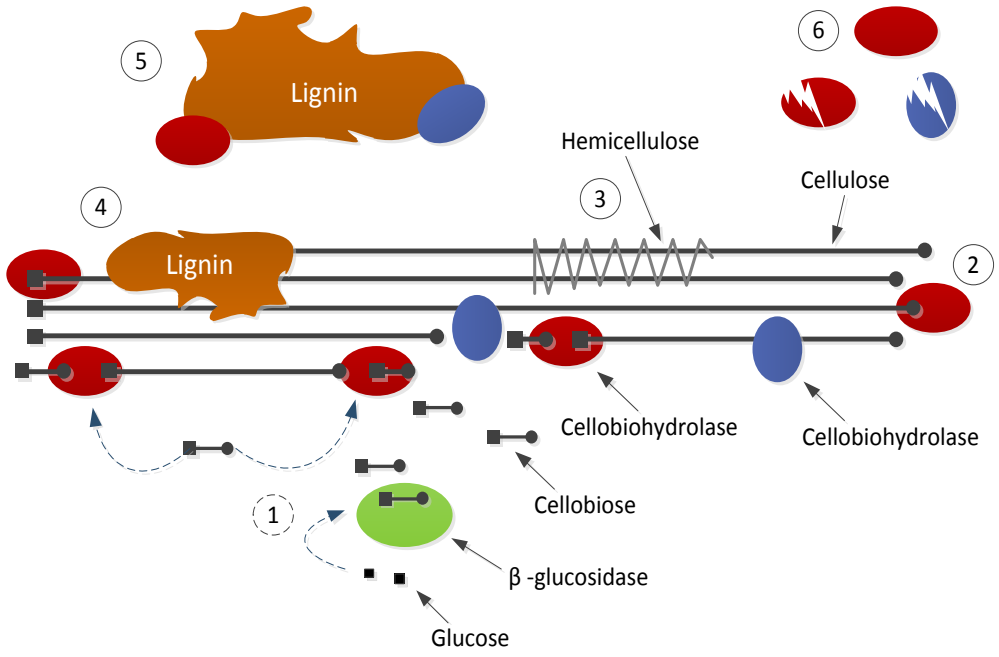


Figure 11: A simplistic overview of the factors that limit the efficient hydrolysis of cellulose. 1: Product inhibition of the cellobiohydrolases and β–glucosidases by cellobiose and glucose, respectively. 2: Unproductive binding of the cellobiohydrolases onto a cellulose chain. Due to the strong binding of the cellulose chain by cellobiohydrolases in their catalytic core, obstacles can make the enzymes pause and thereby become unproductively bound. 3 and 4: Association or covering of the microfibrils by the hemicelluloses and lignin can prevent access of the cellulases to the cellulose surface. 5: Both cellulase and hemicellulase enzymes can adsorb un-specifically onto lignin particles or surfaces. 6: Loss or denaturation of the enzyme activity due to mechanical shear, low thermostability or proteolytic activity (Figure adapted from Fan *et al.* 1981 [52]).

The substrate- and enzyme-related factors are discussed in the sections that follow.

Substrate-related Factors

The rates and yields of enzymatic hydrolysis are highly dependent on the substrate-related factors. Some lignocellulosic substrates show greater recalcitrance during enzymatic hydrolysis than others [44]. This may be related to the origin of the substrate, type and conditions of pretreatment and/or the kind of enzymes that are employed [44].

One of the most important aspects is the concentration of substrate. Performing hydrolysis at high solid loadings (15 - 20 %) leads to an increased ethanol concentration and also reduces energy requirements and costs of product recovery and downstream processes, due to the requirements for distillation [45], [126]. However, when performing hydrolysis at an increased insoluble solids content (150 - 200 $g.L^{-1}$), several process-related problems can occur, such as those associated with cellulase effectiveness (sugar inhibition) and enzyme-substrate mixing [45], [127]. An additional disadvantage of such high solid loadings is the reduced ability of the enzymes to reach the reactions site as this leads to sugar inhibition due to the increased difficulty for the sugars to diffuse away from the catalytic site [45]. Viscosity therefore has a big impact on enzyme-substrate mixing and should be decreased [45].

Besides the solids loading during enzymatic hydrolysis, other structural properties of the substrate depend on the starting feedstock and pretreatment applied: crystallinity and DP of cellulose, the pore size and accessible superficial area, lignin and hemicellulose content (which includes the acetyl content). The two main chemical and physical substrate factors influencing the rate and extent of the hydrolysis of cellulose by means of cellulase enzymes are (i) cellulose crystallinity [121] along with the DP that decrease enzyme efficiency [128], and (ii) the physical barrier that the lignin and polysaccharide matrix provide which prevent the enzymes from gaining access to the cellulose fibril [45].

Cellulose consists of crystalline and amorphous regions each presenting different degree of accessibility to cellulases. Generally , crystalline cellulose is not as accessible to cellulases attack compared to amorphous cellulose [45]. This dual nature may be responsible for the progressive reduction of cellulose conversion during enzymatic hydrolysis [45]. This follows as amorphous cellulose is thought to be hydrolysed right at the beginning of hydrolysis, thereby the material gets enriched in crystalline regions, which therefore increases the recalcitrance of the substrate as enzymatic hydrolysis progresses [45], [129], [130]. The

initial fast hydrolysis is also influenced by changes in the physical properties of native cellulose as can be seen during a cellulases attack, even before measurable quantities of reducing sugars are produced [45], [131]. Such changes include swelling, fragmentation, transverse cracking, the considerable loss of tensile strength and a reduction in the DP[45], [132].

Lignin, one of the cell wall components, affects the accessibility of cellulose to cellulase in more ways than one by acting as a physical barrier in cellulase accessibility [44], [45]. The content thereof, as well as the type of lignin, thus have significant effects on the hydrolysis of a variety of lignocellulosic substrates [45], [133]. Removal of lignin to facilitate hydrolysis even further has a lot of advantages [65]. Some of these include the significant increase in the available cellulose surface area, reduced non-productive enzyme binding which leads to a great improvement in accessibility of the substrate to the different enzymes and subsequent cellulose hydrolysis efficiency [65].

In herbaceous crops such as sorghum and triticale, lignin does not have that much of an impact because of the lower initial content thereof within these feedstocks [134]. Steam-explosion pretreated lignin is also not as reactive as others and their binding capacity to cellulases is lower compared to other treatments and feedstocks, such as softwoods [134]. However, although the lignin within these feedstocks might have a lower impact, the effect will still be significant.

It has also been demonstrated by many authors that the solubilisation of hemicelluloses during the process of pretreatment can facilitate the subsequent cellulose hydrolysis [45], [135]–[137]. Further evidence also exists, showing the employment of cellulases combined with hemicellulases in hydrolysing pretreated substrates to achieve synergistic benefits [37], [45], [135], [138]. In addition, the residual acetyl groups of the hemicellulose within the pretreated fibers interfere with the recognition of enzymes through steric hindrance, thereby restricting the access of cellulase [45], [139], [140]. The rate of enzymatic hydrolysis is thereby slowed down.

Enzyme-related Factors

The enzymatic hydrolysis (EH) of cellulose can be influenced by the following enzyme-related factors [2], [90], [93], [141]:

- Enzyme properties: size, mechanism of action, structure (cellulose binding (CBD) domain)
- Enzyme concentration
- Synergism and loss of synergism during the enzymatic hydrolysis
- Reaction conditions: influence of pH, optimum temperature and stability of the enzymes to temperature and agitation, time.
- Unproductive binding of cellulases to lignin.
- End-product inhibition (glucose) and inhibition by other products of hydrolysis (cellobiose, xylose, xylo-oligomers)
- Inhibition by degradation products and lignin-derived compounds released during pretreatment.

In some cases, the hydrolytic activity of the individual enzymes involved with crystalline substrates is reduced by removal of the catalytic-binding domain [44]. The amorphous cellulose is, however, not affected [44]. Adsorption is therefore more important for crystalline substrates requiring the intensive action of several enzymes in comparison to amorphous substrates which are easier hydrolysed [44].

The efficiency of the enzymatic hydrolysis of lignocellulosic materials mainly depends on the appropriate ratio of the various enzyme components in addition to the presence of all the components [142]. Synergism is defined as the combined action of two or more enzymes which leads to a higher reaction rate than what the sum of their individual actions do [44]. As mentioned previously, cellulose conversion requires at least the presence of endo-glucanases, exoglucanases and β-glucosidases. The enzymatic hydrolysis of hemicellulose is, however, more complicated than that of cellulose, as the former polymer is composed of a mixture of 5 and 6 carbon sugars and requires the use of several different enzymes to break it up [13]. The hydrolysis of the hemicellulose fraction is therefore an essential step in order to facilitate the complete degradation of cellulose [138]. Since xylan is the main hemicellulose in agricultural by-products, the addition of xylanase will thus result in the production of xylose and XOS [138]. The complete degradation of xylan-to-xylose would

therefore ensure a more profitable production of bio-ethanol along with the possibility of fermenting both xylose and glucose to ethanol [138].

T. reesei produces five endo-glucanases, two cellobiohydrolases and two β-glucosidases [41]. Several of these apparent "redundant enzymes" have shown to exhibit synergy either by having different affinities for the diverse sites of attack, or hydrolysing different ends of the cellulose chains [41]. Optimisation of the synergies between enzymes, e.g. endo-endo, endo-exo, exo-exo and endo- or exoglucanases and β-glucosidases by altering the composition of the enzyme mixtures is important [15]. Exo-exo synergism is explained by the fact that CBH II hydrolyses cellulose from the non-reducing end whereas CBH I hydrolyse from the reducing end [71]. The two cellobiohydrolases is thus able to display new hydrolytic sites for each other by removing the cellulose chains coating the chain ends [71].

The synergistic interaction that occurs between different cellulases is of great benefit when the hydrolysis rates of complex sugars are increased [44]. The extent to which synergism is exhibited by these cellulases as well as the optimal composition of enzyme mixtures, however, depends on the specific substrate [15], [44].

The ratio of these enzymes could vary through the EH with subsequent reduction in the synergism. Another factor that could influence the reduction in cellulose conversion is the end-product inhibition, i.e. cellulases by the formation of cellobiose and β-glucosidases by the formation of glucose [13]. Other inhibitors such as acetic acid, furfural and xylo-oligomers can also have an effect on hydrolysis, as discussed in the previous section [4], [81].

2.6.2.3 Research in Enzyme Technology for Lignocellulose Conversion

Enzyme research in the bio-ethanol field has two pressing needs [3]: The first need is for optimisation of enzyme cocktails by improving the understanding of which proteins or enzymes are essential for deconstructing lignocellulose [3]. Attempts to build a synthetic enzyme mixture which will define their essential lignocellulosic activities and optimal ratios, thus has to be addressed [3]. A second need in enzyme research involves having a method that can realistically evaluate new alternative enzymes [3]. This comes from the fact that mono-component assays do not reflect the hydrolytic potential of enzymes and other important characteristics such as degree of synergism (DS) [3]. Assays that are based on synthetic substrates also do not effectively reflect the behaviour of enzymes against pretreated native lignocellulose, which represents real substrates [3].

Optimisation of Enzyme Cocktails

Approaches such as protein engineering and "bio-prospecting" for superior key enzymes and high-level expression in plants are currently being investigated [3]. The main focus is, however, on the development of synthetic enzyme mixtures, which would aid in identifying the specific ratio in which these hundreds of enzymes operate and which of the enzymes are important [3].

Enzymes most commonly used for commercial cocktails originate from the fungus *T. reesei* which is known nowadays as *Hypocrea jecorina* [24]. Commercial enzyme mixtures normally consists of cellulases (EG and CBH) and are supplemented by β-glucosidase to diminish product inhibition of the cellulases by the action of cellodextrins [40]. The need for tailor-made enzyme mixtures is a necessity due to the fact that preparations of commercialised broad spectrum cellulase lack sufficient β-glucosidase as well as xylanase activities that are a pre-requisite for the efficient release of monomeric sugars [87].

When assembling a core set of enzymes, it should contain enzymes that are required for a minimum reasonable release of glucose as well as xylose from the specific biomass [143]. For cellulose hydrolysis, this entails including at least one endo-glucanase, one cellobiohydrolase as well as one β-glucosidase in the core set [143]. For hemicellulose, where xylose is the main sugar, the core set requires the presence of an endo-xylanase as well as a β-xylosidase [143]. Addition of xylanases to the enzyme cocktail could decrease the inhibition that is triggered by XOS, as the xylanases supply β-glucosidases that help to remove these compounds [8].

In a study by Meyer *et al.*(2009), pretreated barley straw was used as material [97]. The four main cellulases of *T. reesei:* EG I, EG II, CBH I and CBH II were assessed. The data indicated the following results [97]:

1. The activity of CBH I is very important for hydrolysis and, when compared to the other enzymes, is thus required in the highest amounts.
2. The level of CBH II that is present, vary from 17 to 27 %. High levels thereof are also required.
3. With respect to the level of endo-1,4-β-glucanase activity required (elevated to 37 - 38 %), the optimal profile varied between different pretreatments.

4. Compared to the profile secreted by native *T. reesei*, the optimal profile required for the pretreated material differed remarkably when looking at the required levels of endo-1,4-β-glucanase activity.
5. Lastly, the highest glucose yields where obtained after enzymatic hydrolysis when the straw was impregnated with H_2SO_4 during pretreatment, followed by SE.

Although minimisation of the dosage and selection of additional enzymes is still necessary, data confirmed the possibility of profiling and selecting mono-active enzymes for lignocellulosic materials [97]. Additionally, data indicated that different enzymatic profiles for the optimisation of hydrolysis are required for differently pretreated substrates [97].

The minimal enzyme cocktail approach can thus promote a more rational utilisation of biomass-upgrading enzyme activities along with stimulating a more efficient enzymatic hydrolysis [97]. One of the aspects addressed in this thesis is the optimisation by statistic design of the combination of cellulase and hemicellulase in order to improve the yields.

Methods for Evaluation of Digestibility and Enzyme Preparations

The realization that lignocellulose's potential for use as an industrial feedstock lies in the digestibility thereof, is a great barrier to overcome [144], [145]. Reliable analysis of the saccharification properties of the material should therefore be able to repeat these steps on small scale in order to evaluate the hydrolysis in bigger and more diverse sample populations [145].

There are mainly two things that drive the need for this kind of methodology: The large number of substrates and feedstock-pretreatment combinations that can be studied as well as the increasing number of enzymes that are being developed through metagenomics and transcriptomics [24], [146], [147]. The hydrolytic effectiveness and high costs of enzymes are also major factors that restrict commercialisation of the processes involved in biomass conversion [148]. The development of a rapid assay for lignocellulosic hydrolysis has therefore been of great interest to address this need [149].

A major challenge during the development of such a system is the difficulty in handling the material at milligram scale, as lignocellulosic biomass is a heterogeneous and insoluble material [145]. The material of the biomass is ground to a very small particle size to facilitate the handling and distribution thereof [145]. A reduction in size, however, represents the

initial step in a conversion process and can in itself be seen as a mechanical pretreatment with the potential to increase digestibility and greatly improve enzymatic hydrolysis, due to a decrease in the crystallinity of cellulose and an increase in accessible surface area [145], [150]–[152]. In one study, Decker *et al.* reported that milling the material to a mesh particle size of 20 - 80 µm did not affect the digestibility of the substrate [145], [153]. A further reduction in particle size would however, lead to increased saccharification and thereby be able to mask possible differences that might occur between different materials [145]. To distribute the biomass in a reproducible and accurate manner, several alternative methods have been proposed, e.g. the direct pipetting of biomass slurry or dispensing fabricated handsheets of lignocellulosic material into the wells of micro-plates [145], [149], [151].

A micro-assay represents a rapid method for testing how newly selected cellulase enzymes perform on pretreated lignocellulosic substrates [148]. This process involves the formation of handsheets from the pretreated lignocellulosic material [149]. These handsheets are cut into small discs that are distributed into the various micro-titre plates [149]. Cellulose hydrolysis to glucose can thereafter be estimated with the use of an enzyme-coupled spectrophotometric assay [149].

The use of this assay shows substantial time and cost benefits when compared to standard procedures that are performed in large scale shake flasks [149]. It is also more relevant to lignocellulosic hydrolysis than the methods which use synthetic substrates [149]. Additional advantages thereof include its potential to evaluate multiple variables with relative ease, its capability to screen cellulase preparations, test enzyme supplementations and also its ability to discriminate between enzymatic preparations [148], [149].

2.7 Fermentation

Once all the polysaccharides have been hydrolysed into fermentable sugars i.e. glucose and xylose, they are subjected to a fermentation step by a fermentative organism. The main goal during fermentation is reaching an ethanol yield closest to the maximum theoretical value of 0.51 $g_{ethanol}.g^{-1}_{consumed\ sugar}$ [71]. The conversion of these sugars into ethanol needs to take place as efficiently as possible in order to make the process economically feasible [71].

S. cerevisiae is considered the main micro-organism in starch- and sucrose-based ethanol production [15]. The advantages of this organism are its production of high ethanol yields (> 0.45 $g.g^{-1}$), its high tolerance to ethanol (> 100 $g.L^{-1}$), its tolerance to inhibitors that are produced during biomass pretreatment as well as its high specific rates of carbon consumption (1.3 $g.g^{-1}_{cell\ mass}.h^{-1}$) [13], [15]. This yeast can, however, only ferment hexose sugars such as glucose, mannose and galactose [13]. Pentoses e.g. xylose and arabinose, being the main building blocks of hemicelluloses, can thus not be fermented [13]. The development of micro-organisms that efficiently ferment these hemicellulose sugars is, however, rapidly progressing [52].

Xylose-fermenting yeasts, e.g. *Pichia stipitis* and *Candida shehatae*, can therefore be beneficial for use in the fermentation of materials that have high xylan content [15]. The only disadvantages are, however, their low tolerance to inhibitory compounds within undetoxified lignocellulose hydrolysates and their need for very low and well-controlled oxygen supply for the efficient fermentation of xylose [15].

In general, organisms that are to be used for the production of ethanol should give high productivity, a high ethanol yield and also be able to resist high concentrations of ethanol in order to maintain low distillation costs [15]. Additionally the temperature, pH and inhibitor tolerance along with the ability to utilise multiple sugars are essential during fermentation [15].

In whatever way fermentation takes place, it remains important to maintain high carbohydrate concentrations during hydrolysis, in order to reach high ethanol concentrations during fermentation [13]. When following the route of enzymatic hydrolysis, four different fermentation-hydrolysis configurations for pretreated lignocellulose are apparent, namely: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and lastly, consolidated bioprocessing (CBP) [71]. Figure 12 gives a schematic overview of bio-ethanol production from lignocellulosic materials and illustrates the different possible process schemes with different degrees of integration of the biological events: enzyme production, enzymatic hydrolysis and fermentation. These configurations are discussed further in the sections below.

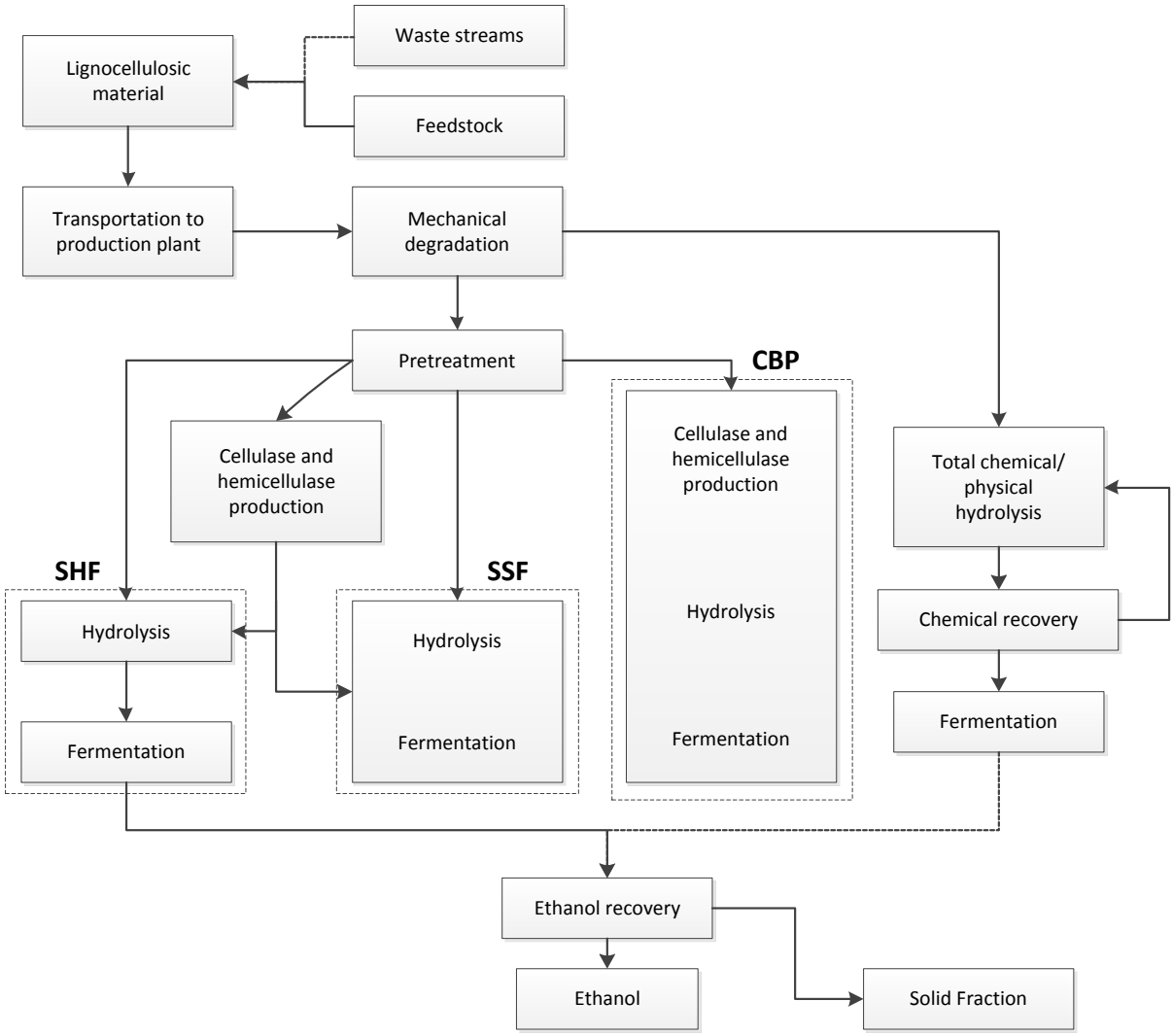


Figure 12: A schematic overview of bio-ethanol production from lignocellulosic material [33]. SHF - Separate hydrolysis and fermentation, SSF - Simultaneous saccharification and fermentation and CBP - Consolidated bioprocessing (Figure adapted from Vermerris et al. 2008 [71]).

2.7.1 Separate hydrolysis and fermentation

Enzymatic hydrolysis and fermentation can be performed in two different reactors under optimum conditions with regard to their individual pH, temperature and design of equipment requirements in the SHF configuration [13], [15]. The maximum activity of cellulases takes place around 50 ^{o}C, while fermenting organisms such as *S. cerevisiae* cannot tolerate temperatures above 37 ^{o}C [13]. The need for two separate vessels is thus obvious.

Drawbacks of SHF include end-product inhibition of cellulases by glucose and cellobiose, which leads to a decrease in productivity and reduction in the reaction rate with the increase

in sugar concentration and concomitant increase in the required enzyme dosage [13]. Another disadvantage is the loss of sugars that occur after enzymatic hydrolysis when separation of the solids and liquids takes place [13].

2.7.2 Simultaneous saccharification and fermentation

Instead of performing fermentation subsequent to enzymatic hydrolysis, another option is to perform the latter together with fermentation [15]. This idea is called simultaneous saccharification and fermentation and was introduced by Gauss *et al.* in a patent from 1976 [15], [154].

SSF is considered one of the most advantageous schemes for fermentation as it combines the cellulase enzymes as well as the fermentable microbes in the same reactor [5], [15]. This dramatically improves the economics of bio-ethanol production. This simultaneous hydrolysis and fermentation leads to one of the main advantages, being the immediate conversion of the released sugars formed during enzymatic hydrolysis to ethanol [13], [15]. A low sugar concentration in the broth is thus maintained, thereby alleviating cellulase end-product inhibition and diminishing the risk for infections [13]. This could also hold the potential for the application of lower enzyme dosages. SSF is therefore seen as superior to SHF and a better alternative for the production of ethanol [17].

SSF also holds the advantage that glucose, following a separate enzymatic hydrolysis step, does not need to be separated from the lignin fraction [15]. A potential loss of sugar can therefore be avoided [15]. Combining hydrolysis and fermentation also decreases the number of reactors that are required, thereby reducing the investment costs [13], [15]. This is an important aspect, since economic analysis has indicated that a high-solid SSF process will reduce the operating cost of ethanol production by up to 20 % [15], [155]. Yeasts can, however, not be recycled during the SSF process due to the presence of lignin [13], [15]. Similarly, enzyme recycling and reutilisation is also difficult. This thus leads to financial losses [15].

Several of the compounds that are present in the pretreatment hydrolysates and inhibit enzymatic hydrolysis can also be converted by the fermenting organisms [15]. This might explain the higher reported ethanol yields that are obtained in SSF in comparison to SHF [15]. The formation of inhibitors can therefore be withstood to a higher extent in SSF processes [15], [81]. The yeast is also capable of partly detoxifying the slurry and thereby

adds to increasing the productivity of enzymatic hydrolysis [13]. Higher overall ethanol productivity may follow, resulting in a lower total reactor volume [13].

Incomplete hydrolysis of the solid lignocellulosic fraction is a huge disadvantage during SSF [15]. Possible reasons for this include unproductive enzyme adsorption, enzyme deactivation and increased crystallinity with the conversion of pretreated cellulose or decreased availability of chain ends [15].

One of the main drawbacks of SSF involve the optimum temperature for enzymatic hydrolysis being higher than the temperature used for fermentation [15], [17], [39]. A compromise thus had to be found between the optimal temperatures for the yeast and cellulolytic enzymes [15]. As the optimal temperature for *S. cerevisiae* is at 30 ^{o}C and the temperatures for cellulolytic enzymes are around 55 ^{o}C, a temperature of 37 ^{o}C was regarded as a suitable solution [15]. The ethanol yield within the fermentation process can be improved by making use of thermotolerant yeasts, e.g. *Kluveromyces marxianus* [39]. Advantages of these yeasts include rapid growth at temperatures above 40 $^{\circ}$C, high saccharification yields as well as a decrease in the risk of contamination [39].

Ethanol concentrations that enter the distillation stage should be at least 4 % ($v.v^{-1}$) in order for the process to be economically feasible [8]. For the majority of lignocellulosic materials, this would require solid concentrations of 16 % ($dw.v^{-1}$) or above [8]. In a SSF process, this is one of the many technical difficulties that will have to be overcome, since hydrolysis at such high solid contents may lead to problems related to sufficient mixing and mass transfer [8]. Application of new configuration processes, including previous prehydrolysis at higher temperatures to those applicable in a SSF, would ensure optimal activity [156]. The prehydrolysis allows for a rapid reduction in viscosity of the high solid content substrate and enables better homogenisation for the inoculation of the fermenting organism [13].

It, however, remains a challenge to produce high concentrations of ethanol [13], [15]. A few ways to achieve this include: performing enzymatic hydrolysis or SSF at high concentrations of dry mass, utilising all sugars (including fermentation of pentoses) that are present in the pretreated material, which will lead to increased ethanol concentrations along with decreased production costs and lastly, to separate the solid and liquid fractions and to only use the solid fraction for the production of ethanol [13].

2.7.3 Simultaneous saccharification and co-fermentation

The SSCF process represents greater integration than SSF [25]. The pretreated lignocellulose (slurry) containing the hemicellulosic-derived sugars and the cellulose-enriched fraction are hydrolysed and fermented simultaneously within a single unit [25]. The use of the same unit, coupled with the circumvention of filtering the slurry and reduction of waste streams, offers operational saving costs. A key factor in this process is the utilisation of an ethanol-producing micro-organism that can efficiently assimilate pentoses (xylose), released as a result of the hydrolysis of hemicelluloses during pretreatment, along with hexoses (glucose) from enzymatic hydrolysis [25]. The co-fermenting micro-organisms that are used need to be compatible in terms of operating temperature as well as pH, and must be able to utilise glucose and xylose simultaneously under micro-aerobic or anaerobic conditions [17].

Although progress is rapid within the field of xylose fermentation, a few industrial yeast strains still have to prove their capability to efficiently ferment xylose in lignocellulosic hydrolysates [15]. Results on the SSF of lignocellulosic materials have primarily been reported for the industrial pentose fermenting *S. cerevisiae* strain TMB3400 [15]. Other pentose utilising yeasts such as *Pichia stipitis* have also been evaluated in SSCF, but higher yields as well as ethanol concentrations were still achieved with *S. cerevisiae* TMB3400 [15].

2.7.4 Consolidated bioprocessing

Another process that is receiving increased recognition as a prospective breakthrough for low-cost biomass processing and thought to be the future of lignocellulosic ethanol, is CBP or direct microbial conversion (DMC) [3], [17], [95]. In this process the production of saccharolytic enzymes, the hydrolysis of the polysaccharides present in pretreated biomass, as well as the fermentation steps are carried out in the same reactor by a single microbe or microbial consortium, able to ferment both pentose and hexose sugars [3], [95].

Currently, there is no natural micro-organism with the necessary traits for CBP although significant research is on-going for their development, such as engineering an existing ethanologen micro-organism for enzyme production or engineering an enzyme producer with the ability of fermentation. The use of Prokaryotic CBP microbes such as *Zymomonas mobilis* is better suited when using bacterial enzymes, whereas a eukaryotic CBP microbe like *S. cerevisiae* will work better for fungal enzymes [3]. *S. cerevisiae* is also an interesting

micro-organism for CBP because of its general robustness in industrial process conditions [157].

Ideally, CBP would require no costs for the purchase of enzymes or the production thereof [17]. In practise, not all the required enzymes and/or the required dosages for complete conversion can be produced by the engineered microbe. Nonetheless, the use of such micro-organism will allow for significant reduction of the externally added enzymes. It is, however, not an efficient process due to the long fermentation periods and poor ethanol yields involved [17].

2.8 Commercialisation of Second Generation Ethanol

The transition to a new economic model that incorporates renewable energy such as biomass is driven by the development of alternative and renewable sources of energy to partially or totally replace fossil-derived fuels in order to reduce global warming and guarantee national energy supply. Liquid biofuels obtained by biomass transformation are one of the few alternatives to substitute petrol in the transport sector at larger scale, given its abundance and its reasonable compatibility to the current distribution systems.

However, biofuels production faces numerous obstacles that hamper their incorporation into the energy mix. In order to promote the use of bio-energy such as biofuels, it is necessary to identify the opportunities of such systems and the barriers within a specific country. Although many of these barriers are common for all renewable energies, those involved in the biomass production chain is more complex. The biomass deployment consists of several steps: biomass production, harvesting, its distribution and transformation by multiple conversion technologies in order to provide the specific energetic service. This complex chain implies the collaboration of numerous and different sectors (energetic, transport, agricultural and forestry industries, automobile industry, research and development, etc.) whose cooperation is paramount to implement their production at larger scale. Besides the previous barriers, there are other more specific for 2G ethanol owing to the intrinsic nature of the biomass. Lignocellulose conversion to ethanol requires a complex processing technology compared to that applied on sugar-rich or grain biomass for 1G bio-ethanol production [152]. Although this fact has held back the commercial production of 2G bio-ethanol, the continuous progress in research and development is highlighted by the

increasing number of on-going projects and large scale facilities in construction. The current and planned facilities for commercial production of 2G ethanol are listed in Table 8.

Table 8: Commercial plants for second generation (2G) ethanol production (Table adapted from [158]).

Company/Project	Commercial Plants	Millions of gallons						Location	Feedstock	Technology
		2012	2013	2014	2015	2016	2017			
Abengoa	3		15 - 26					USA, Brazil	Corn stover, bagasse	Enzymatic hydrolysis
Beta-Renewables	2		20					USA, Italy	Mixed biomass	Enzymatic hydrolysis
BlueSugars	1	-	-	-	15			Brazil	Stover	Enzymatic hydrolysis
BlueFire Renewables	1				19			USA	Municipal Solid Wastes	Acid Hydrolysis
BP Biofuels	2		36			36 - 72		USA	Bagasse, miscanthus	Enzymatic hydrolysis
COFCO/Sinopec	1		13	26				China	Corn stover	Enzymatic hydrolysis
Dupont Biofuels Solutions	2		27.5					USA	Corn cob	Enzymatic hydrolysis
GraalBio Investimentos	6	-	21.6					Brazil	Bagasse	Enzymatic Hydrolysis
Inbicon	2	-	-		18 - 19			Denmark	Straw	Enzymatic Hydrolysis
Lignol	1	0.02						Canada	Wood wastes	Enzymatic Hydrolysis
Mascoma	1	-		20	40			USA	Hardwood	Consolidated Bioprocessing
POET-DSM	9	-	20	25				USA	Corn stover, cobs	Enzymatic Hydrolysis
MO Renewables - COFCO/CNOOC	1	-			10 - 12			China	Cellulosic Ethanol	Enzymatic Hydrolysis
Weyland / Statoil Hydro	1	-			5 - 8			Norway	Cellulosic Ethanol	Enzymatic Hydrolysis

The biochemical production of ethanol from lignocellulose primarily involves the following steps: pretreatment, enzymatic hydrolysis and fermentation. The major limitations when applying enzymes in the breakdown of celluloses are therefore the high costs involved (primarily due to the excess amount thereof that is needed to compensate for its low specificity) as well as the extensive processing time that is required (because of the chemical and physical recalcitrance of lignocellulose along with the slow hydrolysis rates to obtain degradation results that are reasonable) [3], [111], [159]. A recent NREL report stated that enzymes can contribute up to 25 % of the conversion cost (which excludes the cost of the feedstock) [111].

As the cost of enzyme production and its use are one of the major contributors in the final cost, substantial research is being directed towards the improvement of enzymatic hydrolysis. Among the different areas of research and aspects which are directly involved in the hydrolysis efficiency, the following should be pointed out [160]:

- Search of new feedstocks with desirable properties for the process: higher productivity per hectare, low inputs requirement for production, high carbohydrate content, improved digestibility, etc.
- Enhancement of pretreatments and its efficiency so that there is a reduction in total enzyme requirements for carbohydrate hydrolysis. The partial removal or relocation of lignin during pretreatment minimises the unproductive binding of cellulases.
- Near-site or in situ enzyme production.
- Reduction of enzyme production cost:
  - Improvements in cellulase expression [112]
  - Use of cheaper carbon sources, including lignocellulosic substrates.
- Reduction of the enzyme dosage by:
  - Search for new enzymes with improved properties or improvement of properties of existing enzymes: higher thermostability and specific activity, optimal binding capacity (i.e. presence of CBD to facilitate adsorption to crystalline cellulose but also contributes to unproductive binding to lignin), and low susceptibility to end-product inhibition, inhibitors generated during pretreatment and the ethanol from fermentation.

- Enhancement of enzyme synergism by development of tailor-made enzyme combinations adapted to specific feedstock-pretreatment. The boost effect of enzymes in the appropriate ratio can lead to reduction of the final protein concentration needed for complete hydrolysis of the carbohydrates. These enzyme combinations can incorporate not only cellulases but also a set of accessory enzymes (hemicellulases, ligninases) that promote cellulose conversion.
- Reduction of unproductive binding to lignin by supplementation with non-catalytic additives such as surfactants, polymers and proteins.
- Process integration:
  * Application of a CBP configuration where the externally added enzyme is reduced partially.
  * SSF alleviates end-product inhibition of cellulases.
- Improvements in cellulose hydrolysis rates by optimising reaction conditions via process engineering [113].

Regarding the fermentation step, research and development is driven towards increments in ethanol yield, productivity and final ethanol concentration. A minimum ethanol concentration of 4 % ($v.v^{-1}$) has been established for an economically feasible distillation [161]. Concentrations higher than 15 % ($dw.v^{-1}$) of pretreated fibers are required to provide enough glucose that could yield this bench mark. Performing hydrolysis and/or fermentation using the whole slurry at high solid loadings leads to higher sugar concentrations and therefore ethanol concentrations. Given that the slurry contains hexoses (glucose, galactose, mannose) and pentoses (xylose, arabinose), the development of micro-organisms with the ability to ferment them all will improve the lignocellulose conversion to ethanol. The slurry, however, contain also compounds that are toxic for the fermentative micro-organism, therefore another trait that is being targeted, is increased tolerance to inhibitors produced during pretreatment.

In terms of process configurations, the SSF process has been recognised as a more efficient route to accomplish enzymatic hydrolysis and fermentation [160]. This SSF configuration process reduces the end-product inhibition of the enzymes by the continuous uptake of the sugars by the fermentative micro-organism. However, high solids loading

entails problems of mixing and high viscosity that are being addressed by development of bioreactors with advanced mixing capacity and reduced energy consumption [162], by operating fed-batch processes [163] and application of a liquefaction step at higher temperatures prior to SSF [164].

Overall, the economic feasibility of conversion technology of lignocellulose to ethanol must entail achieving a high ethanol yield at high solid loadings and reduced enzyme requirements over short periods of time.

2.9 References

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3. Aims and Interest of the Study

The present master thesis has been carried out within the area of research of *Bioprocess Engineering* of the Department of Process Engineering and the *Biofuels Research Chair* of Stellenbosch University, based on biomass conversion by fermentation processes to produce fuels and chemicals. The thesis was framed within the larger research project entitled “Pretreatment, hydrolysis and fermentation of sorghum and triticale lignocellulosic biomass for cellulosic bio-ethanol production” and financed by the Technology Innovation Agency (TIA).

Current commercial production of ethanol, 1G ethanol, is based on sugar- and starch-rich feedstocks designated to the food market [1]. Regardless of the available technology, 1G ethanol is considered to be of restricted benefit and prospective in the long term, owing to several potential negative impacts such as competition for arable land and resources (water, fertilizers, etc.) with food crops, limited production capacity in agriculture to guarantee biomass supply, reduction of biodiversity, etc. [2]. In this context, the utilisation of other plant residues and feedstocks could help to mitigate the limitation of land and biomass availability, and thus represents a better alternative for large scale bio-ethanol production than just 1G technologies [2]. Bio-ethanol that is produced from such plant residues and feedstocks, in particular lignocellulose, is referred to as 2G bio-ethanol or cellulosic ethanol [3]. Lignocellulose is in much greater abundance in nature than the feedstocks used for 1G biofuels production [4]. Moreover, lignocelluloses can be generated as residue from activities of agricultural, forestry and municipal sectors, or from dedicated energy crops grown on marginal lands [5]-[7]. Furthermore, 2G biofuels have been proven to provide additional environmental benefits such as GHG emission reduction [3], [8].

Economical and efficient conversion of polysaccharides of plant cell walls into their components (monomers) is crucial for the integral use of the lignocellulosic feedstock [3]. Their intrinsic resistance to breakdown is a major bottleneck for the development of 2G biofuels production based on biochemical technologies [9], [10]. Efficiency of cell wall saccharification is influenced by many factors comprising feedstock properties, type and conditions of pretreatment and hydrolysis conditions, including the enzyme combinations

employed (Figure 13). In this context, the main objective of the larger TIA project was to maximise the ethanol production from sorghum bagasse and triticale straw in an integrated manner, by optimising each of these steps: selection of varieties, pretreatment conditions, enzyme combinations and fermentation strategies.

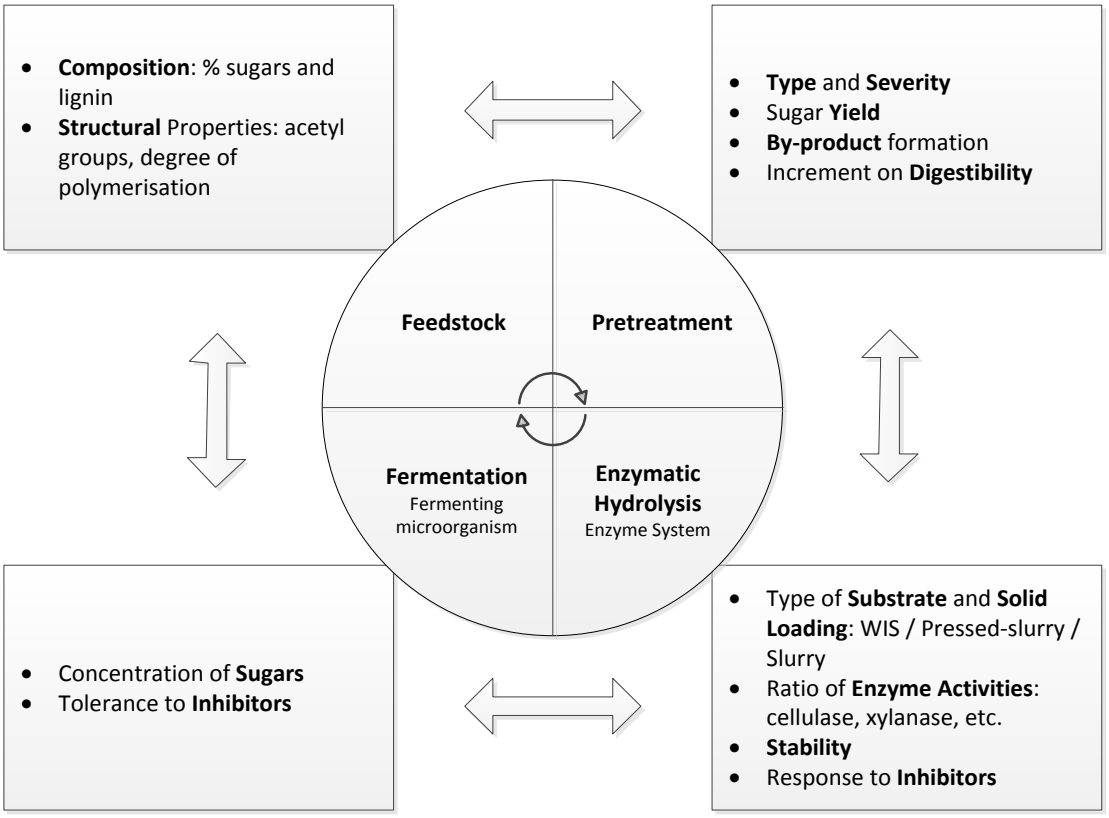

Figure 13: Interaction between the different steps and elements in an enzyme-based process for production of second generation (2G) ethanol.

Different kinds of lignocellulosic feedstocks can be used for bio-ethanol production, such as perennial grasses, wood biomass (hardwood or softwood) or crop residues. The projected increment in bio-ethanol demand worldwide makes the extension of raw material sources with less expensive feedstocks, necessary. Alternative crops that are adapted to climate and soil conditions are also preferable. In this study, lignocellulosic residues from sorghum and triticale, bagasse and straw respectively, were selected as raw materials. These feedstocks offer several advantages over conventional crops. For example, triticale is able to grow in marginal soils with minimum nitrogen inputs. Similarly, sorghum presents high biomass yields with limited use of water.

Figure 14 highlights the most important aspects of each step in bio-ethanol production.

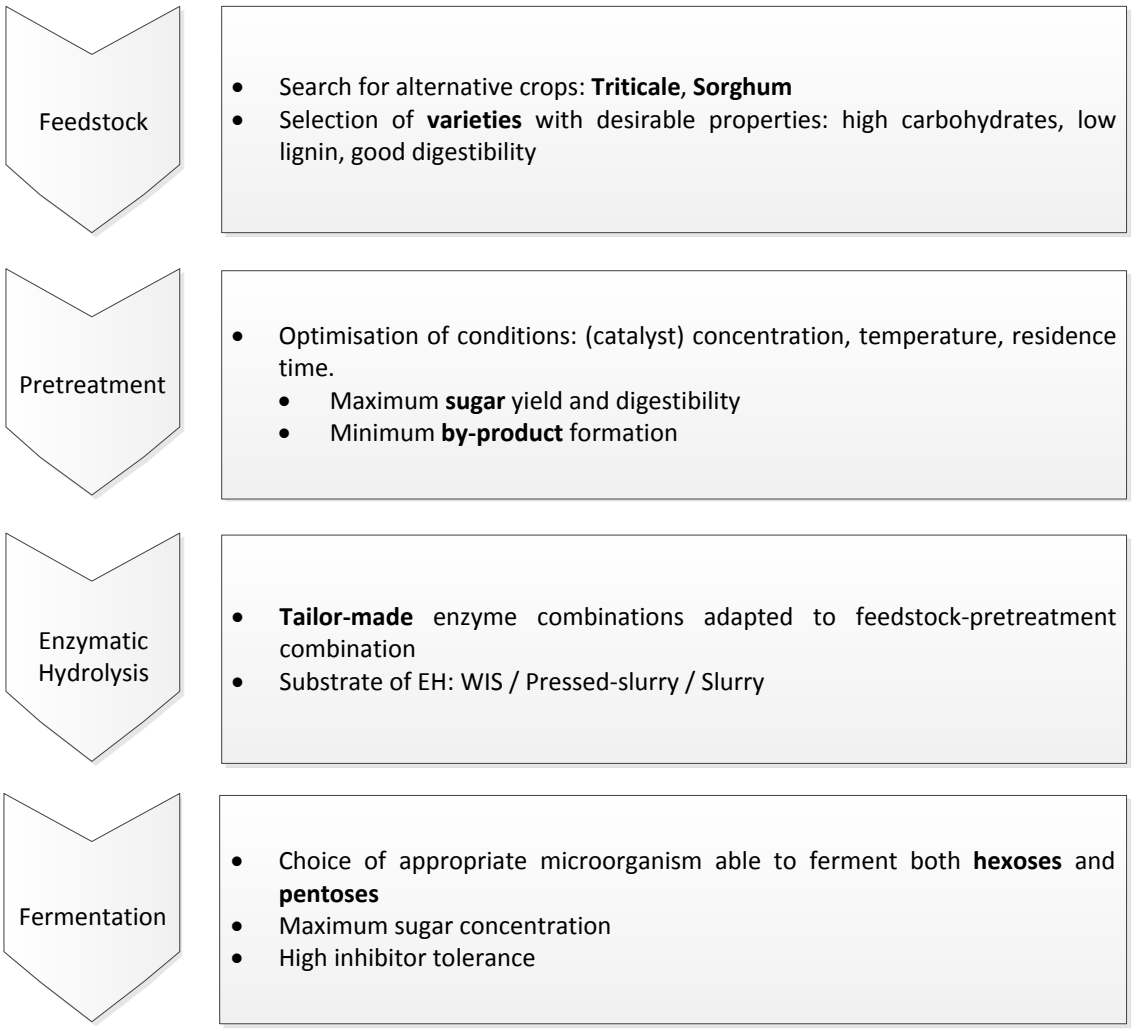

Figure 14: Aspects that impact on the feasibility of the commercial production of second generation (2G) ethanol that were considered within the larger research project to which the present study contributed. This thesis is focussed on the enzymatic hydrolysis part.

The composition and structure of lignocelluloses is variable due to the intrinsic heterogeneity of the biomass coupled with other factors that affect the composition of the same feedstock such as age, harvest period and tissue. The selection of the varieties, which are more digestible, can help in developing a cost-efficient pretreatment and saccharification process. This study is based on selected varieties of sorghum and triticale based on carbohydrate content and their response to standard pretreatment.

The next step towards reducing cost is the further optimisation of pretreatment conditions of selected varieties in terms of sugar recovery and polysaccharides conversion. Steam-explosion pretreatment was chosen since it has shown to be effective in herbaceous biomasses. Optimised pretreatment conditions were applied to the selected varieties. The pretreated material or slurry consists of a solid fraction, also designated as water-insoluble solids (WIS), enriched in cellulose and lignin and a liquid fraction or pre-hydrolysate containing the sugars solubilised during the pretreatment, mainly hemicelluloses-derived sugars. Depending on the severity of the pretreatment, the sugars can be further degraded into furans that, coupled with the solubilised lignin and acetic acid released from the hemicelluloses, impact negatively on the biological transformation. Although current research is focussed on the development of detoxification processes and robust micro-organisms that are able to effectively employ hexoses and pentoses of the slurry into bio-ethanol, the majority of the studies still separate the slurry in the two fractions in order to optimise the conversion of each one into biofuels. Moreover, the solid fraction is subjected to a washing step to remove residual inhibitors that remain soaked in the fibers. However, in an industrial process the use of the whole slurry should be desirable since the filtration and washing steps are avoided with subsequent cost savings. One of the aspects that was addressed in this thesis is the use of three different substrates, namely WIS, pressed-slurry and whole slurry to determine its impact on enzyme saccharification and sugar yield.

Improvements on pretreatment technologies and reduction on enzyme production costs through biotechnology have led to several-fold reduction of enzyme loading for lignocellulose hydrolysis. Nonetheless, enzyme production and its use are still considered one of the main cost contributors of cellulosic ethanol production processes. Several strategies have been proposed to reduce the amount of enzyme required for complete enzymatic hydrolysis. These strategies can be grouped in terms of enhancement of the enzyme systems and process development. The first group includes development of enzyme-producing strains with improved protein-titres, development of enzymes with improved specific activity by protein-engineering of known enzymes or by bio-prospecting of new enzymes. Alternatively, there is a trend to use tailor-made enzyme combinations adapted to feedstock and pretreatment. In this approach, combinations of different enzyme preparations (characterised as cellulase, xylanase, pectinase, etc.) or individual components

and their ratios can be studied and optimised statistically for a particular biomass-pretreatment combination. Further, the supplementation with non-catalytic additives such as surfactants, polymers and proteins has also been shown to enhance enzyme performance and, therefore, reduce the enzyme loading. In this study, new commercial enzyme preparations of cellulases, xylanases and pectinases, and the surfactant Polyethylene Glycol (PEG)-4000 were evaluated in enzymatic hydrolysis of steam-exploded triticale straw and sorghum bagasse and compared with a control enzyme combination. The best enzyme preparations were optimised by experimental design in order to reach a target cellulose conversion of at least 80 %.

A number of factors that have an influence on enzymatic hydrolysis are illustrated in the diagram in Figure 15.

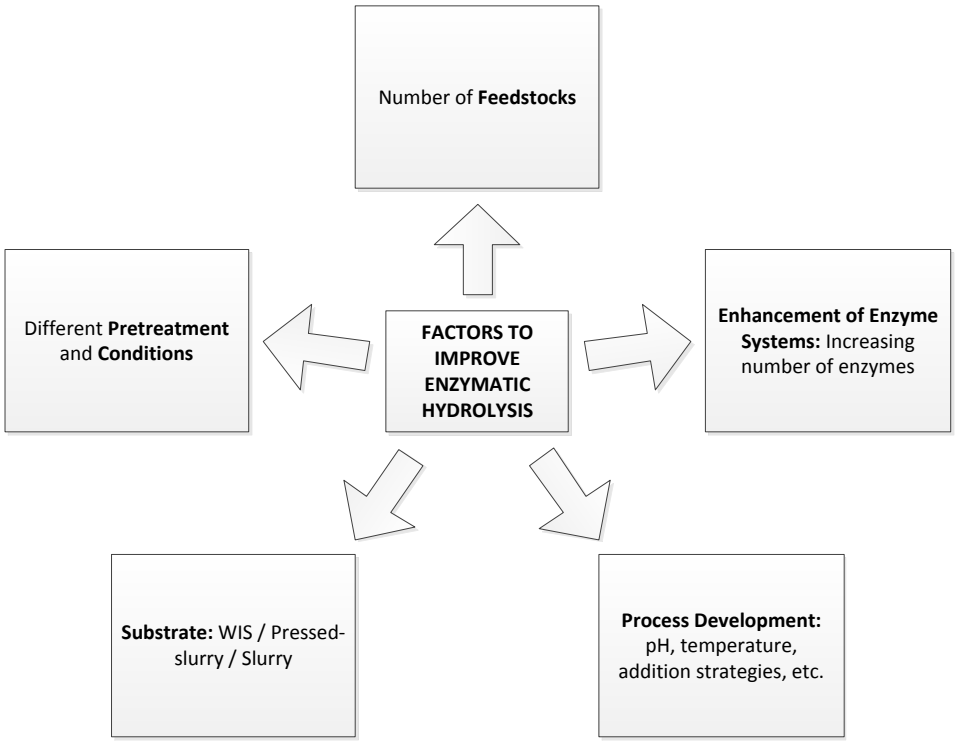

Figure 15: Factors that contribute to efficient enzymatic hydrolysis.

Based on the previous considerations, the general aim of this thesis is the optimisation of enzymatic hydrolysis integrated with feedstock and pretreatment condition selection, thus the optimisation of enzyme combinations for selected varieties of sorghum bagasse and triticale straw pretreated under optimum conditions of SE. With the former as main task, the following studies were carried out:

1. Enzyme characterisation of conventional and new commercial enzyme preparations in terms of enzyme activities on model substrates (filter paper, carboxy-methyl cellulose (CMC), xylan, cellobiose, mannan) and protein concentration (see Chapter 5).
2. Evaluation of new commercial cellulase preparations in enzymatic hydrolysis of steam-exploded triticale straw and sorghum bagasse (see Chapter 6, Addendum A).
3. Application of a fractional design to determine the effect of accessory enzymes (xylanase, pectinase) and surfactants (PEG-4000) that significantly increase the enzymatic conversion of cellulose by cellulases (see Chapter 6, Addendum A).
4. Optimisation of a combination of enzyme preparations selected in the fractional design by central composite design (CCD) in order to reach a considerable good cellulose conversion (target of 80 %) with minimum enzyme loading.
5. Study the effect of solids loading with the selected enzyme combination (see Chapter 6, Addendum A).

The growing amount of enzymes involved in the degradation of lignocellulose, along with the amount of factors that should be taken into account when optimising enzymatic hydrolysis of specific biomass-pretreatment combinations, requires fast and accurate evaluation methods of enzymatic hydrolysis. Performing enzyme activity assays in model substrates such as filter paper, cellobiose or Birchwood xylan is not representative of the real hydrolytic potential of enzyme preparations on lignocelluloses [11]. The enzyme requirements will, however, also differ depending on the specific feedstock, selected pretreatment and conditions. In this context, the application of micro-assays using lignocellulosic substrates could save reagents, time and therefore costs, thereby allowing the study of multiple factors [11]. The main challenge in the development of such systems derives from the heterogeneous nature of lignocellulose, which hinders the ability to have representative samples and its handling at milligram scale [12]. There have been many attempts to develop high-throughput systems where the lignocellulose biomass is ground or milled fine (0.25 - 0.5 mm) in order to facilitate the distribution of the biomass [12]–[15]. However, the milling alters the structural properties of the initial substrate, possibly improving its digestibility and thereby masking possible differences in performance among enzymes combinations and/or feedstock-pretreatment combinations [12].

One of the activities developed within the thesis is to set up a micro-assay methodology based on handsheets to study new enzyme preparations as well as the digestibility of steam-exploded agricultural by-products during enzymatic hydrolysis. The main objective is to determine if such a method is able to distinguish between different feedstocks, pretreatments and enzyme combinations. This part of the thesis included the following studies:

1. Application of a homogenisation step of the WIS fraction in a liquidiser at different rpm prior to handsheets-making to determine its effect on (see Chapter 7):
    a. Composition
    b. Fiber distribution
    c. Material digestibility.
2. Compare results of cellulose conversion between micro-scale and small scale with the conventional and the optimised enzyme combination when using WIS, pressed-slurry and slurry as substrate (see Chapter 7).

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4. Materials and Methods

The methodologies applied for the studies carried out in this thesis are schematised in Figure 16. Selected varieties were pretreated under optimum conditions. The slurry attained was pressed to obtain solid and liquid fractions. The pressed-slurry was further washed to remove inhibitors. The slurry, pressed-slurry and water-insoluble solids (WIS) were subjected to enzymatic hydrolysis with different dosages of conventional and new commercial enzyme preparations. The best enzyme preparations were further optimised by experimental design.

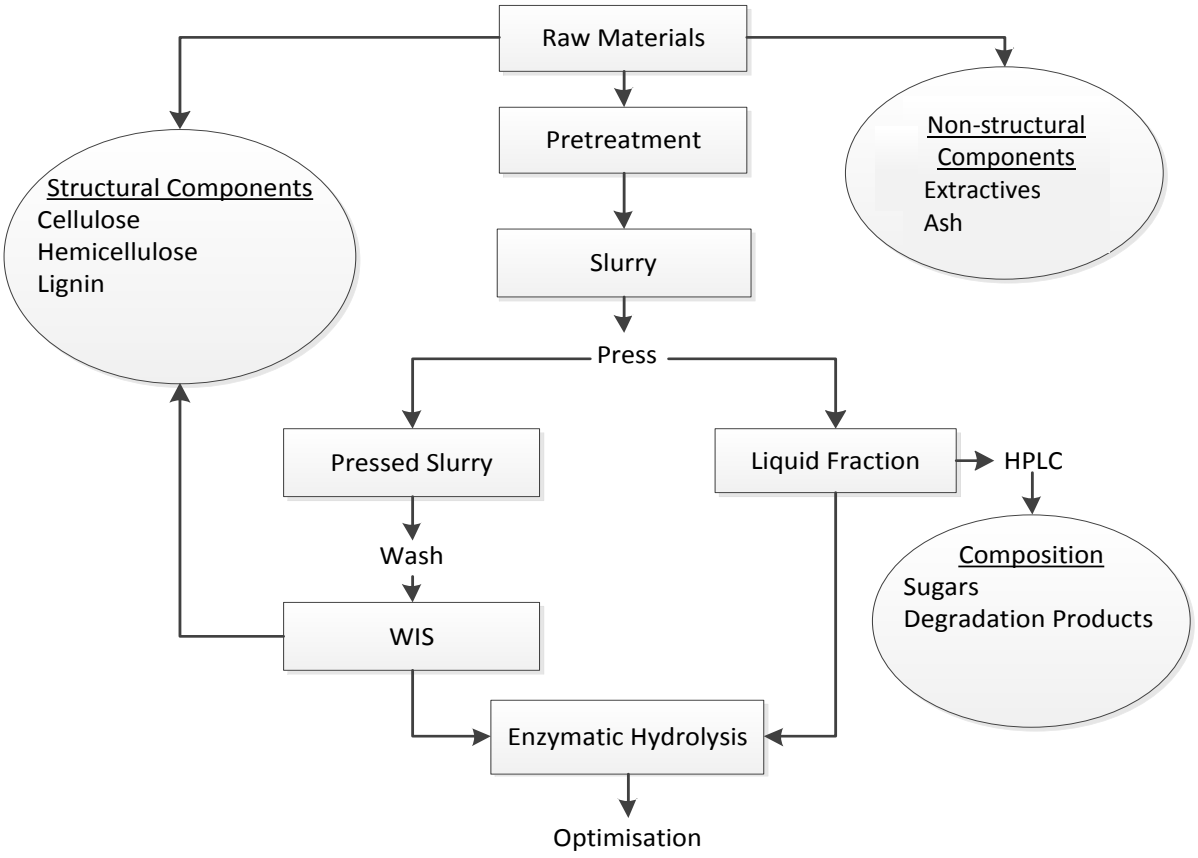

Figure 16: Simplified scheme of the experimental layout used in this thesis.

4.1. Enzyme Preparations

The enzyme preparations used in this study are listed in Table 9. Companies such as Genencor, Novozymes and Dyadic have developed different enzymes for application on lignocellulosic materials for bio-ethanol production. The enzyme preparations employed include cellulases (Cellic® CTec2, Spezyme CP, Accelerase 1500, Optiflow, Alternafuel CMAX), beta-glucosidase (Novozyme 188), xylanases (Cellic® HTec2, Multifect Xylanase), pectinase (Pectinex Ultra) and laccase (LACCASE).

Table 9: Enzyme preparations evaluated in this study.

Company	Name of enzymes formulation	Main Activity	Remarkable properties
Novozymes	Novozymes 188	β-glucosidase	CONTROL combination Enzyme combination that has been used by lignocellulosic group Dosage: 15 FPU + 15 IU β-glucosidase.g^{-1} WIS
Genencor	Spezyme CP*	Cellulase + β-glucosidase	
Novozymes	Cellic® CTec2	Cellulase + β-glucosidase	Developed for an industrial process (expected very good performance)
	Cellic® HTec2	Hemicellulase	
Genencor	Accelerasse 1500	Cellulase + β-glucosidase	Improved cellulase
	Optiflow*	Cellulase + β-glucosidase	No need for β-glucosidase supplementation
Dyadic	Alternafuel CMAX (Liquid)	Cellulase + β-glucosidase + hemicellulase	Cellulase with accessory enzyme activities: hemicellulases.
	Alternafuel CMAX (Powder)		
Genencor	Multifect Xylanase	Xylanase	Synergism with cellulase
	Pectinex Ultra	Galacturonidase	
ZA Biotech	Laccase	Ligninase	Possible use to reduce toxicity of pretreated material

* FPU – Filter Paper Units
* IU – International Unit

4.2. Enzyme Characterisation

4.2.1. Activity determination

Most of the enzyme preparations are classed as cellulases, but they are normally comprised of diverse enzymes. For example, for cellulose degradation enzymes such as endo-glucanases, cellobiohydrolases and β-glucosidases are required. Moreover, the enzyme preparations generally include side activities involved in the degradation of other polysaccharides. Therefore, the hydrolytic potential of the different enzyme preparations was evaluated in model substrates. It is worth noting that although these assays do not represent the hydrolytic capacity of real lignocellulosic substrates, they are useful in terms of determination of the required dosages and comparison with other studies.

The assays applied to establish the cellulolytic capacity of an enzyme system were based on filter paper, carboxy-methyl cellulose and β-glucosidase. The determination of these activities was carried out according to the International Union of Pure and Applied Chemistry (IUPAC) [1]. Likewise, the hemicellulolytic capacity of the preparations was evaluated in xylan [2] and mannan [3].

The enzyme activity unit (IU) is defined as the activity of the enzyme responsible for the formation of 1 µmol.min^{-1} of reducing sugars, measured as glucose/xylose/mannose equivalents, under the specific conditions of the assay.

4.2.1.1. Cellulase activity on filter paper

The enzyme preparations were first diluted with 0.05 M citric acid buffer with a pH of 5.0. The buffer was also supplemented with 2.0 % sodium azide to prevent any microbial growth. The enzyme preparations were then incubated for 1 h at 50 °C in the presence of 50 mg of Whatman n°1 filter paper [1], [4]. Thereafter, the enzyme reaction was stopped by the addition of 3 mL dinitrosalicylic acid (DNS). The samples were then boiled for 5 minutes, which unleashes the colorimetric reaction. After cooling down in cold water and waiting for the pulp to settle, 200 µL of the reaction was mixed with 2 mL of distilled water. The absorbance of the samples was then read with the help of a spectrophotometer at a wavelength of 540 nm.

The concentration of reducing sugars, as equivalents of glucose, was determined by the DNS method [5]. Owing to the absence of linearity between absorbance and high concentrations of reducing sugars, the enzyme preparations need to be diluted to a concentration that will release an absolute amount of 2 mg of reducing sugars for the described assay conditions. At these concentrations, the relation between absorbance and glucose concentration is approximately linear. These amounts correspond to 0.37 µmoles of glucose released per minute and millilitre of dilution (0.37 $IU.mL^{-1}$ of enzyme dilution).

4.2.1.2. Activity on cellobiose (β-glucosidase)

Different dilutions of the enzyme preparations were incubated with a 25 mM cellobiose solution for 30 minutes [1], [4]. Thereafter, the reaction was stopped by boiling the samples for 5 minutes. The residual cellobiose and the glucose released were determined by high-performance liquid chromatography (HPLC) analysis as described in section 4.11. In this case, the enzyme preparation needed to be diluted with 0.05 M citrate buffer (pH 5.0) in order for it to release 1 mg of glucose (absolute amount). This corresponded to 0.0926 µmoles of glucose released per minute and mL under the described conditions (0.0926 IU β-glucosidase per mL of dilution).

4.2.1.3. Activity on carboxy-methyl cellulose

For activity determination on carboxy-methyl cellulose (CMC), the enzyme preparations were first diluted prior to incubation at 50 ˚C in 0.05 M citrate buffer (pH 5.0) in the presence of CMC at 0.5 % ($w.v^{-1}$) [2]. After 5 minutes, the reaction was stopped by the addition of DNS and boiled for 5 minutes. The absorbance of the sample was measured at 540 nm after cooling down in a cold bath. This unit of CMCase activity corresponded to the production of 1 $\mu mol.min^{-1}$ of reducing sugars, measured as reducing glucose, under the described conditions.

4.2.1.4. Activity on hemicellulose

The hydrolytic potential on xylan [2] and mannan [3] was also determined. Different dilutions of the enzymes were incubated in the presence of commercial Beechwood xylan (1 % $w.v^{-1}$) or commercial mannan (0.25 % $w.v^{-1}$) for 5 minutes. Reactions were stopped by the addition of DNS and boiled for 15 minutes. After cooling down, the absorbance was

measured at 540 nm. The unit of activity of xylanase or mannanase corresponded with the production of 1 µmol.min^{-1} of reducing sugars, measured as reducing xylose or mannose, under the described conditions.

4.2.2. Protein content determination

The protein concentration of the enzyme preparations was determined using the bicinchoninic acid (BCA^{TM}) assay (Kit BCA-Compat-Able Protein Assay kit, ref 23229, Pierce, Rockford IL) with bovine serum albumin as standard [4]. Different dilutions of the enzyme preparations were firstly subjected to precipitation. The precipitates were re-suspended in ultrapure water and the samples were incubated with the protein reagent for 30 minutes at 37 °C. The absorbance was measured at 562 nm.

4.3. Raw Material

Sweet sorghum bagasse and triticale straw were provided by the University of KwaZulu-Natal (Ukhulinga Experimental Farm) and Department of Genetics at Stellenbosch University (Mariendahl Experimental Farm), respectively.

The average moisture content of both materials was very similar, approximately 7.3 %. The feedstock materials were coarsely ground with a Condux-Werk type mill (Wolfgang bei Honou, Germany) and sieved. The material with a particle size between 0.38 and 10 mm was collected for pretreatment. The chopped bagasse and straw were then sealed in plastic bags containing 600 g of raw material each and stored in a conditioning room (23 ˚C, relative humidity of 50 %) until further use. The composition of raw material was determined as described in section 4.5.1.

Commercial Avicel® PH Microcrystalline Cellulose, Beechwood xylan and mannan was used during enzyme characterisation. Avicel was also used as control during hydrolysis tests.

4.4. Pretreatment

Steam-explosion pretreatment was performed at Process Engineering by applying Masonite technology in a pilot SE unit operated by batches and equipped with a 19 L

reaction vessel, a boiler capable of supplying saturated steam up to 40 bars and a cyclone tank for decompression and collection of exploded material (Figure 17). The reactor was loaded with approximately 600 g (dry matter) of feedstock per batch. Thereafter, the material was directly heated with saturated steam to a temperature of 190 °C or 200 °C, for triticale and sorghum respectively, and maintained for 5 minutes before being suddenly depressurised.

Figure 17: Steam-explosion unit at the Process Engineering facilities.

After the explosion, the material (slurry) was recovered in a cyclone, cooled to about 40 °C and then pressed for liquid and solid recovery as illustrated in Figure 16. The pressed-slurry was thoroughly washed (10x the volume of the sample) with distilled water to obtain the WIS fraction. The slurry was characterised in terms of water-soluble solids (WSS), WIS and total solids content [6]. The WIS and liquid fractions were then subjected to composition analysis as described in section 4.5.

4.5. Raw Material and Pretreated Material Characterisation

Figure 18 illustrates all the analysis procedures to be done with the raw, pretreated and hydrolysed material.

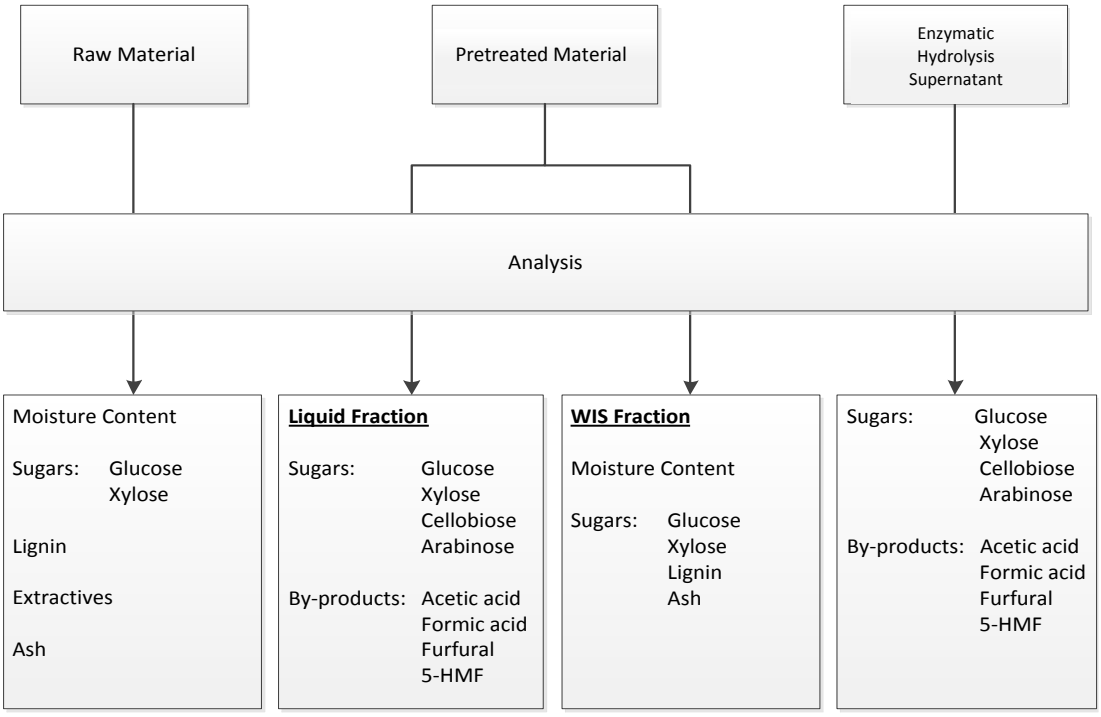

Figure 18: Analysis procedures of raw material, pretreated material and hydrolysed material (enzymatic hydrolysis supernatant).

4.5.1. Raw material/WIS

The composition of the raw material was analysed following the scheme in Figure 19 by using the laboratory analytical procedures (LAPs) of the NREL for determination of extractives, sugars, lignin and ash [6]–[10]. The same LAPs were used for the composition of the water-insoluble solids (WIS) fraction of the pretreated material, except for the extractives determination.

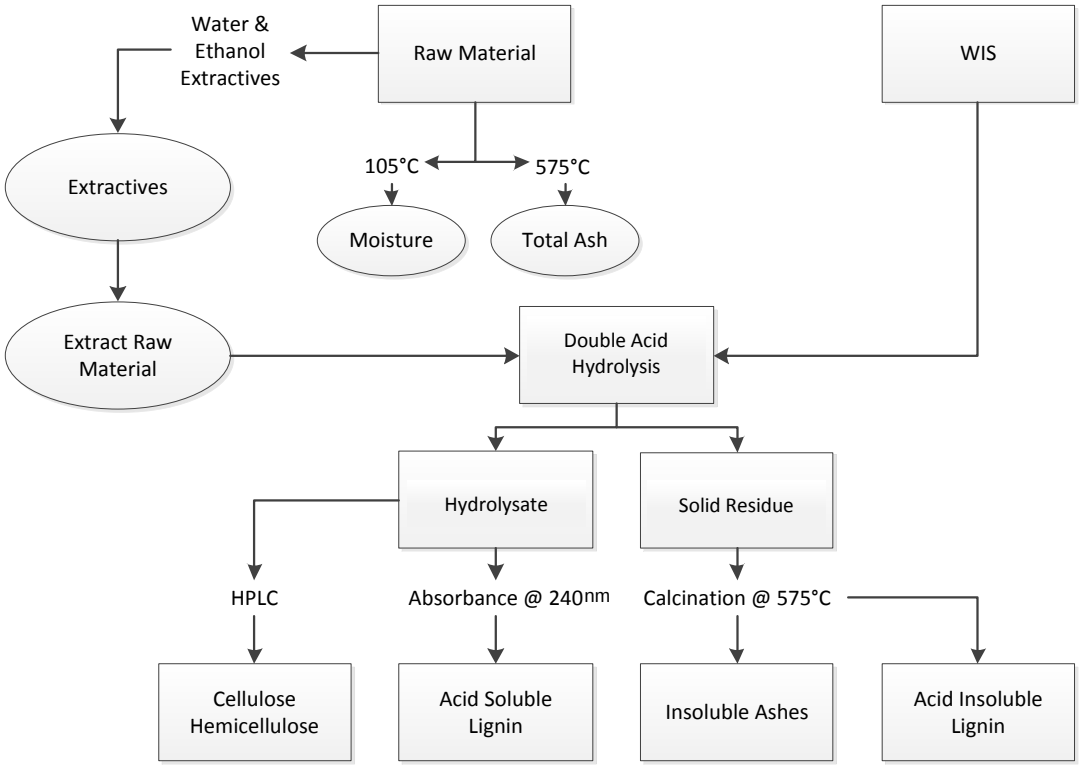

Figure 19: Procedure used for analysis of the composition of raw material and WIS [6]–[10].

Determination of the moisture content or total solids is necessary in order to discuss the results on a dry weight basis. The moisture content of the samples was determined by oven drying until constant weight was achieved at 105 °C (approximately 24 h).

The extractives are the soluble non-structural materials in a biomass sample and correspond with the fraction of biomass that is soluble in water and ethanol. They remain as residue after an exhaustive extraction in the Soxhlet (LAP-010) [9].

The determination of carbohydrates content is based on hydrolysis of the polysaccharides (cellulose and hemicellulose) into monosaccharides. The extractive-free samples are subjected to an initial acid hydrolysis with concentrated H_2SO_4 72 % ($w.w^{-1}$) followed by a second hydrolysis with diluted H_2SO_4 acid 4 % ($w.w^{-1}$). The hydrolysate is then analysed by HPLC as described in section 4.11. It is assumed that the sugars are in polymeric form in the raw material and WIS. For this reason, cellulose and hemicellulose content is estimated taking into account the weight of a molecule of water that is released during the formation of glycosidic links, by applying a conversion factor of 1.11 for hexoses and 1.13 for pentoses.

The acid soluble lignin is measured by Ultra Violet (UV)-Vis spectroscopy at 205 nm (LAP-003) [7]. The insoluble lignin is determined by weighing the remaining solid residue after total acid hydrolysis (LAP-004) [7]. In the case of ash content, which represents the mineral and inorganic material in the biomass, this method is based on calcination at 550 °C (LAP-005) [7].

4.5.2. Liquid fraction (monomers, oligomers, by-products)

Steam-pretreatment partially or totally solubilises the hemicellulose fraction into acetic acid, monomeric sugars (mainly xylose) and oligosaccharides of different DP. In order to determine the fraction of sugars in oligomeric form, the liquid fraction is subjected to a mild acid hydrolysis with H_2SO_4 at 4 % ($w.w^{-1}$) at 121 °C for 30 minutes. The liquid fraction was analysed for sugars, acetic acid and by-products (formic acid, furfural and 5-HMF) and its hydrolysate only for sugars [10]. The difference between total monomeric sugars before and after the hydrolysis was assumed to be in oligomeric form.

4.6. Enzymatic Hydrolysis Assays: Small scale

Small scale enzymatic hydrolysis studies were performed in 250 mL screw cap Erlenmeyer flasks (100 mL working volume) with 0.05 M citrate buffer pH 5.0 and the corresponding enzyme preparations and dosages. The flasks were incubated in a water bath or an incubator at a fixed temperature of 50 °C and agitation of 90 or 150 rpm, depending on the assay. Optimisation studies were conducted at a substrate loading of 2 % ($dw.v^{-1}$). The substrates employed were slurry, pressed-slurry or the WIS from steam-exploded triticale straw and sorghum bagasse. Additionally, control assays of enzymatic hydrolysis were performed using commercial cellulose (Avicel) as substrate. Samples were taken periodically over 120 h, centrifuged for 2 minutes at 10 000 rpm and the supernatant liquid was treated with 35 % ($w.v^{-1}$) perchloric acid (PCA) and 7 N potassium hydroxide (KOH) to precipitate proteins and other impurities. Removal of these substances helps extend the life of the column used during HPLC. Thereafter the samples were analysed by HPLC as described in section 4.11.

4.7. Handsheet Preparation

Handsheets of the WIS fraction were made according to a modified procedure from TAPPI [11] as illustrated in Figure 20.

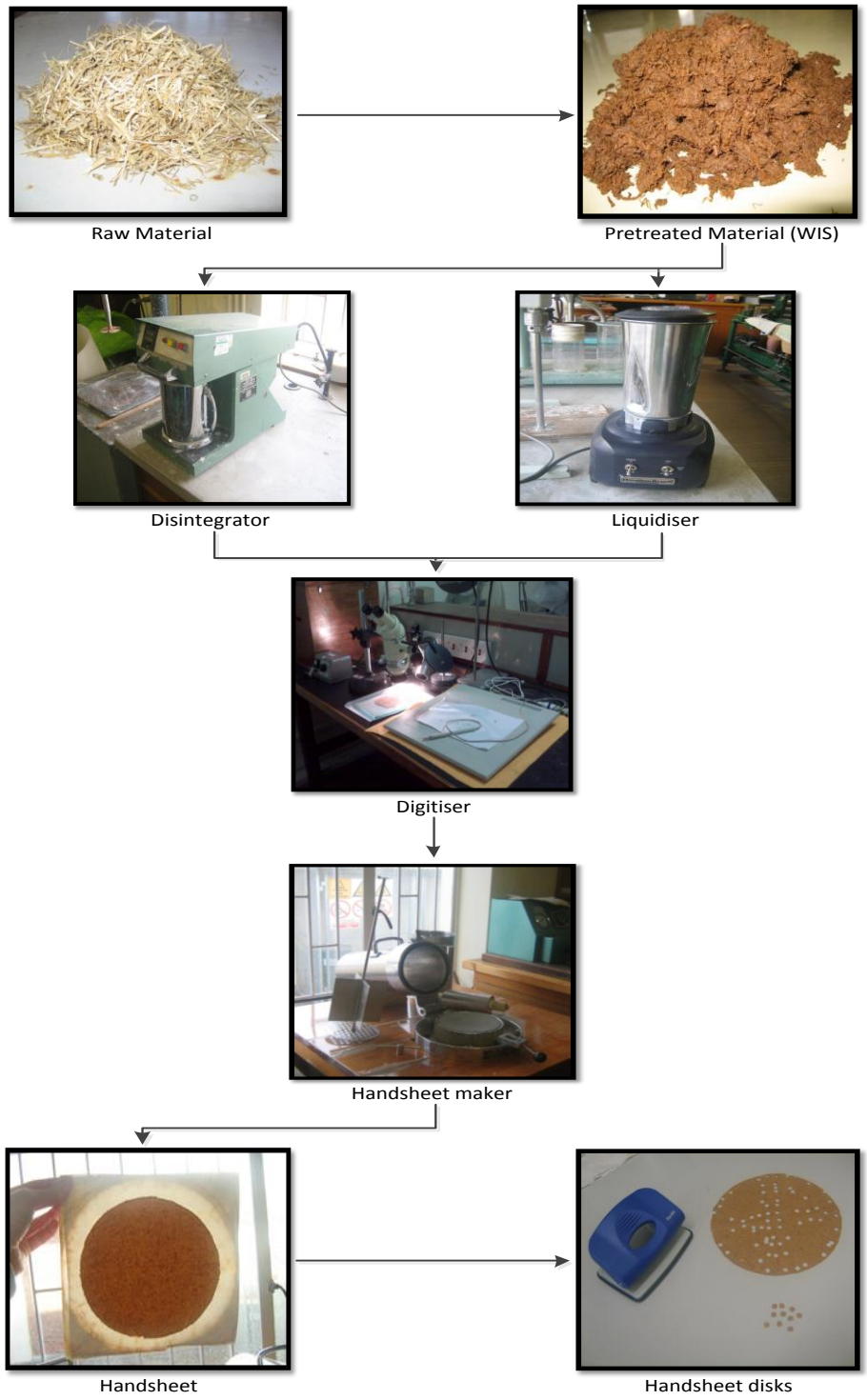

Figure 20: The modified version of the TAPPI standard methodology for making handsheets (adapted from the TAPPI standard methodology 1996 [11]).

The WIS was suspended in 2 L of water and subjected to different treatments to ensure homogenisation by combining the use of two pieces of equipment: a liquidiser (Hamilton Beach Rio^{TM} Stainless Steel Commercial Bar Blender Product Code: HBB 250S-RIO) and a disintegrator (British Pulp Evaluation Apparatus Manufactured by Mavis Engineering Ltd. London N.I. England No 7518 D). The different treatments are summarised in Table 10. Rpm was used as an indicative measure of the severity of the treatments.

Table 10: Different fractions of disintegrator and liquidiser up to 37 500 rpm.

Treatment	Disintegrator rpm	Liquidiser rpm
Untreated	-	-
1	37 500	0
2	31 250	6 250
3	25 000	12 500
4	18 750	18 750
5	12 500	25 000
6	6 250	31 250
7	0	37 500

The effect on fiber length and fiber distribution of these treatments was evaluated using a digitiser. In order to ensure that none of the previous treatments alters the digestibility, samples were used as substrate for enzymatic hydrolysis as described in section 4.8. Based on earlier results, the treatment 2 was selected and applied prior to preparation of handsheets based on the TAPPI standard methodology T205 sp-95 [11]. Handsheets were dried overnight at 23 ˚C with a relative humidity of 50 %.

The composition of the handsheets was determined as described in section 4.5.1. Small discs were perforated from selected handsheets and used as substrate for enzymatic hydrolysis at micro-scale (section 4.8).

4.8. Enzymatic Hydrolysis Assays: Micro-scale

Micro-scale enzymatic hydrolysis assays were performed in 2 mL Eppendorf tubes (0.5 mL working volume) with 0.05 M citrate buffer at pH 5.0, 50 °C and 800 rpm in a micro-plate incubator (Heidolph Incubator 1000). The small discs obtained with a paper perforator were used as substrate of the micro-assays. Figure 21 schematically represents the micro-scale setup.

Figure 21: A schematic representation of the hydrolysis setup at micro-scale; (A) the Heidolph Incubator 1000 and (B) the handsheet-discs (as substrate) and glass beads added in the 2 mL Eppendorf tubes.

Some of the experiments were supplemented with different volumes of liquid fraction to mimic pressed-slurry or diluted slurry with 2 % (dw.v^{-1}) WIS. A small stead bead was included in each assay to favour the mixing. At specific data points, samples were removed from the incubator and boiled to inactivate enzyme activity prior to HPLC analysis.

4.9. Calculations

4.9.1. Conversion rate

The conversion rate as g glucose.L^{-1}.h^{-1}.mL^{-1} enzyme was estimated according to Equation 1 [12]:

$$\textit{Conversion rate} = \frac{\left(\frac{[Glu]_{t3}-[Glu]_{t0}}{3}+\frac{[Glu]_{t6}-[Glu]_{t3}}{3}+\frac{[Glu]_{t12}-[Glu]_{t6}}{6}\right)}{(3\times(ED))} \quad (1)$$

Where:

$[Glu]_{ti}$	Residual glucose concentration (g.L^{-1}) at time point *i*
3, 3, 6 (numerator in main equation)	Amount of hours between time points (0-3; 3-6; 6-12 h)
3 (denominator in main equation)	Number of time intervals studied
ED	volumetric Enzyme Dosage (mL)

4.9.2. Cellulose and xylan conversion

The cellulose conversion as % of the theoretical yield (% digestibility) was calculated using Equation 2 [13]:

$$\textit{Cellulose conversion (\%)} = \frac{[Glucose]+1.053\,[Cellobiose]}{1.111\,f\,[Biomass]} \times 100\,\% \quad (2)$$

Where:

[Glucose]	Residual glucose concentration in the supernatant (g.L^{-1})
[Cellobiose]	Residual cellobiose concentration in the supernatant (g.L^{-1})
[Biomass]	Dry biomass concentration at the beginning of the hydrolysis (g.L^{-1})
f	Cellulose fraction in dry biomass (g.g^{-1})
1.053	Converts cellobiose to equivalent glucose
1.111	Converts cellulose to equivalent glucose

Similarly, the xylan conversion was estimated by using Equation 3:

$$\textit{Xylan conversion (\%)} = \frac{[Xylose]}{1.13\, f_x\, [Biomass]} \times 100\,\% \quad (3)$$

Where:

[Xylose]	Residual xylose concentration in the supernatant ($g.L^{-1}$)
[Biomass]	Dry biomass concentration at the beginning of the hydrolysis ($g.L^{-1}$)
f_x	Xylan fraction in dry biomass ($g.g^{-1}$)
1.13	Converts xylan to equivalent xylose

4.9.3. Degree of synergism

The degree of synergism (DS) between xylanase and cellulase enzymes was calculated using Equation 4 [14]:

$$DS = \frac{GC_{mixture}}{\sum GC_{individual}} \quad (4)$$

Where:

$GC_{mixture}$	Cellulose hydrolysis reached with the cellulase and xylanase together
$\Sigma\ GC_{individual}$	Sum of cellulose hydrolysis reached with the individual enzymes

4.10. Statistical Analysis

The optimisation of enzyme cocktails requires the use of an experimental design that is statistically valid. The experimental data from the CCD of sorghum was thus analysed with the use of the rigorous and user-friendly program Design Expert 8.0.4 (Stat-Ease Inc., Minneapolis, MN, USA). Response surface methodology (RSM) was applied to determine the functional relationship that exists between the two independent variables, Cellic® CTec2 and Cellic® HTec2 and the one response parameter, glucose concentration. The properties of this model were evaluated by means of analysis of variance (ANOVA) calculations.

The Design Expert 8.0.4 software was also used to determine the relationship between the variables Cellic® CTec2, Cellic® HTec2 and hydrolysis time in the triticale feedstock. Here, an equation in terms of actual factors was obtained with which the total amount of sugars present at different combinations of the two enzyme loadings could be determined. This equation was subsequently used in Microsoft Excel and together with Solver, 1000 combinations of the two enzymes, which gave maximum sugar yields at 80 % digestibility of the total amount of sugar present within the feedstock, was determined.

4.11. HPLC analysis

4.11.1. Sugars and by-products

The concentrations of monomeric sugars (arabinose, glucose and xylose), cellobiose, acetic acid as well as the by-products formic acid, furfural and 5-HMF could all be analysed by HPLC using an Aminex HPX-87H Ion Exclusion Column (Hercules, CA) fitted with a cation-H cartridge guard column (Hercules, CA)(Bio-Rad, Johannesburg, RSA). A Refractive Index (RI) detector (Waters 2141, Microsep, Johannesburg, RSA) was used to measure the sugars, whereas a UV detector set at 215 and 280 nm (Waters 2487, Microsep, Johannesburg, RSA) was needed to analyse the by-products. The operating conditions for the column were 65 °C with 5 mM H_2SO_4 at a flow rate of 0.6 mL.min^{-1} which functioned as the mobile phase.

The samples from enzymatic hydrolysis were subjected to a treatment prior to HPLC analysis in order to precipitate the protein and remove salts that could interfere with the analysis. This involved treating the samples with a solution of perchloric acid (PCA) at 35 % (w.v^{-1}) followed by addition of 7 N potassium hydroxide (KOH). The supernatant was filtered through a nitrocellulose membrane with a pore size of 0.22 µm.

Sugar and by-product analysis were performed at least in duplicates and the average value was calculated. The standard deviation of these measurements was below 5 %.

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5. Enzyme Preparation Characterisation

Although substrates such as filter paper, CMC, Beechwood xylan or mannan differ from pretreated lignocelluloses, evaluation of the hydrolytic potential activity of the enzymes on model substrates is still a useful tool to establish dosage for enzymatic hydrolysis and allow for comparison with other studies. The enzymatic activities can be expressed in terms of specific activity when considering the protein concentration. The use of enzyme preparations with high specific activity is desirable in order to reduce the enzyme dosage required for conversion of carbohydrates.

A wide selection of cellulolytic enzymes are commercially available that can be used for enzymatic saccharification of lignocelluloses. In this thesis, different commercial enzyme preparations were first characterised in terms of protein concentration and enzymatic activity in filter paper, CMC, cellobiose, Beechwood xylan and galacto-mannan. The results from these determinations are listed in Table 11. In the case of the Cellic® combinations, CTec2 and HTec2, analysis of activity and protein were not determined due to a confidentiality agreement with Novozymes. The enzyme preparations studied were therefore compared in terms of volumetric dosages ($mL.g^{-1}$ WIS), taking as reference the Spezyme CP enzyme preparation. Values for FPU and cellobiase activity determined for Cellic® CTec2 in a recent paper [1] are also incorporated in Table 11. As can be observed, the various enzyme preparations showed significant differences in required dosages and effective activities; therefore different performance on carbohydrate hydrolysis of lignocellulose was likely.

Table 11: Description of the activities of enzyme preparations used in this study

Company	Name of enzyme formulation	Protein concentration ($mg.mL^{-1}$)	Cellulase activity ($U.mL^{-1}$ or $U.g^{-1}$)			Hemicellulase activity ($U.mL^{-1}$ or $U.g^{-1}$)		Laccase ($U.g^{-1}$)
			FPU	CMC	β-glucosidase	Xylanase	Mannanase	
Novozymes	Novozymes 188	120	0.31	< 0.1	929	< 0.1	0.8	n.d.
	Celluclast 1.5 FG	134.8	55.5	11.6	44	837	1.3	n.d.
Genencor	Spezyme CP*	116.2	59	13.3	40	5 263.4	5.1	n.d.
Genencor	Accelerasse 1500	70	55	12.2	150	269	1.0	n.d.
	Optiflow*	183.4	130	22.8	220	2 895	5.9	n.d.
Dyadic	Alternafuel CMAX (Liquid)	130.2	37	8.3	213	6.2	n.d.	n.d.
	Alternafuel CMAX (Powder)	77.5	148	7.2	n.d.	6.5	n.d.	n.d.
Genencor	Multifect Xylanase	47.7	11.8	< 0.1	n.d.	60 855	0.5	n.d.
	Pectinex Ultra	40	0.37	0.4	n.d.	176.1	23.6	n.d.
ZA Biotech	Laccase	12	9.4	0.4	n.d.	0.1	n.d.	300
Novozymes	Cellic® CTec2 [1]	161.2	120.5	-	2 731*	-	-	-
	Cellic® HTec2 [2]	-	Cellulase background			High specificity towards soluble hemicellulose	-	n.d.

* Activity determined on para-nitro-phenil-beta-glucopiranoside

n.d. – none detected

Standard deviation less than 5 %

As expected, the enzyme preparations characterised as cellulases presented the greater values of filter paper activity (FPA). Optiflow and Cellic® CTec2 exhibited the highest values, 130 and 120.5 FPU.mL^{-1}, respectively. Spezyme CP is one of the most commonly used cellulase preparations and was therefore the cellulase preparation (59 FPU.mL^{-1}) selected as reference point. It is worth to note that Spezyme CP also displayed hemicellulase activity, mainly xylanase (5 263.4 U.mL^{-1}). The presence of xylanase activity in cellulase preparations is advantageous for the removal of residual xylan that remains in the fibers after pretreatment, thereby boosting the cellulase activity. However, given its low β-glucosidase activity (40 IU β-glucosidase.mL^{-1}), Spezyme CP is generally supplemented with Novozyme 188 (920 IU β-glucosidase.mL^{-1}) to prevent cellobiose accumulation in the hydrolysis media. The new cellulases preparations of Alternafuel CMAX and Optiflow contain more than 5 times the amount of β-glucosidase compared to conventional cellulases such as Spezyme CP and Celluclast. Cellic® CTec2 also has a significant amount of β-glucosidase [2], so in principle these three enzyme preparations would not need extra addition. Regarding CMCase activity, which is an estimation of the endo-glucanase activity within the preparation, Optiflow presented the highest values (22.8 U.mL^{-1}).

Additionally, two xylanase preparations were also selected for the studies, namely Multifect Xylanase and Cellic® HTec2. Multifect xylanase exhibited almost 12 times more activity on Beechwood xylan than the Spezyme CP preparation. Cellic® HTec2, besides having considerable activity on soluble hemicelluloses, also contains background cellulase [2].

Other enzymes of interest are ligninases such as lacasses. These enzymes have been used as biological treatment to reduce lignin content [3], or prior to enzymatic hydrolysis and/or fermentation to reduce the toxicity of the pretreated material. A laccase from the South African company, ZA Biotech, was also evaluated [4].

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6. Optimisation of Enzymatic Hydrolysis of Steam-pretreated sweet Sorghum bagasse and Triticale straw

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All experiments were planned in collaboration with Dr M.P. García-Aparicio and Mr D. Diedericks and executed by myself. The results were analysed with the help of Dr M.P. García-Aparicio and D. Diedericks. All co-authors provided input and contributed to draft the final manuscript that will be submitted for publication in the format presented.

Keywords: Lignocellulose, Cellulase, Hemicellulase, Enzymatic Hydrolysis, Response Surface Methodology

Abstract

Sweet sorghum bagasse (SSB) and triticale straw (TS) are considered potential lignocellulosic raw materials for the production of fuel ethanol as an alternative to feedstocks that are mainly starch- or sugar- based.

The main objective of this study was to develop an optimised combination of cellulase (Cellic® CTec2) and xylanase (Cellic® HTec2) enzyme preparations (cocktail) that is adapted to a specific feedstock-pretreatment. The optimum was defined as the minimum total enzyme dosage that is needed for a target glucan conversion of at least 80 %. This was done by using a statistically designed factorial experimental approach. The combined effects of the optimum enzyme loadings and hydrolysis times were investigated further using response surface methodology (RSM).

The optimised enzyme cocktails, consisting of 0.15 mL Cellic® CTec2.g^{-1} WIS and 0.32 mL Cellic® HTec2.g^{-1} WIS for sorghum and 0.1 mL Cellic® CTec2.g^{-1} WIS and 0.2 mL Cellic® HTec2.g^{-1} WIS for triticale, exhibited a high performance in the conversion of both substrates. The amount of glucose produced at a digestibility of 80 % was 8.44 g.L^{-1} for sorghum after 72 h and 6.83 g. L^{-1} for triticale after 48 h. The minimal enzyme loadings which yielded these concentrations were 0.47 mL.g^{-1} WIS and 0.30 mL.g^{-1} WIS for sorghum and triticale, respectively.

The results accentuated the different enzyme requirements for the sorghum and triticale feedstocks, even though they are similar to a large extent (both herbaceous biomasses). These different enzyme loadings can be explained by the difference in pretreatment severity and the resulting different properties of the feedstocks after pretreatment. The efficiency of the optimised enzyme cocktail during the hydrolysis of lignocellulosic substrates was also evident.

6.1. Introduction

The development of alternatives to fossil fuels such as biomass-derived fuels is becoming a pressing global issue and is currently receiving major attention as the world's crude oil reserves are depleting fast and the concerns regarding environmental degradation is growing [1], [2]. Compared to traditional fossil-based products, the bioconversion of products derived from biomass offers environmental and economic advantages [3], [4]. As bio-ethanol is able to reduce crude oil dependence and promises cleaner combustion that leads to an improved environment, it has long been a favourable choice as opposed to fossil fuels [1], [5]. Therefore, bio-ethanol is receiving extensive interest at regional, national as well as international levels [6]. The global market for bio-ethanol production has now entered a rapid, intermediate growth phase [6].

Researchers are currently turning their attention away from 1G ethanol, which is mainly food-based, and focussing on the production of second generation (2G) ethanol which represents non-food-based ethanol from lignocellulosic materials [1]. This is owing to problems associated with the production of 1G biofuels, such as the increase in food prices, net energy losses as well as increased greenhouse gas (GHG) emissions [7].

Lignocellulosic materials are considered the most abundant and cheap biomass that is widely available to the world [3], [8], [9]. These materials can be used for the production of fermentable monomeric sugars (mainly xylose and glucose) through hydrolysis and these sugars can subsequently be used as substrate during fermentation [3], [10].

Various lignocellulosic materials have been assessed for their production of fuel ethanol [11]. Among these are hard- and softwoods, dedicated energy crops, herbaceous material and agricultural residues [11]–[13]. Both sorghum and triticale have been identified as promising feedstocks, mainly due to their high biomass yield, low input requirements, tolerance to drought and the fact that they can produce high yields under a wide variety of conditions [11], [14]–[16]. These feedstocks are also considered to be abundant and renewable [3], [17], [18]. The agricultural by-products (bagasse, straw) from sorghum and triticale are therefore seen as valuable feedstocks which can be used as an alternative to starch-based crops such as barley, maize or wheat, without it having any effect on the food market [11], [19].

Lignocellulosic biomass comprises of hemicellulose, cellulose and lignin [20]. All of these are organised into a firm structure which differ between feedstocks [20]. Monomeric sugars can be obtained through the conversion of lignocellulosic material with the use of enzymes or dilute acid [3]. The enzymatic process seems to be more capable of efficient hydrolysis as it is more specific, milder and produces less inhibitors compared to acid hydrolysis [3], [21]. The enzymatic hydrolysis of cellulosic biomass is also considered to be an eco-friendly method that can be used to replace treatments involving chemicals [8]. When following the enzyme route, the degradation of lignocellulose into monomeric sugars requires the combined action of multiple hemicellulolytic and cellulolytic enzymes [20], [22]. The necessity of adding several enzymes is owed to the chemical composition, complex physical structure and physiochemical properties (cellulose crystallinity, degree of polymerisation (DP)) of these materials [20].

There are three possible substrates for ethanol production when using pretreated lignocellulose: whole slurry, pressed-slurry or water-insoluble solids (WIS). The substrate of

choice will be determined mainly by the feedstock, ability of the fermentative micro-organism to co-ferment pentoses and hexoses and its tolerance to inhibitors. The use of the whole slurry, for example, would be similar to the simultaneous saccharification and co-fermentation process described elsewhere, where both the liquid and solid fractions of the pretreated materials are fermented in a single process step, with both glucose and xylose converted to ethanol. This process options may provide a higher loading of fermentable sugars in the fermentation process, while also saving on production costs since the filtration/pressing/washing step is avoided. However, depending on the feedstock and pretreatment conditions, there is solubilisation and formation of components that can act as inhibitors during the subsequent enzymatic hydrolysis and fermentation steps. Moreover, some of the hemicellulosic-derived sugars are in oligomeric form that has been shown to be inhibitors to cellulases at low concentrations [23]. Due to these disadvantages the separation of solid and liquid from fermentation has been proposed, either by filtration or pressing, resulting in the "pressed-slurry" option, or by extensive washing to remove residual liquid from solids, in the "WIS" option. The pentoses and hexoses liquid fractions from either of these steps would be fermented separately, while either the pressed-slurry or washed WIS will be fed into a simultaneous saccharification and fermentation (SSF) process for hydrolysis and fermentation. The majority of the studies wash the pressed-slurry to remove inhibitors that are soaked in the cellulose fibers, minimising the toxicity during hydrolysis and fermentation. However, this step adds extra cost and generates additional waste-water streams, together with significant dilution of sugars, requiring energy inputs for subsequent concentration.

The process of bioconversion is not economically viable at the moment due to the slow pace at which enzymatic hydrolysis takes place and the high enzyme loading (with the associated high enzyme costs) that is required to obtain rates and yields that are acceptable [3], [22], [24], [25]. According to the latest economic analysis, the high costs associated with enzymes provide a bottleneck in the application of many ethanol production studies [1]. Many other factors, which can be divided into mechanistic and structural substrate factors, can also affect the process of enzymatic hydrolysis [3]. At present, the main focus of research into biotechnology is the search for lignocellulolytic enzymes that are highly effective in the hydrolysis of plant biomass [20]. Lignocellulosic bio-ethanol production costs, however, is still more when comparing it to fossil fuels, because the conversion process

relies greatly on the feedstock, efficient and low cost enzymes as well as effective process design. [6], [7].

The general aim of this study is to optimise a combination of enzymes adapted to specific feedstocks and pretreatment conditions. With this as the main goal, the specific objective will be to optimise the hydrolysis parameters; cellulase and hemicellulase loading as well as hydrolysis time in the case of triticale, to obtain the maximum glucose production at a feedstock digestibility of 80 %.

For sorghum, this will be studied using RSM according to the central composite experimental design (CCD). Especially in the field of bioprocessing, investigators have adopted the use of RSM to quantify the complex interplay existing between parameters that affect biological systems [26]–[30]. RSM has thus shown to be an efficient statistical method and is used to optimise multiple factors in a systematic manner [3], [20]. This study will be done within the minimal amount of experiments, while also maintaining a high degree of statistical significance within the results [3], [31]. In the case of triticale, the equation of total sugars obtained by the use of Design Expert® software will be used together with Solver in Microsoft Excel to determine the minimum enzyme loadings with which to obtain maximum sugar production at 80 % digestibility of the feedstock. The optimisation of parameters (individual cellulase and hemicellulase loadings and hydrolysis time) can help to make hydrolysis more efficient and thereby reduce the hydrolysis time which would directly affect process productivity [26].

6.2. Materials and Methods

6.2.1 Raw materials

Sweet sorghum bagasse and triticale straw were provided by the University of KwaZulu-Natal (Ukhulinga Experimental Farm) and Department of Genetics at Stellenbosch University (Mariendahl Experimental Farm), respectively. Both varieties used in this study were selected based on their response to pretreatment. The triticale EliteM13 variety used was a preferred variety based on preliminary experiments performed in our laboratory [32]. For sorghum, variety MSJH16 was considered a control variety (‘bad’) after preliminary testing done in our laboratory and subsequently used to determine the performance of other ‘better performing‘ varieties [33]. It was, however, still used for the experiments in this study

due to the limited amounts of the better performing varieties that were available, thereby representing weak feedstock properties ('worst case' analysis).

The sorghum feedstock used, although not one of the best performing varieties, is still going to be used for pilot scale bio-ethanol production to see the effect of variety selection on enzyme requirements (which is my main focus). Also to be tested is the effect of variety on the sugar yield during pretreatment, sugar yield during enzymatic hydrolysis and finally, ethanol yield during fermentation.

The raw material was milled and sieved and the material with a particle size between 0.38 and 10 mm was collected. The chopped bagasse/straw were then sealed in plastic bags and placed in a storage container until it was subjected to pretreatment.

6.2.2 Pretreatment of materials

Steam-explosion (SE) pretreatment assays took place in a 19 L SE pilot unit. The reactor was loaded with 600 g (7.34 % moisture) of raw material in a batch pretreatment manner. Different pretreatment conditions were applied for the two feedstocks; 5 minutes at 200 ˚C for the sorghum MSJH16 variety and 5 minutes at 190 ˚C for the triticale EliteM13 variety. These were the optimum pretreatment conditions for these specific varieties according to previous studies [32], [33].

After the material was subjected to SE, the product of pretreatment (also called the slurry) was collected in a cyclone and pressed to separate the liquid fraction from the solid fraction. The solid fraction was subjected to a thorough washing step (10 times) to obtain the WIS, where after this material was analysed along with raw material to determine the chemical composition thereof [34]–[37]. This procedure is described in section 6.2.5.2. The sugar and by-product content of the liquid fraction was determined by high-performance liquid chromatography (HPLC) analysis [38]. The remaining WIS and liquid fractions were then stored at -20 °C until it was used in the subsequent enzymatic hydrolysis experiments.

6.2.3 Commercial enzymes preparations

The commercial enzymes used in this study were the cellulases, Spezyme CP and Cellic® CTec2, and the hemicellulase (endo-xylanase (EX)) Cellic® HTec2. Novozyme 188 was added to the Spezyme CP enzyme preparation during enzymatic hydrolysis to provide sufficient β-glucosidase (BG) activity. Spezyme CP was kindly supplied by Genencor

(Genencor, Leiden, Netherlands) whereas Novozymes (Novozymes A/S, Denmark) kindly supplied Novozyme 188, Cellic® CTec2 and Cellic® HTec2.

6.2.4 Enzymatic hydrolysis of pretreated material

Enzymatic hydrolysis was performed in 250 mL screw cap Erlenmeyer flasks (100 mL working volume) at a 2 % WIS loading ($dw.v^{-1}$). Citrate buffer, supplemented with 2.0 % sodium azide to prevent contamination, was used at a concentration of 0.05 M and pH 5.0.

The choice of substrates that would perform the best during enzymatic hydrolysis was validated by testing the performance of a cellulase enzyme on WIS, pressed-slurry as well as whole slurry for both sorghum and triticale substrates. The experiments from the CCD of sorghum took place with pressed-slurry as substrate. The extensively-washed WIS was supplemented with the amount of liquid fraction embedded in the WIS after pressing the slurry (± 60 % moisture in pressed-slurry). Alternatively, for the triticale feedstock (variety EliteM13) the WIS fraction after washing was used for enzymatic hydrolysis. The solids content for both pressed-slurry and washed WIS substrates only contained insoluble solids.

Hydrolysis was initiated by the addition of the enzymes to the contents of the screw cap Erlenmeyer flasks. The flasks were placed in a water-bath at a temperature of 50 °C and a shaking speed of 90 rpm. At the indicated time points (0, 3, 6, (7.64), 9, 12, 24, 48, 72, (88.36) and 120 h), samples were drawn from the flasks, placed in Eppendorf tubes and boiled for 5 minutes to inactivate the enzymes. These samples were then stored at -20 °C until the sugar content thereof was determined by HPLC analysis as described in section 6.2.5.3. All experiments were performed in duplicate and average results are shown.

6.2.5 Analytical methods

6.2.5.1. Determination of enzyme activities

The control commercial enzyme preparations, Spezyme CP and Novozyme 188, were each subjected to standardised tests in order to determine their protein content and respective activities on model substrates. The activities of cellulase (Filter Paper Units (FPU), carboxy-methyl cellulose (CMC)) and β-glucosidase were determined using the method described by Ghose (1987) and the xylanase activity was measured according to the method of Bailey *et al.* (1991) [39], [40]. Protein content was determined with the use of a

bicinchoninic acid ([BCA]™ assay, BCA-Compat-Able Protein Assay kit, ref. 23229, Pierce, Rockford, IL) using bovine serum albumin as the protein standard.

6.2.5.2. Chemical analysis

Chemical compositions of the raw materials and washed WIS fractions were determined with the use of the standard laboratory analytical procedures (LAP) for biomass analysis (carbohydrates, lignin, ash and extractives) that is provided by the National Renewable Energies Laboratory (NREL) (CO, USA) [34]–[37]. The liquid fraction was analysed according to the LAP for determining sugars solubilised and by-product formation during pretreatment [38].

6.2.5.3. HPLC analysis

The concentrations of sugar monomers (arabinose, glucose and xylose), cellobiose as well as acetic acid and the by-products formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (5-HMF) were analysed by HPLC using an Aminex HPX-87H Ion Exclusion Column (Hercules, CA) fitted with a cation-H cartridge guard column (Hercules, CA)(Bio-Rad, Johannesburg, RSA). A Refractive Index (RI) detector (Waters 2141, Microsep, Johannesburg, RSA) was used to measure the sugars, whereas an Ultra-Violet (UV) detector set at 215 and 280 nm (Waters 2487, Microsep, Johannesburg, RSA) was needed to analyse the by-products. Both the sugar and by-product content of the test samples were quantified with the use of a standard stock of the respective, combined components that were prepared at the suitable concentration. The operating conditions for the column were 65 °C with 5 mM H_2SO_4 at a flow rate of 0.6 mL.min^{-1}, which functioned as the mobile phase.

The amount of sugars present in oligomeric form within the liquid fraction was determined by applying a mild acid hydrolysis [38]. In this process, a calculated amount of 72 % (w.w^{-1}) dilute sulphuric acid (H_2SO_4), based on the pH of the sample, is added to 5 mL of the liquid fraction of the feedstock (both the raw material and WIS, respectively), which brings the concentration of the H_2SO_4 down to 4 % (w.w^{-1}). This mixture is then autoclaved for 30 minutes at 121 °C. During this period in the autoclave, all the sugars that are present within that amount of liquid fraction are hydrolysed into monomers. Samples were filtered through a 0.22 µm micro-filter before subjecting them to HPLC analysis. The amount of oligomers was calculated as the difference between the total monomers after acid hydrolysis and the initial monomers concentration.

6.2.6. Experimental design and statistical analysis

Cellulase and xylanase enzyme loadings (and hydrolysis time) were chosen as independent variables and glucose concentration was the response parameter evaluated to reach the target of 80 % cellulose conversion.

For sorghum, the high and low level of these variables (in which they had the maximum effect on the single response parameter) were obtained from literature as well as from studies of volumetric dosages done with the cellulase enzyme [41]. Similarly it was necessary to determine a realistic range of cellulase and xylanase dosages for triticale to be applied in the CCD for optimisation. For this purpose, screening experiments were performed with increasing cellulase dosage at a fixed xylanase loading and vice versa. These dosages of cellulase and xylanase which provided the maximum glucose concentrations were selected as the centre point values in the oncoming experimental design.

Once the minimum and maximum enzyme loadings for both feedstocks were determined, it could be implemented in the subsequent experimental design. The experimental design for the sorghum feedstock consisted of a 2^2 CCD, with four star points and the centre point in triplicate. For triticale, a CCD with three factors, 6 star points and two central points were employed to include the effect of time. These experimental setups can be seen in Table 12 and Table 13, respectively.

Table 12: A 2^2 factorial central composite experimental design (CCD) for pretreated Sorghum bagasse from cultivar MSJH16 employed during optimisation studies of Cellic® CTec2 (cellulase) and Cellic® HTec2 (xylanase) loading.

Run	Cellulase loading ($mL.g^{-1}$ WIS)	Xylanase loading ($mL.g^{-1}$ WIS)
	Factorial points	
1	0.05	0.15
2	0.05	0.44
3	0.15	0.15
4	0.15	0.44
	Star points (Cellulase, Xylanase)	
5	0.0293	0.295
6	0.1707	0.295
7	0.1	0.0899
8	0.1	0.5001
	Centre points	
9 (CP)	0.1	0.295
10 (CP)	0.1	0.295
11 (CP)	0.1	0.295

Table 13: The 2^3 factorial central composite design (CCD) employed during optimisation studies of pretreated Triticale straw from cultivar EliteM13 for three factors, namely the enzymes Cellic® CTec2 (cellulase loading), Cellic® HTec2 (xylanase loading) and Time (h).

Run	Cellulase loading (mL.g^{-1} WIS)	Xylanase loading (mL.g^{-1} WIS)	Time (h)
	Factorial points		
1	0.100	0.200	24
2	0.100	0.200	72
3	0.100	0.400	24
4	0.100	0.400	72
5	0.200	0.200	24
6	0.200	0.200	72
7	0.200	0.400	24
8	0.200	0.400	72
	Star points (Cellulase, Xylanase and Time)		
9	0.079	0.300	48
10	0.221	0.300	48
11	0.150	0.159	48
12	0.150	0.441	48
13	0.150	0.300	7.64
14	0.150	0.300	88.36
	Centre points		
15(C)	0.150	0.300	48
16 (C)	0.150	0.300	48

Additionally, samples were taken at 0, 3, 6, 9, 12, 24, 48, 72 and 120 h to study the kinetics (see Addendum A, section 6.6.1.) and also because differences in interaction are better seen at initial stages where end-product inhibition is reduced.

The use of multiple centre points is employed to validate the analysis of variance (ANOVA) and thereby justify the repeatability of the experiment. Also, a completely randomised run order was chosen in order to prevent confusion between all the factors that can contribute to unexplained variation within the response parameter (glucose concentration).

An enzyme cocktail can be optimised by the application of a CCD, which incorporates linear and interaction effects of independent variables. The experimental data from the CCD of sorghum was thus analysed with the use of the rigorous and user-friendly program Design Expert® version 8.0.4 (Stat-Ease Inc., Minneapolis, MN, USA). RSM was applied to determine

the functional relationship that exists between the two independent variables, Cellic® CTec2 and Cellic® HTec 2 and the one response parameter, glucose concentration. The Design Expert® software was also used to determine the relationship between the variables Cellic® CTec2 dosage, Cellic® HTec2 dosage and hydrolysis time in the triticale feedstock.

6.3. Results and Discussion

6.3.1. Composition of raw and pretreated material

During SE pretreatment, the chemical composition of the lignocellulose is altered by exposure to the high temperatures and sudden pressure changes. The pretreatment conditions, slurry (pretreated material) properties and chemical composition of the untreated (raw material) and treated (WIS) substrates are shown in Table 14 and Table 15 [32], [33]. The properties and composition of the materials are represented as it will have an impact on the performance of enzymes during enzymatic hydrolysis. The variety of sorghum bagasse used was the worst performer among the selected varieties based on pretreatment response [33], while the triticale straw variety was one of the preferred varieties [32].

Table 14: Properties of Sorghum bagasse MSJH16 and Triticale straw M13 from pretreatment.

Pretreatment Conditions		
	Sorghum MSJH16	**Triticale M13**
Impregnation	-	Water-impregnated
Temperature (˚C)	200	190
Time (min)	5	5
Severity Factor * [42]	3.64	3.35
Pretreated material (slurry) properties		
pH liquid fraction	3.50	3.95
% WIS	25.81	34.30
% WSS	11.49	10.62
Insoluble solids recovery %	61.74	77.06

* The **severity factor** of a substrate is a parameter that gives an idea of the severity of the pretreatment and is estimated considering the temperature and residence time.

WIS – Water-Insoluble Solids

WSS – Water-Soluble Solids

Table 15: Chemical composition of the raw material and WIS of the pretreated material of both Sorghum bagasse and Triticale straw in % dry weight.

Component	Sorghum bagasse MSJH16		Triticale straw M13	
	Raw Material (% dry weight)	**WIS** (% dry weight)	**Raw Material** (% dry weight)	**WIS** (% dry weight)
Cellulose	37.95	52.36	35.56	46.40
Hemicellulose	19.63	9.35	18.63	14.46
Lignin	18.26	24.98	17.40	21.92
Ash	2.23	1.77	2.62	0.47
Acetyl groups	5.20	0.04	2.28	0.05
Extractives	7.66	-	9.34	-

Standard deviation less than 5 %

Sorghum bagasse/triticale straw were comprised of 54.19 - 57.58 % carbohydrates, 17.40 - 18.26 % lignin, 2.23 - 2.62 % ash, 2.28 - 5.20 % acetyl groups and 7.66 - 9.34 % extractives on a dry weight basis, which is in agreement with values reported in literature for sorghum bagasse [1] and triticale straw [43].

Although sorghum bagasse and triticale straw presented very similar chemical compositions, the application of water-impregnation for triticale straw and/or possible structural differences between the two feedstocks, led to their requirement for different pretreatment conditions in order to yield material that is optimally digestible [32], [33]. The pretreatment of sorghum was more severe than for triticale, as indicated by its higher severity factor (Table 14). These harsh conditions during pretreatment could also have led to the decreased % WIS and % insoluble-solids recovery of sorghum compared to triticale.

The composition of the WIS fraction of both pretreated materials is indicated in Table 15. Both sorghum bagasse and triticale straw were enriched in cellulose during pretreatment, although triticale to a lesser extent, with a large amount of hemicellulose (xylan) still present within the WIS fraction after pretreatment. This may have been related to the less severe pretreatment required to maximise cellulose hydrolysis for triticale straw.

The lignin contents of sorghum bagasse and triticale straw were significantly lower than that of softwood and hardwood feedstocks (Table 15) [44], although the lignin contents were somewhat higher compared to other herbaceous crops. The lignin within the feedstocks might therefore be able to act as a physical barrier or be involved in non-productive binding of the enzymes during enzymatic hydrolysis [45]. However, SE lignin has

proven to be less reactive to cellulases due to their low affinity compared to other pretreatments that actually remove the lignin, such as organosolv pretreatment [46].

Both feedstocks also had very low ash content (Table 15). In lignocellulosic material, low ash contents has potential benefits for the process of enzymatic hydrolysis as it is able to increase the efficiency of the hydrolysis, especially in feedstocks with a low cellulose concentration [47]. The acetyl content of both feedstocks decreased after pretreatment and was only present in small amounts (Table 15).

Pretreatment contributed to the formation of inhibitors within the liquid fraction. The composition of the sugars and by-products present in the liquid fraction of the pretreated materials are summarised in Table 16. Among the inhibitors present in the liquid fraction, the concentrations of formic and acetic acid were the highest for both sorghum bagasse MSJH16 and triticale straw M13, with 5-HMF (derived by degradation of six carbon sugars) and furfural only present in very small quantities (Table 16). Acetic acid (formed by hydrolysis of acetyl groups) concentrations above 2 $g.L^{-1}$ have been reported to inhibit enzymatic hydrolysis significantly [48]. For sorghum bagasse, this could be problematic when using whole slurry as substrate as a large amount of acetic acid (5.29 $g.L^{-1}$) was present within the liquid from pretreatment of this feedstock. Formic acid is also a very toxic substance as it can inactivate enzymes of the cellulase complex when present at concentrations larger than 11.5 $g.L^{-1}$ [48]. In this study, however, the concentration of formic acid was low (0.31 – 1.40 $g.L^{-1}$), despite a four times higher concentration thereof in the liquid from sorghum bagasse pretreatment. Concentrations of the inhibitors furfural and 5-HMF above 2 $g.L^{-1}$ may have similar negative effects of enzymatic hydrolysis [48], but these furans were only present in small amounts (0.12 – 0.59 $g.L^{-1}$ furfural and 0.04 – 0.19 $g.L^{-1}$ 5-HMF) within both feedstocks. It is also worth to note that the liquid fraction was rich in xylose in oligomeric form due to the method of auto-hydrolysis present in SE pretreatment. Xylo-oligomers have shown to be stronger inhibitors of the cellulases than other end-products of enzymatic hydrolysis such as cellobiose and glucose [23].

Overall, the concentration of inhibitors was higher in the liquor from pretreatment of sorghum bagasse than for pretreatment of triticale straw. This could be due to the more susceptible hemicellulose component thereof and/or the more severe pretreatment conditions applied to sorghum bagasse pretreatment.

Table 16: The composition of all the sugars and by-products present within the liquid fraction of Sorghum bagasse and Triticale straw after steam-explosion pretreatment.

Sugar and By-product content	Chemical species in the liquid fraction from steam-explosion ($g.L^{-1}$)	
	Sorghum MSJH16	Triticale M13
Monomeric glucose	0.45	0.26
Oligomeric glucose	5.14	8.03
Monomeric xylose	6.46	1.31
Oligomeric xylose	39.36	23.88
Acetic acid	5.29	1.31
Formic acid	1.40	0.31
Furfural (215 nm)	0.59	0.12
5-HMF	0.19	0.04

Standard deviation less than 5 %

6.3.2. Characterisation of commercial enzyme preparations

The activities and protein concentrations of the control enzyme preparations on model substrates (filter paper, CMC, cellobiose and Beechwood xylan) are summarised in Table 17. Evaluation of the hydrolytic potential activity of enzymes on model substrates is a useful tool to establish a dosage which can be used during enzymatic hydrolysis and also to allow for comparison with other studies. Numerous enzymes were characterised and based on their values and preliminary results, only those presented in Table 17 were chosen for further experiments.

Table 17: Enzyme activities and protein concentration of the commercial enzyme preparations.

Enzyme preparations	Enzyme activities				Protein concentration ($mg.mL^{-1}$)
	FPA ($FPU.mL^{-1}$)	CMC ($U.mL^{-1}$)	β-glucosidase ($U.mL^{-1}$)	Xylanase ($U.mL^{-1}$)	
Cellic® CTec2 * [49]	120.5	-	2 731	-	161.2
Cellic® HTec2	-	-	-	-	
Spezyme CP	58.7	13.3	40.2	5 263.4	116.2
Novozyme 188	0.31	< 0.1	929.0	< 0.1	120.0

* values obtained from literature
FPA - filter paper activity
Standard deviation less than 5 %

The use of Spezyme CP in an enzyme cocktail requires the addition of a small volume of Novozyme 188 to supply a sufficient amount of β-glucosidase to the mixture and thereby prevent product inhibition from occurring. The Cellic® enzymes supplied by Novozymes reportedly require only a fraction of the dosage used by its predecessor enzymes and work with a wide variety of feedstocks and pretreatment methods [50], [51]. They are therefore thought to have a superior performance compared to other enzymes. Two enzyme combinations were tested for the saccharification of the pretreated materials, i.e. the Spezyme CP/Novozyme 188 cocktail at a dosage of 15 FPU.g^{-1} WIS (0.28 mL.g^{-1} WIS) as control and the newer Cellic® CTec2 and Cellic® HTec2 cocktail. The second combination consisted of Cellic® CTec2 and Cellic® HTec2, for which the dosages and ratio between the two components were optimised separately for pretreated sorghum bagasse and triticale straw.

All the subsequent hydrolysis experiments with the selected cocktails were performed at a WIS loading of 2 % (dw.v^{-1}). Thereby end-product inhibition could be reduced and it was able to distinguish better between the different enzyme preparations. Digestibility studied during pretreatment as well as feedings during fed-batch fermentation (simultaneous saccharification and fermentation) were also performed at a low solids loading of 2 % (dw.v^{-1}) WIS. Enzymatic hydrolysis experiments could therefore be compared to these experiments at this baseline.

6.3.3. Process options for saccharification of pretreated lignocellulose

There are three possible substrates that can be applied during enzymatic hydrolysis, namely the WIS fraction, pressed-slurry and whole slurry. For both feedstocks, all of these substrates were tested with three different cellulase loadings, using only the Cellic® CTec2 enzyme. Results of glucan conversion, xylan conversion and total sugars (glucose, xylose, cellobiose) concentration for pretreated sorghum bagasse and triticale straw are illustrated in Figure 22 and Figure 23, respectively. Conversion was evaluated to see if the sugar content/inhibitors within the liquid fraction would have an impact on the conversion of the glucan/xylan from the WIS fraction and oligomers (gluco-oligomers, xylo-oligomers) from the liquid fractions. The original amount of sugar within each sample at time point 0 h was subtracted from all the values obtained at the subsequent time points. The sugar concentration considered all the sugars; those provided by the liquid fraction as well as those that were released from enzymatic hydrolysis. Control experiments, with only the

liquid fraction, were also performed to monitor if the hydrolysis conditions (temperature and pH) would hydrolyse the oligomers. This was, however, not the case.

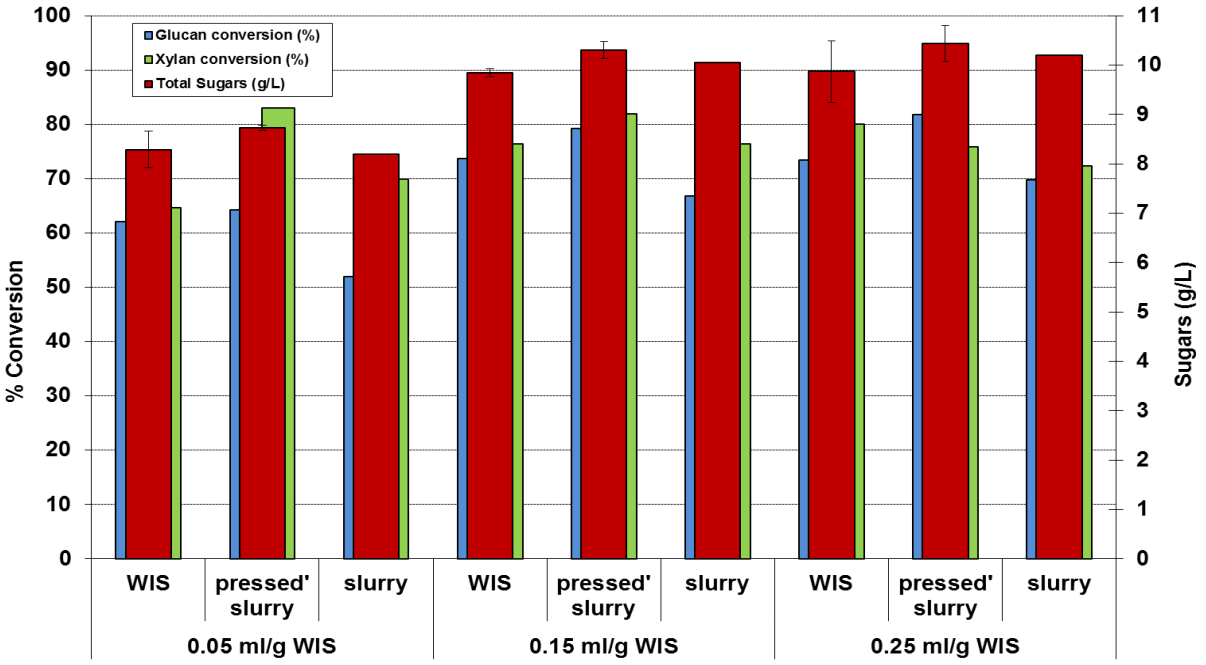

Figure 22: Results of the Glucan conversion (%), Xylan conversion (%) and Total Sugars concentration (g.L^{-1}) of Sorghum MSJH16 at different dosages of Cellic® CTec2 (mL.g^{-1} WIS) after 72 h at a solids loading of 2 % (dw.v^{-1}) WIS.

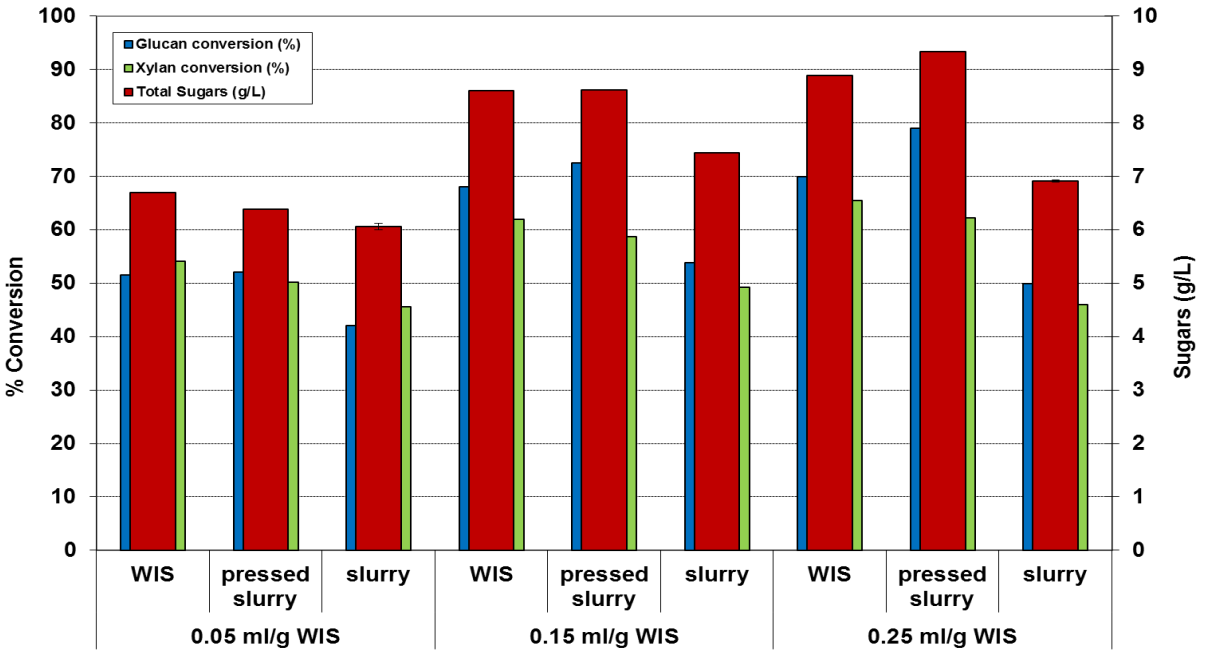

Figure 23: Results of the Glucan conversion (%), Xylan conversion (%) and Total Sugars concentration (g.L^{-1}) of Triticale M13 at different dosages of Cellic® CTec2 (mL.g^{-1} WIS) after 72 h at a solids loading of 2 % (dw.v^{-1}) WIS.

For sorghum it was observed that pressed-slurry had the highest total sugars concentration as well as a relatively high glucan and xylan conversion, when compared to both the washed WIS and whole slurry fractions. Using pressed-slurry, the sorghum substrate therefore not only contained the solid fraction (WIS) enriched in cellulose, but also some sugars, mainly xylose, that are retained in it after pressing. The use of pressed-slurry was also preferred as it represents a substrate that is closer to the commercial approach. From Figure 22 it is also observed that the cellulase loading could be limited at 0.15 $mL.g^{-1}$ WIS as no significant increase in glucose conversion, xylose conversion or overall total sugars concentration were seen at a higher cellulase loading (0.25 $mL.g^{-1}$ WIS).

Conversely, the WIS fraction of triticale was the desired substrate at low cellulase dosages as it yielded a higher total sugar concentration, comparative glucan conversion and higher xylan conversion compared to pressed-slurry and whole slurry (Figure 23). The WIS fraction was therefore selected as the preferred substrate for subsequent optimisation of enzymatic hydrolysis of pretreated triticale straw.

6.3.4. Enzyme cocktail optimisation

6.3.4.1. Preliminary experiments prior to CCD

The range of enzyme dosages that were applied during the experiments with sorghum were obtained from preliminary volumetric studies of the cellulase enzyme (see Addendum A, section 6.6.2.) as well as values obtained from literature [41]. A suitable range of cellulase and xylanase dosages for the subsequent CCD optimisation were determined for pretreated triticale straw through screening experiments. First, the xylanase dosage was fixed at 0.3 $mL.g^{-1}$ WIS and the cellulase dosage varied between 0.05 - 0.25 $mL.g^{-1}$ WIS. Thereafter the cellulase dosage was fixed at 0.15 $mL.g^{-1}$ WIS and a xylanase dosage range of 0.05 - 0.44 $mL.g^{-1}$ WIS was tested. These results are illustrated in Figure 24A and 24B. These screening experiments indicated preferred dosages of 0.15 and 0.3 $mL.g^{-1}$ WIS for Cellic® CTec2 (cellulase) and Cellic® HTec2 (xylanase), respectively (Figure 24). These enzyme dosages were selected as centre point for the subsequent CCD experimental design and optimisation for pretreated triticale straw.

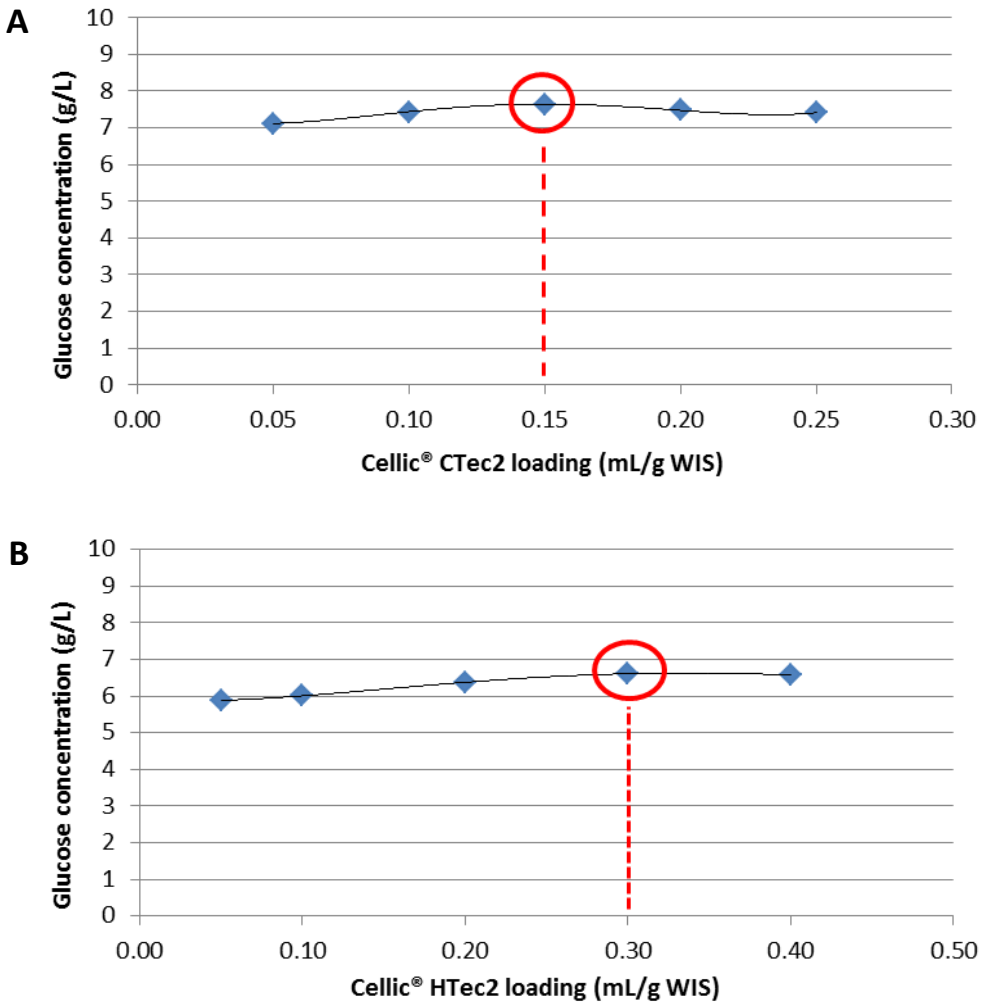

Figure 24: Screening data of the (A) Cellic® CTec2 and (B) Cellic® HTec2 loadings for Triticale at 72 h to obtain the optimum values for both enzymes to be used as the centre point in the subsequent CCD experiments. Optimum values for both are indicated by the red circles.

6.3.4.2. Optimisation via CCD

The required dosage for enzymatic hydrolysis of pretreated sorghum bagasse and triticale straw was minimised with the use of CCD optimisation. The goal of the CCD optimisation was to achieve 80 % hydrolysis of the polysaccharides present in the pretreated materials. This target was selected based on preliminary experiments, meeting both the requirements for efficient hydrolysis and avoiding the use of excessively large enzyme dosages (see Addendum A, section 6.6.3.). As 100 % digestibility would require too much enzyme, a digestibility of 80 % was established as the baseline for comparison between cocktails.

As per CCD methodology, hydrolysis of pretreated materials was performed at factorial, star and centre point conditions. The glucose released during enzymatic hydrolysis is

illustrated in Table 18 and Table 19. The factorial point conditions were chosen in such a way that all possible combinations between the two independent variables (high and low values of both cellulase and xylanase) were considered. The values for sorghum ranged between 0.05 – 0.15 mL Cellic® CTec2.g^{-1} WIS and 0.15 - 0.44 Cellic® HTec2.g^{-1} WIS whereas for triticale the ranges were 0.1 – 0.2 mL Cellic® CTec2.g^{-1} WIS and 0.2 – 0.4 mL Cellic® HTec2.g^{-1} WIS (as illustrated in the factorial designs in Table 12 and Table 13). These values were obtained from literature and screening experiments, respectively [41].

Table 18: Glucose concentrations ($g.L^{-1}$) obtained during hydrolysis of sweet Sorghum bagasse with the Cellic® CTec2 and Cellic® HTec2 enzymes at a solids loading of 2 % ($dw.v^{-1}$) WIS. (The glucose concentrations shown in the table below have already subtracted the amount of glucose present at time point 0 h).

Run no.	Enzyme loading		3 h ($g.L^{-1}$)	6 h ($g.L^{-1}$)	9 h ($g.L^{-1}$)	12 h ($g.L^{-1}$)	24 h ($g.L^{-1}$)	48 h ($g.L^{-1}$)	72 h ($g.L^{-1}$)	120 h ($g.L^{-1}$)
	Cellulase ($mL.g^{-1}$ WIS)	Xylanase ($mL.g^{-1}$ WIS)								
	Factorial points									
1	0.05	0.15	4.04	5.19	5.84	6.73	7.18	7.62	8.46	9.35
2	0.05	0.44	4.39	5.40	6.20	6.87	7.23	8.02	8.83	9.74
3	0.15	0.15	4.32	4.32	6.29	6.86	7.48	8.29	8.82	9.63
4	0.15	0.44	5.76	6.64	7.57	8.21	8.89	9.39	9.95	10.44
	Star points (cellulase, xylanase)									
5	0.0293	0.295	3.81	5.21	5.92	6.54	7.07	7.28	7.58	7.82
6	0.1707	0.295	5.06	6.19	7.09	7.59	7.91	8.68	9.28	10.21
7	0.1	0.0899	4.15	5.40	6.26	6.89	7.73	8.28	8.77	9.44
8	0.1	0.5001	6.76	6.19	7.09	7.59	7.91	8.68	9.28	10.21
	Centre points									
9 (C)	0.1	0.295	4.75	5.77	6.67	7.29	7.82	8.42	9.00	9.94
10 (C)	0.1	0.295	4.53	5.42	6.54	7.11	7.55	8.16	8.85	9.75
11 (C)	0.1	0.295	4.51	5.48	6.49	6.99	7.71	8.26	8.67	9.54

Standard deviation less than 5 %

Table 19: Glucose concentrations ($g.L^{-1}$) obtained during hydrolysis of Triticale straw at a solids loading of 2 % ($dw.v^{-1}$) WIS with Cellic® CTec2, Cellic® HTec2 and Time (h) as the three variables. (The glucose concentrations indicated in this table have already taken into account the amount of glucose present at time point 0 h).

Run no.	Enzyme loading		3 h ($g.L^{-1}$)	6 h ($g.L^{-1}$)	7.64 h ($g.L^{-1}$)	9 h ($g.L^{-1}$)	12 h ($g.L^{-1}$)	24 h ($g.L^{-1}$)	48 h ($g.L^{-1}$)	72 h ($g.L^{-1}$)	88.36 h ($g.L^{-1}$)	120 h ($g.L^{-1}$)
	Cellulase ($mL.g^{-1}$ WIS)	Xylanase ($mL.g^{-1}$ WIS)										
	Factorial points											
1 and 2	0.1	0.2	2.81	4.03		4.36	5.01	6.55	7.48	7.28		7.26
3 and 4	0.1	0.4	3.75	4.91		5.10	5.18	6.04	7.50	7.93		7.84
5 and 6	0.2	0.2	2.05	3.48		3.26	3.94	4.78	6.39	6.43		6.55
7 and 8	0.2	0.4	3.33	4.98		5.06	5.95	6.65	7.91	8.42		6.76
	Star points (cellulase, xylanase and time)											
9	0.079	0.300	3.46	4.27		5.04	5.25	6.68	7.58	7.84		7.91
10	0.221	0.300	3.98	4.57		5.68	5.96	6.53	8.01	8.39		8.51
11	0.150	0.259	3.25	4.58		5.16	5.69	6.98	8.40	7.35		8.04
12	0.150	0.441	3.29	4.33		5.43	5.64	6.40	8.60	7.24		8.47
	Centre points											
13 and 14	0.150	0.300	3.11	3.81	4.85	4.97	5.59	6.92	8.03	6.56	6.99	8.12
15 (C) and 16 (C)	0.150	0.300	3.40	4.73		4.96	5.67	6.69	7.95	7.10		8.38

Standard deviation less than 5 %

As observed in both Table 18 and Table 19, a minimum cellulase loading along with a larger amount of xylanase was required to reach the maximum glucose concentrations. Longer hydrolysis times also resulted in improved hydrolysis yields. A target of 80 % was therefore applied for minimisation of the enzyme dosages.

6.3.5. Statistical analysis

The ANOVA information for the glucose released during enzymatic hydrolysis of steam-pretreated sorghum bagasse and triticale straw, as calculated with the use of the Design-Expert® software, are shown in Table 20 and Table 21, respectively. The sum of squares, mean sum of squares, F-value and p-value of each term is indicated.

Table 20: Analysis of variance (ANOVA) for glucose production as dependent variable of the two enzymes Cellic® CTec2 and Cellic® HTec2 during the hydrolysis of pretreated Sorghum bagasse at 72 h with a solids loading of 2 % (dw.v^{-1}) WIS.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (Probability factor)
Model	2.50	2	1.25	11.10	0.0049
A – Cellic® CTec2	1.88	1	1.88	16.72	0.0035
B – Cellic® HTec2	0.62	1	0.62	5.48	0.0473
Residual sum of squares	0.90	8	0.11	-	-
Lack of fit	0.85	6	0.14	5.17	0.1710
Pure error	0.055	2	0.027	-	-
Total	3.40	10	-	-	-

Confidence level of 95 %

Table 21: Analysis of variance (ANOVA) for glucose production as dependent variable of the three factors Cellic® CTec2, Cellic® HTec2 and Time during the 48 h hydrolysis of pretreated Triticale straw at a solids loading of 2 % (dw.v^{-1}) WIS.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value (Probability factor)
Model	15.56	6	2.59	11.26	0.0010
A – Cellic® CTec2	0.000998	1	0.0009982	0.004335	0.9489
B – Cellic® HTec2	1.38	1	1.38	5.99	0.0369
C - Time	6.79	1	6.79	29.48	0.0004
AB	1.72	1	1.72	7.47	0.0231
A^2	2.83	1	2.83	12.30	0.0066
C^2	4.44	1	4.44	19.30	0.0017
Residual sum of squares	2.07	9	0.23	-	-
Lack of fit	2.07	8	0.26	101.08	0.0768
Pure error	0.00256	1	0.00256	-	-
Total	17.64	15	-	-	-

Confidence level of 95 %

The respective p-values for the models, predicting hydrolysis of pretreated sorghum bagasse and triticale straw, indicated that both these models were statistically significant (p-value < 0.05). The lack of fit, which describes the variation of the data around the fitted models, was not significant, implying good agreement between the predicted and experimental values at a 95 % confidence level.

Hydrolysis of pretreated sorghum bagasse was primarily influenced by the cellulase enzyme Cellic® CTec2 with its p-value of 0.0035. It was therefore expected that the model of this feedstock would show a linear relationship with respect to only the cellulase enzyme. The xylanase also had a significant effect on glucose concentration, but to a lesser extent. This could be due to the lower residual xylan content in the sorghum feedstock after pretreatment and apparent presence of limited amounts of cellulase activity in Cellic® HTec2.

For pretreated triticale straw, the linear function of hydrolysis time had the most significant influence on the glucose concentration response. A significant two-factor interaction was also observed between Cellic® CTec2 and Cellic® HTec2 indicating that it would be better to change both parameters together rather than only one at a time. The quadratic interaction of time indicated that with time, an optimum value could be reached.

6.3.6. Statistical model validation

The contour plot as well as the response surface plot for optimisation of enzymatic hydrolysis of pretreated sorghum bagasse at 72 h is shown in Figure 25. The model was directed using a backwards process to prevent it from over-fitting the data. It was concluded that enzyme dosages of 0.15 mL Cellic® CTec2.g^{-1} WIS and 0.32 mL Cellic® HTec2.g^{-1} WIS were the minimum requirement to achieve 80 % hydrolysis of steam-exploded SSB (indicated by the red circles). However, this corresponded with 1.7 times more enzyme dosage than for the Spezyme CP/Novozyme 188 control mixture. After 72 h the control cocktail, however, only reached a glucose concentration of 6.21 g.L^{-1} as opposed to the glucose concentration of 8.44 g.L^{-1} with the optimised cocktail.

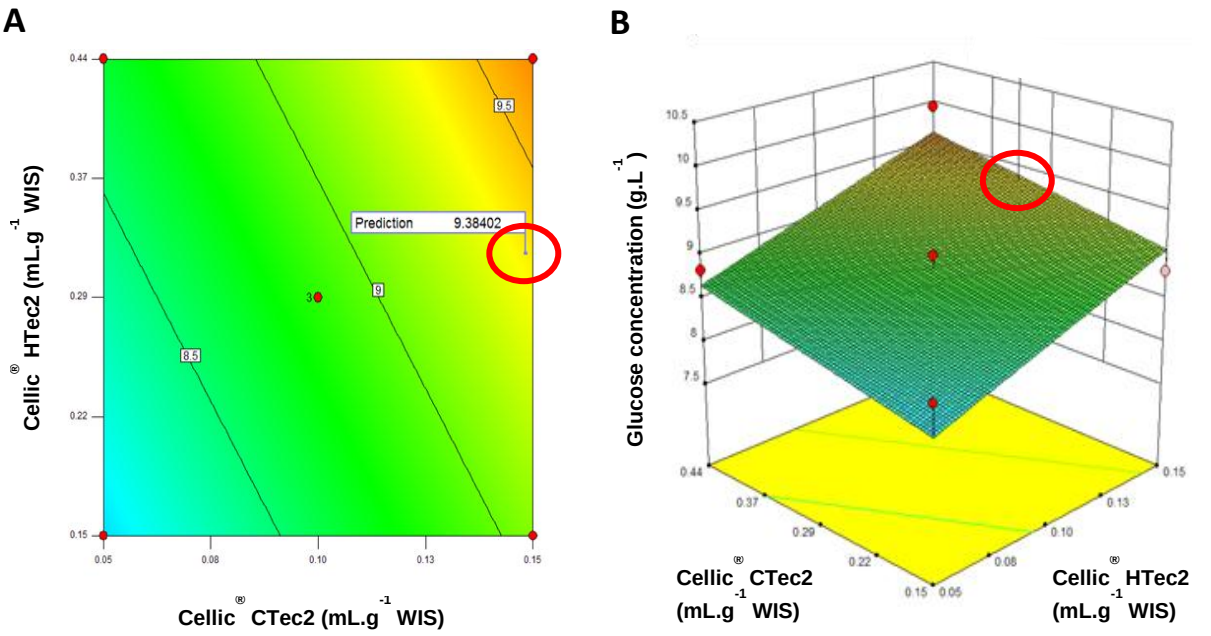

Figure 25: (A) Contour plot and (B) Response surface plot of the glucose concentrations of sweet Sorghum bagasse showing the effect of interaction between Cellic® CTec2 and Cellic® HTec2 loadings at 72 h with a solids loading of 2 % (dw.v^{-1}) WIS.

RSM was also applied to triticale. The contour plot and response surface plot after optimisation of 48 h are illustrated in Figure 26. The response surface plot shows the optimum that was reached due to the quadratic interaction of time as a factor.

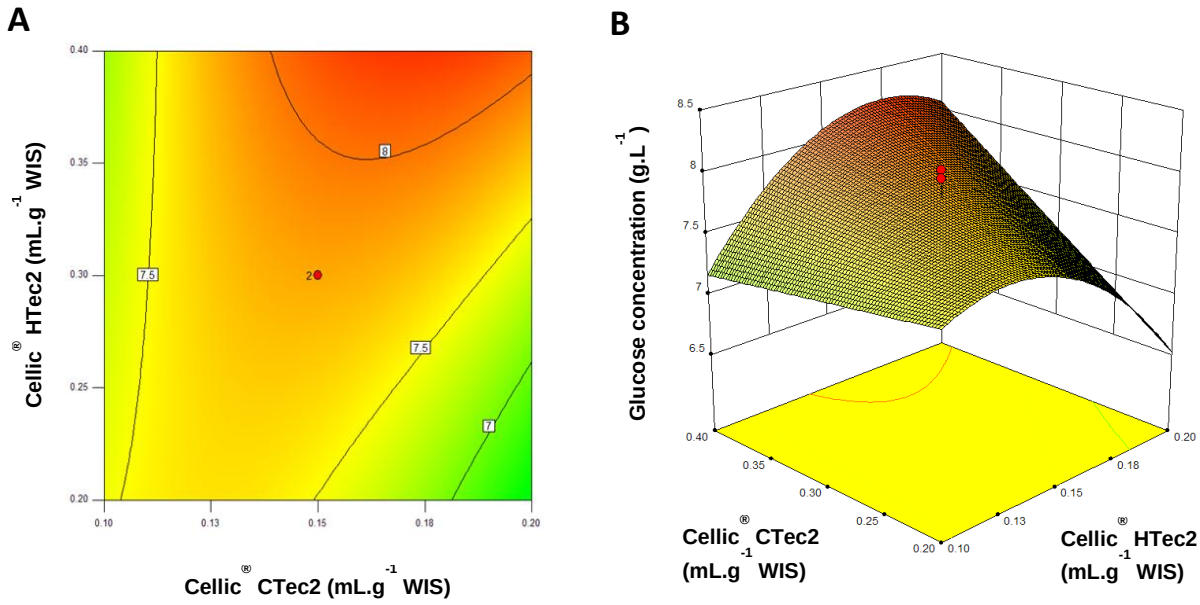

Figure 26: (A) Contour plot and (B) Response surface plot of the glucose concentrations of Triticale straw showing the effect of interaction between Cellic® CTec2 and Cellic® HTec2 loadings at 48 h with a solids loading of 2 % (dw.v^{-1}) WIS.

Further tests, however, had to be performed to determine the optimum enzyme dosages for the triticale enzyme cocktail. For triticale, an equation in terms of actual factors was obtained when the data from the CCD was evaluated with Design Expert® software version 8.0.4. The relationship between the different parameters is given in Equation 5 below.

$$\begin{aligned} Total\ Sugars = {} & 2.6 + (3.2 \times 10^{1} \times Cellic^{®}CTec2) - (1.1 \times 10^{1} \times Cellic^{®}HTec2) \\ & + (1.3 \times 10^{-1} \times Time) + (9.3 \times 10^{1} \times Cellic^{®}CTec2 \times Cellic^{®}HTec2) \\ & - (2.0 \times 10^{2} \times (Cellic^{®}CTec2)^{2}) - (1.1 \times 10^{-3} \times Time^{2}) \end{aligned} \quad (5)$$

Equation 5 was then used in a Microsoft Excel document in combination with the Solver programme which provided up to a thousand combinations of the two enzyme loadings that would yield the maximum amount of sugar at 80 % digestibility of the feedstock.

Figure 27 schematically represents all of these data points which gave the glucose yield corresponding to 80 % digestibility at different time points. The general trend (indicated by the black arrow) seemed to be that the highest enzyme loadings reached the maximum amount of glucose (at 80 % digestibility) in the shortest time (indicated by the blue dots). This was also true for the opposite; as the amount of enzyme is decreased, the time in which the maximum glucose concentration was reached, extended (moving from the red to the green and purple dots). This was, however, expected as material will always be hydrolysed faster when there is a sufficient amount of enzyme present. This is true until a saturation point is reached. It was also observed that some combinations required more enzyme than other combinations for maximum hydrolysis, but that it took longer to reach those values (indicated by the orange dots). This was contradictory to the general trend where larger enzyme loadings require reduced time. A possible reason for this could be that a large amount of enzymes are able to block the active sites of each other, causing a hindrance and thereby taking longer to reach the same amount of glucose as smaller enzyme dosages. As there was still a large amount of residual xylose present within the WIS fraction of triticale after pretreatment, another reason could be that a larger amount of Cellic® HTec2 enzyme (representing an endo-xylanase) was therefore needed to obtain 80 % digestibility of the feedstock. Alternatively, this occurrence could also be explained by

general model phenomena and thus indicates the necessity for validation experiments that need to be performed in order to confirm this data.

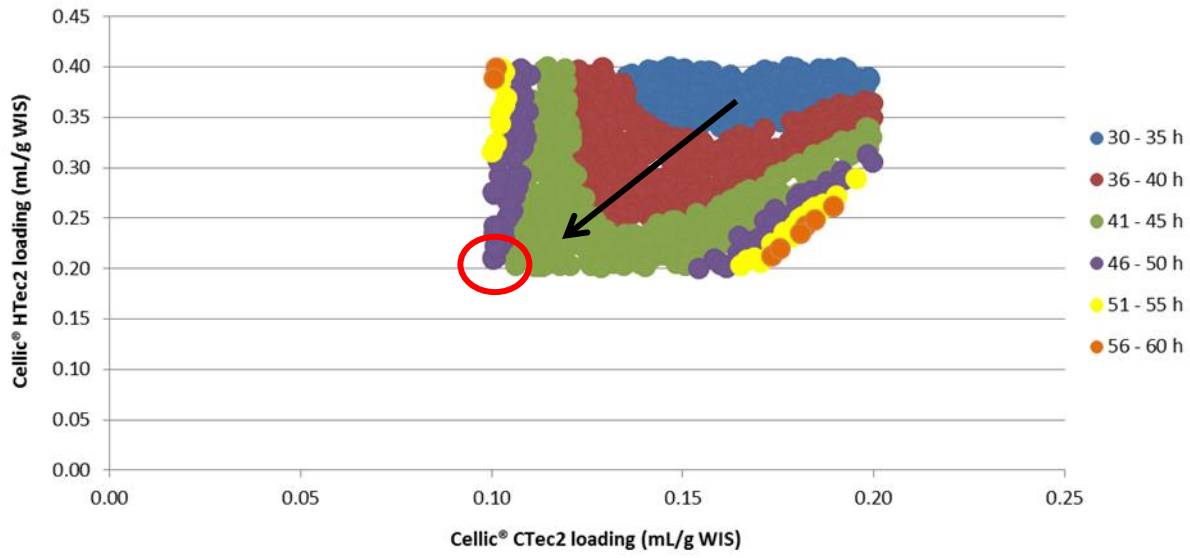

Figure 27: A schematic representation of all the data points that gave the maximum sugar concentration at a digestibility of 80 % with different Cellic® CTec2 and Cellic® HTec2 loadings at specific time points with a solids loading of 2 % (dw.v^{-1}) WIS.

From the model represented in Figure 27, the optimum cocktail for triticale was therefore determined as 0.1 mL Cellic® CTec2.g^{-1} WIS and 0.2 mL Cellic® HTec2.g^{-1} WIS (indicated by the red circle). These were the minimum dosages where the maximum amount of glucose (at a digestibility of 80 %) could be reached within the shortest amount of time. The enzyme loadings corresponded to 0.30 mL.g^{-1} WIS when volumetrically compared to the 0.28 mL.g^{-1} WIS of the control cocktail (Spezyme CP/Novozyme 188). After 48 h of hydrolysis, a glucose concentration of 5.36 g. L^{-1} (57.76 % yield) was reached with the control cocktail whereas for the optimised cocktail, 6.83 g. L^{-1} (73.60 % yield) could be reached.

After evaluating the model graphs, validation experiments were conducted. Figure 28 represents a line graph showing the difference in glucose concentrations during hydrolysis when the control cocktail (Spezyme CP/Novozyme 188) was compared to the new optimised cocktail (Cellic® CTec2/ Cellic® HTec2) with WIS for triticale as substrate and pressed-slurry for sorghum. As expected, the optimised cocktail performed the best in both feedstocks.

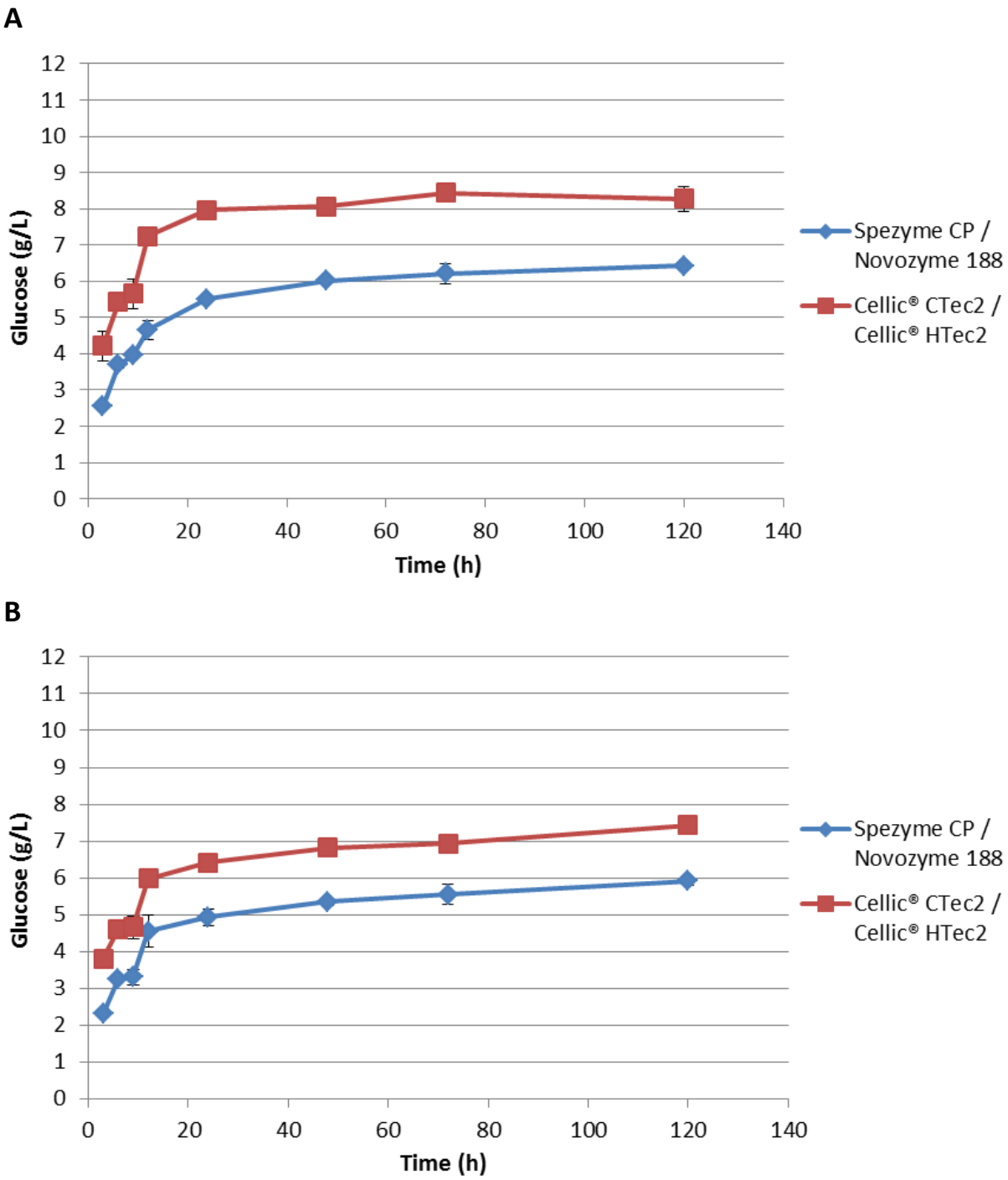

Figure 28: Hydrolysis profiles illustrating the difference in glucose concentration ($g.L^{-1}$) obtained with the Spezyme CP/Novozyme 188 and Cellic® CTec2/Cellic® HTec2 cocktails in both feedstocks at a solids loading of 2 % ($dw.v^{-1}$) WIS. The yields obtained with the control and new cocktail are shown in brackets, respectively. (A) Glucose concentrations of the Sorghum feedstock after 72 h when pressed-slurry was used as substrate during hydrolysis (52.90 %) (71.89 %). (B) Glucose concentration of the Triticale feedstock after 48 h with WIS as substrate (57.76 %) (73.60 %).

The results of the validation experiments not only indicated that the optimised cocktail helped increase the sugar concentration, but also that the use of these optimised

parameters improved the productivity of the process as higher sugar concentrations were obtained in reduced time.

6.3.7. Degree of synergism

One of the strategies to enhance the enzymatic hydrolysis of carbohydrates present in lignocellulosic materials is the development of enzyme combinations that include different activities, thereby providing a more efficient conversion than the application of each of these enzymes individually. It is believed that a combination of enzymes acting in synergism could result in a reduction of the total enzyme dosage and/or reduction of hydrolysis time. For example, the addition of endo-xylanases has been proven to promote cellulose conversion by cellulases [41]. In this study, the combination of xylanase with cellulase provided better conversion of cellulose as well as a reduction in the time required to reach the target cellulose conversion.

In order to evaluate if this improvement was due to true synergism or to an additive influence, the DS was estimated considering the cellulose conversion obtained when only the cellulase or xylanase preparation was applied for enzymatic hydrolysis.

It was observed that there was strong synergism for glucose production mainly at early stages of the enzymatic hydrolysis and for those combinations where the cellulase loading was low (runs 1, 2 and 5 in Table 22). The highest DS (2.15) was determined for the combination with lower cellulase dosage (run 5) at 3 h of enzymatic hydrolysis (EH). The DS decreases as the hydrolysis progress, giving similar values at the end of EH for all enzyme combinations (Table 22).

The maximum difference (22.7 %) was obtained at 72 h for the combination of 0.029 mL Cellic® CTec2 with 0.295 mL Cellic® HTec2.g^{-1} WIS (run 5). This improvement by addition of xylanase is lower than the one reported for steam-treated sorghum bagasse (32 %) of similar composition hydrolysed with a combination of 0.02 mL cellulase.g^{-1} WIS and 0.41 mL xylanase.g^{-1} WIS [41].

However, when considering glucose and cellobiose to estimate cellulose conversion, no DS was detected (Table 23). The EH performed with just xylanase presented cellobiose accumulation at all dosages evaluated (0.0293 - 0.5001 mL.g^{-1} WIS) (data not shown). Similarly, the combination of cellulase with the highest xylanase loading also showed cellobiose accumulation compared with the rest of the cocktails. Although Cellic® HTec2 is characterised by endo-xylanase, it also contains a cellulase background [52]. Similarly, the cellulase preparation Cellic® CTec2 also contains hemicellulase activity. Therefore it is

difficult to estimate the real synergistic effect between the different components. Furthermore, the Cellic® CTec2 preparation includes other accessory enzymes such as glycoside hydrolases family 61 (GH61), an oxidative enzyme that has been proven to boost cellulose conversion [49].

Table 22: Degree of synergism (DS) for glucose production ($g.L^{-1}$).

Run no.	Enzyme loading		Ratio	3 h ($g.L^{-1}$)	6 h ($g.L^{-1}$)	9 h ($g.L^{-1}$)	12 h ($g.L^{-1}$)	24 h ($g.L^{-1}$)	48 h ($g.L^{-1}$)	72 h ($g.L^{-1}$)	120 h ($g.L^{-1}$)
	Cellulase ($mL.g^{-1}$ WIS)	Xylanase ($mL.g^{-1}$ WIS)	Cellulase : Xylanase								
	Factorial points										
1	0.05	0.15	1:3	1.93	1.55	1.22	1.71	1.25	0.89	0.80	0.71
2	0.05	0.44	1:8.8	1.74	1.35	1.04	0.96	0.75	0.64	0.61	0.58
3	0.15	0.15	1:1	1.25	0.82	0.92	0.88	0.72	0.67	0.62	0.60
4	0.15	0.44	1:2.9	1.48	1.12	0.94	0.88	0.71	0.63	0.60	0.57
	Star points (cellulase, xylanase)										
5	0.0293	0.295	1:10.1	2.15	1.80	1.40	1.32	0.97	0.70	0.62	0.53
6	0.1707	0.295	1:1.73	1.19	1.06	0.89	0.84	0.65	0.60	0.57	0.55
7	0.1	0.0899	1:1.1	1.46	1.26	1.10	1.08	0.89	0.78	0.73	0.65
8	0.1	0.5001	1:5	1.45	0.94	0.81	0.76	0.60	0.54	0.53	0.51
	Centre points										
9 (C)	0.1	0.295	1:2.95	1.44	1.14	0.95	0.89	0.70	0.60	0.58	0.56
10 (C)	0.1	0.295	1:2.95	1.38	1.07	0.93	0.87	0.68	0.59	0.57	0.55
11 (C)	0.1	0.295	1:2.95	1.37	1.08	0.92	0.86	0.69	0.59	0.56	0.54

Standard deviation less than 5 %

Table 23: Degree of synergism (DS) for cellulose conversion ($g.L^{-1}$) (considering glucose and cellobiose release during enzymatic hydrolysis).

Run no.	Enzyme loading		Ratio	3 h ($g.L^{-1}$)	6 h ($g.L^{-1}$)	9 h ($g.L^{-1}$)	12 h ($g.L^{-1}$)	24 h ($g.L^{-1}$)	48 h ($g.L^{-1}$)	72 h ($g.L^{-1}$)	120 h ($g.L^{-1}$)
	Cellulase ($mL.g^{-1}$ WIS)	Xylanase ($mL.g^{-1}$ WIS)	Cellulase : Xylanase								
	Factorial points										
1	0.05	0.15	1:3	0.93	0.81	0.71	0.74	0.68	0.62	0.59	0.59
2	0.05	0.44	1:8.8	0.83	0.77	0.67	0.66	0.63	0.60	0.59	0.58
3	0.15	0.15	1:1	0.75	0.52	0.61	0.61	0.56	0.57	0.54	0.55
4	0.15	0.44	1:2.9	0.89	0.76	0.68	0.67	0.62	0.60	0.59	0.57
	Star points (cellulase, xylanase)										
5	0.0293	0.295	1:10.1	0.93	0.90	0.79	0.80	0.75	0.62	0.60	0.54
6	0.1707	0.295	1:1.73	0.74	0.69	0.62	0.61	0.54	0.54	0.54	0.54
7	0.1	0.0899	1:1.1	0.94	0.87	0.76	0.78	0.68	0.63	0.60	0.57
8	0.1	0.5001	1:5	0.91	0.65	0.60	0.59	0.54	0.53	0.53	0.52
	Centre points										
9 (C)	0.1	0.295	1:2.95	0.80	0.70	0.63	0.63	0.59	0.55	0.55	0.55
10 (C)	0.1	0.295	1:2.95	0.76	0.66	0.62	0.61	0.57	0.54	0.54	0.54
11 (C)	0.1	0.295	1:2.95	0.76	0.66	0.61	0.60	0.58	0.54	0.53	0.53

Standard deviation less than 5 %

6.3.8. Comparison of different feedstocks

Pretreated triticale straw was more easily hydrolysed than pretreated sorghum bagasse, as it reached its maximum sugar concentration at 80 % digestibility of the feedstock by 48 h already. However, this higher conversion percentage is explained by the lower total glucan content of this feedstock. The fact that a much larger enzyme loading was needed to reach 80 % digestibility of the sorghum feedstock than for the triticale (0.47 $mL.g^{-1}$ WIS compared to 0.30 $mL.g^{-1}$ WIS) can be explained by the different properties of the feedstocks (Table 15)

and the different forms of substrate of the respective feedstocks (pressed-slurry vs. WIS) that were used.

Lastly, the overall sugar yield (glucose and xylose) per ton of bagasse/straw was determined for sorghum and triticale, respectively. These results are illustrated in Figure 29. The total sugar yields obtained with combined pretreatment hydrolysis with the control and optimised cocktail of sorghum were 328.2 and 401.0 kg sugar.ton^{-1}, respectively. For triticale, the enzyme control yielded 275.5 kg sugar.ton^{-1} and the optimised combination provided 320.7 kg sugar.ton^{-1} straw. It could therefore be concluded that the optimised cocktail did indeed improve sugar production significantly for both feedstocks.

The improvement in overall sugar yield between the optimised enzyme combination and the control was higher in sorghum bagasse than triticale straw. However, as the triticale feedstock was a preferred variety, it is not surprising that even with the control enzyme combination a good sugar yield was obtained. This fact highlights the need for feedstock evaluation for ethanol production, not only in terms of digestibility, but also in terms of composition and response to pretreatment. Another factor that needs to be integrated in the evaluation is the fermentability of the hydrolysates. Based on the sugar yields obtained with the optimised enzyme combination, an ethanol production of 224.0 and 179.0 L.ton^{-1} of bagasse/straw could be theoretically produced when considering an ethanol yield of 0.44 g ethanol.g^{-1} sugar.

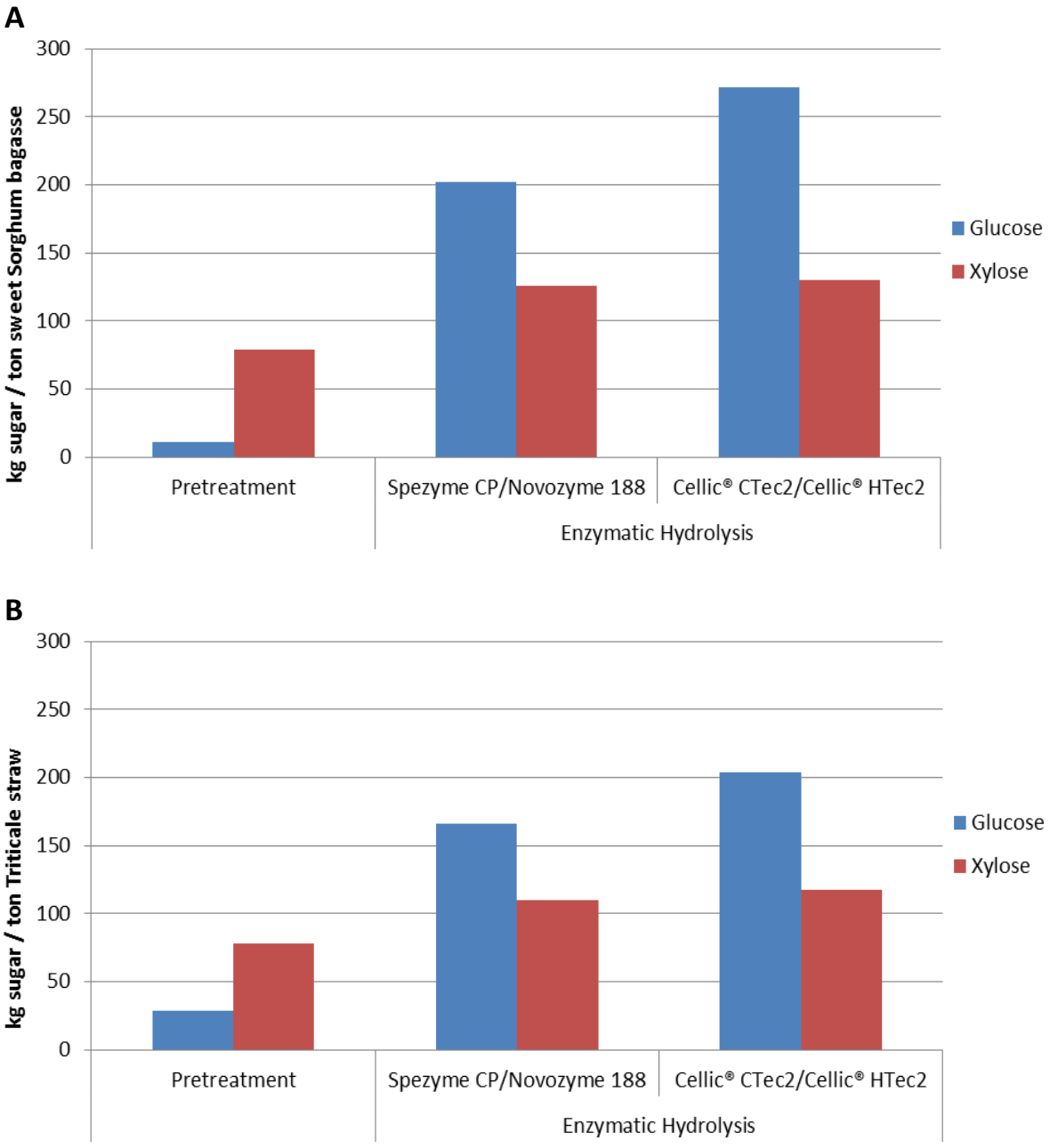

Figure 29: A graph illustrating the overall sugar yield per dry ton of (A) sweet Sorghum bagasse and (B) Triticale straw.

6.4. Conclusions

Sweet sorghum bagasse and triticale straw are promising feedstocks for the production of bio-ethanol as they are abundant, renewable and require low inputs. In spite of being similar feedstocks in terms of composition, their optimum pretreatment conditions were

different. RSM was shown to be an effective way of providing models for optimisation of the total sugar concentration during the enzymatic hydrolysis of both feedstocks. The addition of endo-xylanase seemed to improve glucan conversion.

In conclusion, it was possible to construct an enzyme cocktail to minimise the dosage required for a yield of 80 % by using a statistically experimental design method after screening experiments, optimisation and a verification stage. This data confirmed the conception that substrates which are pretreated under different conditions, appear to require different enzymatic profiles for their optimal hydrolysis, even for similar feedstocks. It was also found that the optimal combination of enzymes could promote a more efficient enzymatic hydrolysis in different feedstocks.

6.5. References

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6.6. Addendum A

These results are presented as screening experiments to determine a few baseline parameters for subsequent optimisation studies.

6.6.1. Conversion rates

For both feedstocks, samples were taken at early hours in order to study the kinetics during the initial 12 h. Figure 30 and Figure 31 illustrate the kinetics of glucose production in sorghum and triticale, respectively, when applying the particular enzyme dosages. In both feedstocks the highest glucose concentrations were reached with addition of the maximum xylanase loadings. Sorghum yielded the highest sugar concentrations, possibly due to the larger cellulose fraction thereof in the WIS. For the sorghum feedstock, the graphs of only cellulase (Cellic® CTec2) and only xylanase (Cellic® HTec2) loadings were also included (Figure 30).

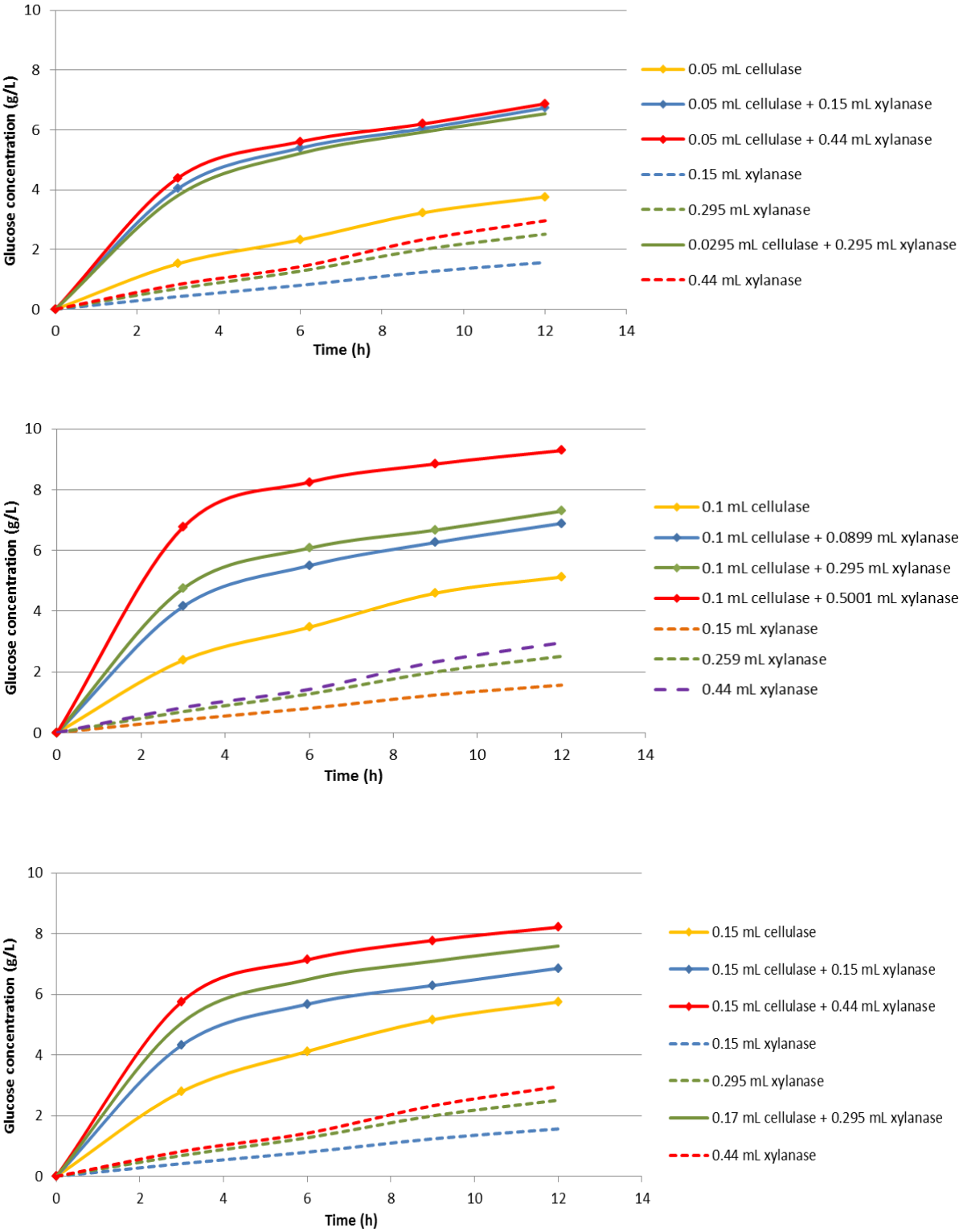

Figure 30: Line graphs representing the glucose concentrations reached when applying the respective enzyme dosages with sweet Sorghum bagasse as feedstock.

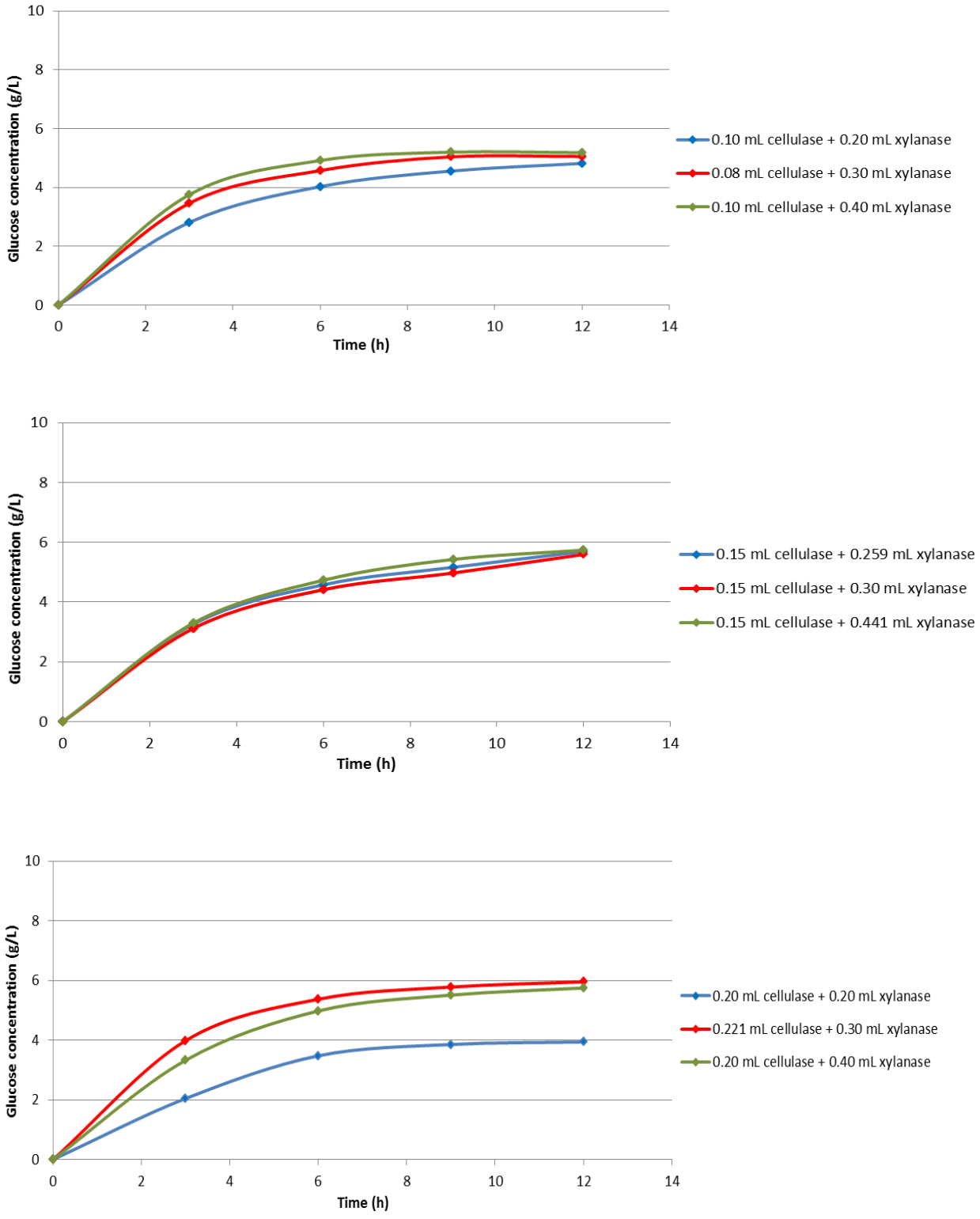

Figure 31: Line graphs for Triticale straw, representing the glucose concentrations reached with each of the enzyme dosages, respectively.

The conversion rate of cellulose into glucose (as described in detail within section 4.9.1.) was estimated during the first 12 hours. The conversion rate was normalised based on the volumetric dosage (mL enzyme in the hydrolysis media). These values are indicated in Table 24 and Table 25 for sorghum and triticale, respectively.

Table 24: Table indicating the conversion rates during the first 12 h of hydrolysis of sweet Sorghum bagasse.

Run	Enzyme dosage (mL.g^{-1} WIS)		Rate of conversion during the initial 12 h (g glucose.L^{-1}.h^{-1}.mL^{-1})
	Cellulase	Xylanase	
1	0.05	0.15	3.37
2	0.05	0.44	1.41
3	0.15	0.15	2.32
4	0.15	0.44	1.45
5	0.0293	0.295	1.11
6	0.1707	0.295	1.68
7	0.1	0.0899	3.62
8	0.1	0.5001	1.62
9	0.1	0.295	1.88

Standard deviation less than 5 %

Table 25: The conversion rates of Triticale straw during the first 12 h of enzymatic hydrolysis.

Run	Enzyme dosage (mL.g^{-1} WIS)		Rate of conversion during the initial 12 h (g glucose.L^{-1}.h^{-1}.mL^{-1})
	Cellulase	Xylanase	
1	0.1	0.2	1.64
2	0.1	0.4	1.12
3	0.2	0.2	1.03
4	0.2	0.4	0.99
5	0.08	0.3	1.41
6	0.221	0.3	1.21
7	0.15	0.259	1.39
8	0.15	0.441	0.98
9	0.15	0.3	1.23

Standard deviation less than 5 %

Overall, the conversion rates for the sorghum feedstock were higher than that for triticale. This could be due to the larger amount of cellulose present within the sorghum feedstock. There was thus more cellulose available for the enzymes to hydrolyse.

The first 12 hours was chosen specifically to evaluate the initial kinetics. At these early time points, end-product inhibition only plays a minor role and differences in performance of the respective enzymes can be easier observed. This follows as different enzyme

combinations, which are not considered optimal, are able to reach the same yield as with optimum enzyme combinations when hydrolysis are performed over long time periods.

All of the experiments were also performed at a solids loading of 2 % (dw.v^{-1}) for a number of reasons:

1. The use of a low solids loading limits end-product inhibition,
2. A 2 % (dw.v^{-1}) substrate loading was the loading used by the group during evaluation of the effect that pretreatment has on digestibility, and
3. SSF experiments were conducted using a fed-batch strategy with 2 % (dw.v^{-1}) feedings at a time

A baseline was therefore attained by which pretreatment, hydrolysis and fermentation could all be compared.

6.6.2. Enzymatic hydrolysis of lignocellulose substrates

Spezyme CP supplemented with Novozyme 188 was selected as the control enzyme cocktail and used as baseline. The new cellulases from Dyadic (Alternafuel CMAX) and Novozymes (Cellic® CTec2 and HTec2), were selected for evaluation of enzymatic hydrolysis of steam-pretreated SSB and triticale straw. Given that 15 FPU.g^{-1} WIS is one of the standard enzyme dosages used in enzymatic hydrolysis (for example in evaluation of pretreatment on digestibility) [53]–[55], the corresponding volumetric dosage of 0.25 mL.g^{-1} WIS based on the activity of Spezyme CP was accepted for further comparisons between the cellulase preparations. Since the new cellulase preparations are expected to perform better, two lower enzyme loadings: 0.15 and 0.05 mL.g^{-1} WIS, were also studied (Figure 32, Table 26 and Table 27).

EH was performed at 2 % (dw.v^{-1}) of WIS for both feedstocks. The time course for glucose release from sorghum and triticale are represented in Figure 32 (A-C) and Figure 32 (D-F), respectively. The low solids loading was selected to prevent end-product inhibition and in order for the difference in performance between the enzyme preparations to be more evident. As the Alternafuel CMAX enzyme preparation from Dyadic performed very poorly in triticale (Figure 32 (D-F)), it was not used in any of the further experiments.

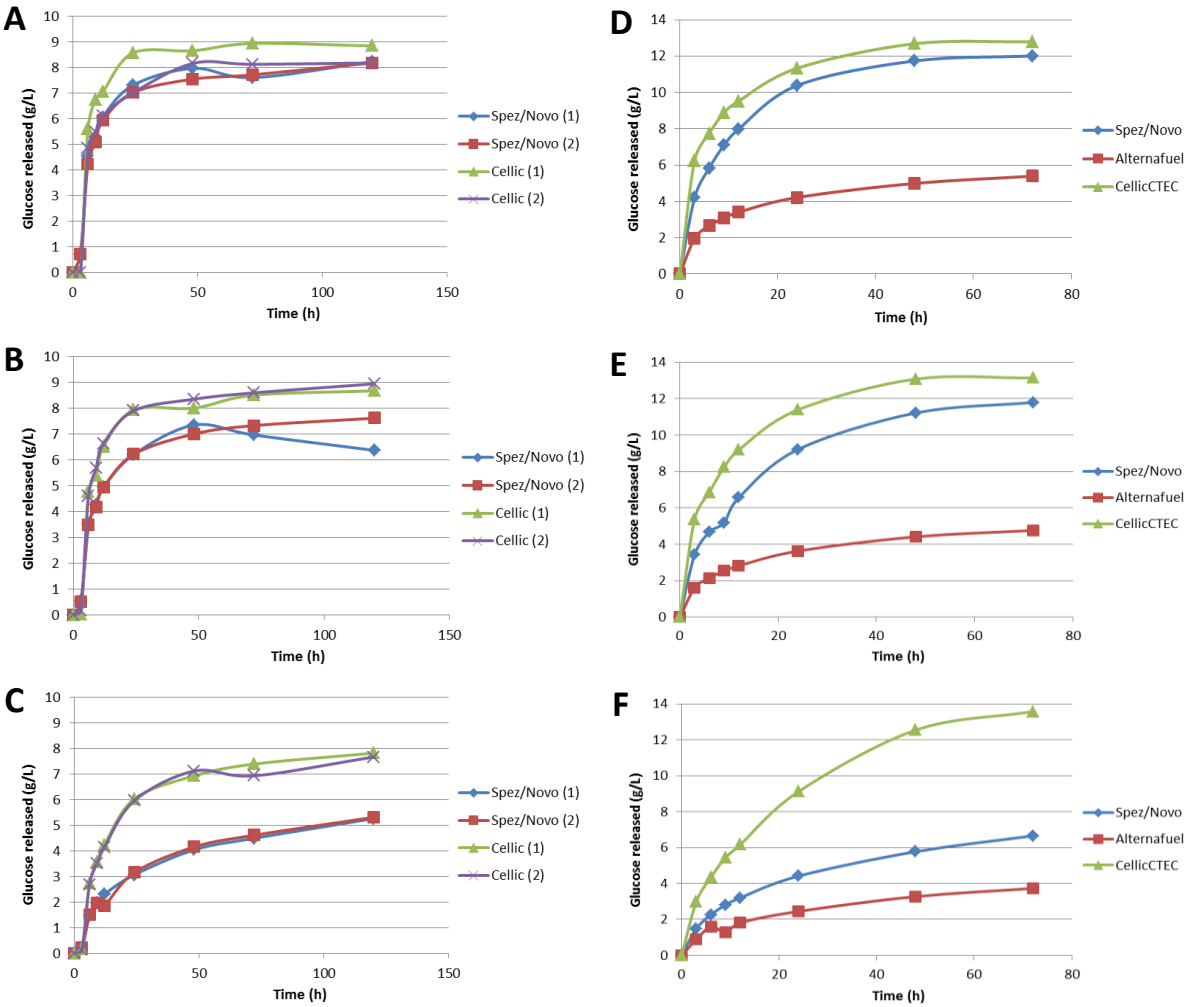

Figure 32: Glucose release during hydrolysis of pretreated Sorghum bagasse and Triticale straw at enzyme loadings (mL.g^{1} WIS) of (A) 0.25, (B) 0.15, (C) 0.05 and (D) 0.25, (E) 0.15, (F) 0.05, respectively.

The initial kinetics during the first 12 hours was studied by determining the cellulose conversion rate (g glucose.h^{-1}.mL^{-1} enzyme) [56]. This conversion rate is an estimation of the speed of cellulose conversion into glucose between the intervals 0-3, 3-6 and 6-12 h, normalised for the volume of enzyme dosage. These values for sorghum are indicated in Table 26 and for triticale in Table 27.

Table 26: The conversion rates of pretreated Sorghum bagasse during the first 12 h of enzymatic hydrolysis with different enzyme dosages. Maximum cellulose conversion (within the shortest time) is also presented.

	Rate of conversion during initial 12 h ($g\ glucose.L^{-1}.h^{-1}.mL^{-1}$)		Maximum Cellulose conversion (%)	
Enzyme dosage ($mL.g^{-1}$ WIS)	Spezyme CP/ Novozyme 188	Cellic® CTec2	Spezyme CP/ Novozyme 188	Cellic® CTec2
0.05	3.87	7.74	54.98 (120 h)	80.19 (120 h)
0.15	3.12	4.16	78.47 (120 h)	91.63 (120 h)
0.25	2.26	2.81	84.29 (120 h)	91.52 (72 h)

Table 27: The conversion rates of pretreated Triticale straw during the first 12 h of enzymatic hydrolysis with different enzyme dosages. The maximum cellulose conversion obtained in the shortest time within the feedstock is also presented.

	Rate of conversion during initial 12 h ($g\ glucose.L^{-1}.h^{-1}.mL^{-1}$)			Maximum Cellulose conversion (%)		
Enzyme dosage ($mL.g^{-1}$ WIS)	Spezyme CP/ Novozyme 188	Cellic® CTec2	Alternafuel	Spezyme CP/ Novozyme 188	Cellic® CTec2	Alternafuel
0.05	6.07	11.67	3.79	69.12 (72 h)	94.41 (24 h)	38.78 (72 h)
0.15	4.16	5.94	1.84	98.04 (24 h)	95.46 (12 h)	49.04 (72 h)
0.25	3.07	3.84	1.35	85.18 (12 h)	98.95 (12 h)	60.96 (72 h)

When looking at Table 26 and Table 27, 0.05 $mL.g^{-1}$ WIS provided the highest conversion rates for both sorghum and triticale. However, the enzyme cocktails cannot be selected based only on the results from the initial 12 hours. Therefore, the maximum conversion during the process also had to be taken into account (also shown in Table 26 and Table 27).

The quicker (earlier) the maximum cellulose conversion is reached, the more cost effective it will be on industrial scale. However, the use of minimum enzyme loadings is required in this study, with the disadvantage that it would take much longer. As it can be observed for both feedstocks, higher yields in terms of maximum cellulose conversion were obtained in shorter time periods when the new cellulase preparation of Novozymes, Cellic® CTec2, was applied (Table 26 and Table 27). This enzyme was superior to the conventional SpezymeCP/Novozyme 188 combination as well as Alternafuel (in the case of triticale) at all enzyme dosages tested.

6.6.3. Fractional design

One of the approaches adopted in order to improve the enzymatic hydrolysis and/or reduce enzyme requirements, is the enhancement of enzyme synergism by constructing tailor-made enzyme combinations adapted to specific feedstock-pretreatment. The

increased effect of enzymes in the appropriate ratio can lead to a reduction of the final enzyme loading needed for complete hydrolysis of the cellulose. The different cellulase enzymes (endo-glucanase (EG), exoglucanase and β-glucosidase) can be combined with accessory enzymes such as hemicellulases and pectinases that have been proven to promote cellulose conversion [57]. The incorporation of hemicellulases could also allow for the integral use of both cellulose and hemicellulose fractions. Further, the supplementation with non-catalytic additives such as surfactants, polymers and proteins have also been shown to enhance enzyme performance and therefore, reduce the enzyme loading [58]. An additional advantage of surfactants is that they favour the recycling of the enzymes.

In this context, the effect of xylanase, pectinase and surfactant Polyethylene Glycol (PEG)-4000 on glucose released during enzymatic hydrolysis of pretreated lignocellulose was evaluated statistically by a factorial experimental design. The aim here was to determine what factors can enhance the enzymatic hydrolysis for the lower cellulase dosage established in previous experiments (volume ranges). The factorial experimental design was thus proposed as a step prior to applying an experimental design for optimisation to check which variables have a significant effect and thereby are able to reduce the amount of runs to get preliminary information.

Two different factorial designs were applied, one for the conventional enzyme combination and one for the new cellulase preparation as enumerated in Table 28 and 29. The range of these variables was selected based on the maximum and minimum values reported in literature [59]–[63]. All these studies were performed with the WIS from steam-pretreated sorghum bagasse at a low solids loading to prevent end-product inhibition.

Table 28: A set of experiments for the conventional cellulase enzyme (Spezyme CP).

Design: 2**(5-2) design (Sorghum bagasse)					
Run	Spezyme CP (mL.g^{-1} WIS)	β-glucosidase Novozyme 188 (mL.g^{-1} WIS)	Multifect Xylanase (mL.g^{-1} WIS)	Multifect pectinase (mL.g^{-1} WIS)	PEG (g.g^{-1} WIS)
8	0.15	0.10	1.70	0.10	0.10
3	0.10	0.10	0.05	0.05	0.10
4	0.15	0.10	0.05	0.10	0.01
2	0.15	0.01	0.05	0.05	0.01
5	0.10	0.01	1.70	0.10	0.01
6	0.15	0.01	1.70	0.05	0.10
1	0.10	0.01	0.05	0.10	0.10
7	0.10	0.10	1.70	0.05	0.01

Table 29: A set of experiments for the Cellic® CTec2 cellulase preparation.

Design: 2**(4-1) design (Sorghum bagasse)				
Run	Cellic® CTec2 (mL.g^{-1} WIS)	Cellic® HTec2 (mL.g^{-1} WIS)	Pectinase (mL.g^{-1} WIS)	PEG (g.g^{-1} WIS)
5	0.025	0.05	0.10	0.10
6	0.150	0.05	0.10	0.01
1	0.025	0.05	0.05	0.01
8	0.150	1.70	0.10	0.10
2	0.150	0.05	0.05	0.10
3	0.025	1.70	0.05	0.10
4	0.150	1.70	0.05	0.01
7	0.025	1.70	0.10	0.01

The statistical significance of the influence of the selected cellulase dosages supplemented by xylanase, pectinase and surfactant PEG-4000 on glucose release during enzymatic hydrolysis was determined by ANOVA. These results can be visualised in the pareto charts represented in Figure 33 for different hydrolysis times (24, 72 and 120 h). These graphs indicate that, except for the Spezyme CP at 24 h, the supplementation with xylanases was the only factor influencing positively on the glucose release at a confidence level of 95 % for the range of conditions studied. Cellic® HTec2 showed to have an improved effect on Cellic® CTec2, compared to the Multiffect Xylanase over Spezyme CP. As expected, xylose release was also influenced by xylanase supplementation (data not shown). However, this effect was considerably lower than for the glucose release.

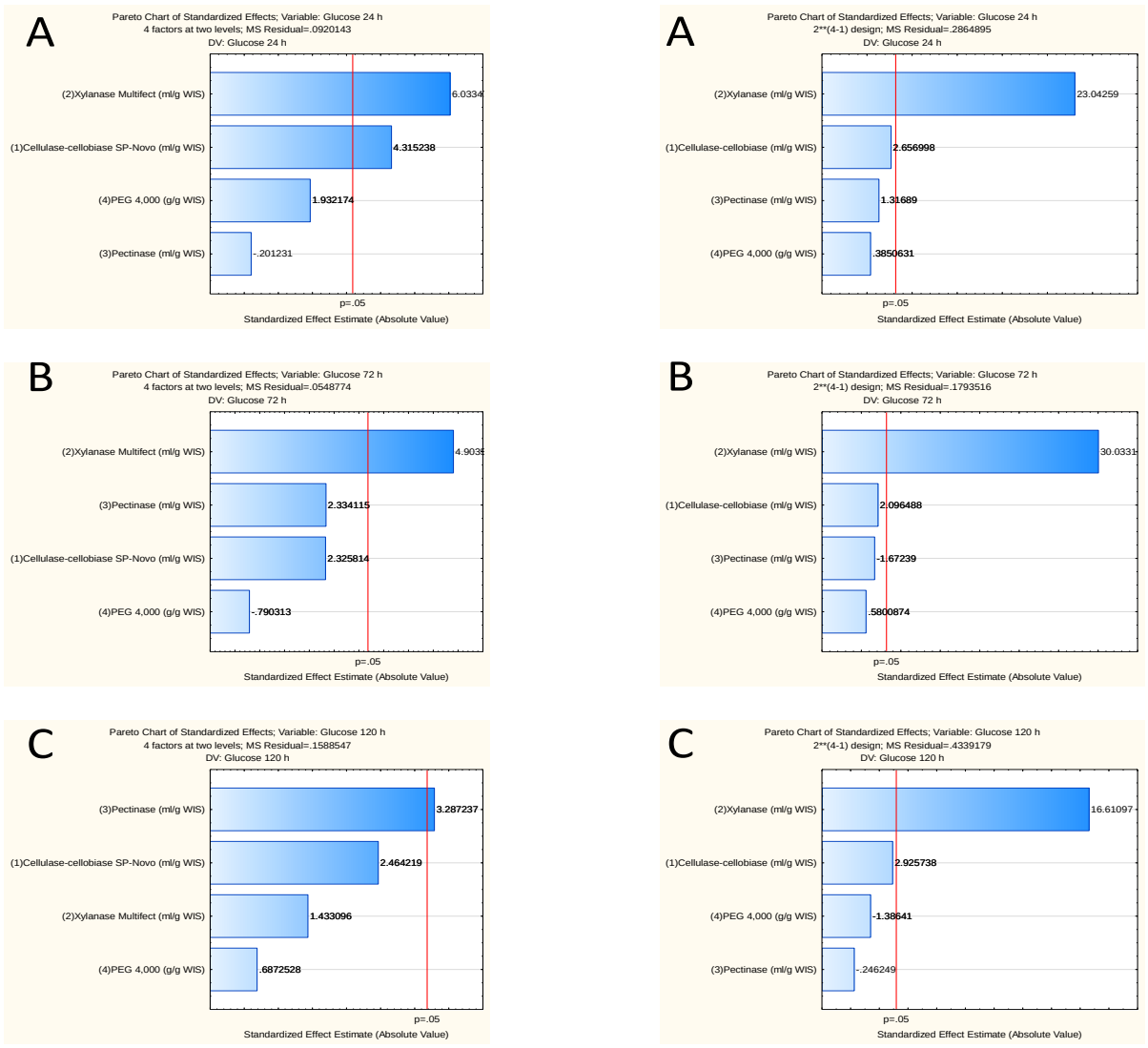

Figure 33: Pareto charts showing the influence of factors on glucose release at (A) 24 h, (B) 72 h and (C) 120 h for the conventional cellulase Spezyme CP (left) and the new cellulase preparation Cellic® CTec2 (right).

From the above data it can be concluded that xylanase was the main factor influencing both glucose and xylose release during enzymatic hydrolysis of steam-pretreated sorghum bagasse. The influence of xylanase could be due to the removal of residual xylan present in the pretreated bagasse (9.35 %), thereby increasing the accessibility of cellulose to cellulases. Therefore, this factor together with cellulase dosage was selected to perform the experimental design for optimisation of enzymatic hydrolysis. Cellic® HTec2 was used as endo-xylanase source instead of Multifect xylanase, as the former yielded better results.

6.6.4. Solids loading

Ethanol concentrations of at least 4 % ($v.v^{-1}$) are necessary for the distillation process to be economically feasible [11]. For the majority of lignocellulosic materials, this requires solid concentrations of 16 % ($dw.v^{-1}$) or higher [11]. However, a high solids loading entails many technical difficulties such as adequate mixing and mass transfer, in addition to the associated increased end-product inhibition [11]. It is well known that glucan conversion is reduced when increasing the substrate concentration. However, in order to achieve a high concentration of ethanol, the solids loading must be increased to reach a high enough sugar concentration in the hydrolysis media. In this part of the study, the effect of solids loading on sugar concentration (glucose, cellobiose and xylose) and cellulose conversion (%) was investigated.

Enzymatic hydrolysis was carried out at different WIS loadings (4, 8, 10, 12, 16 and 20 % ($dw.v^{-1}$)) using a standard enzyme mixture that will be further compared with the optimised enzyme combination. Figure 34 shows these values for different concentrations of WIS from pretreatment.

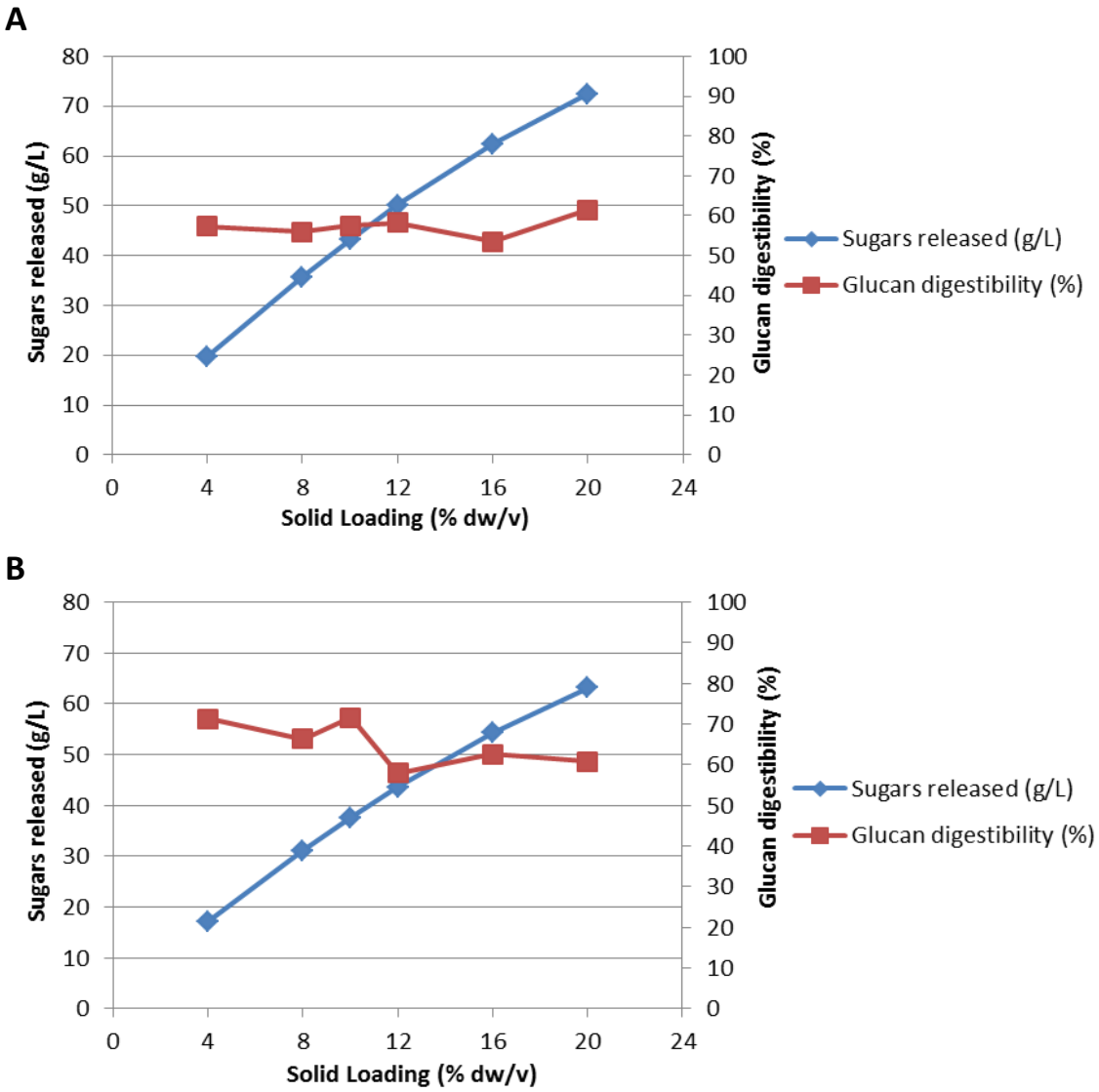

Figure 34: The effect of loading of pretreated solids on glucan concentration (glucose, xylose and cellobiose) after 72 h of hydrolysis for the control enzyme mixture Spezyme CP/Novozyme 188 with (A) Sorghum and (B) Triticale.

Based on these results, there was still room for improvement in terms of preparing an optimised enzyme cocktail and testing different feeding strategies (substrate fed-batch, the sequential/split addition of enzymes, etc.). At higher solids loading it may also be of benefit to investigate the effect of surfactant again.

7. Development and Validation of a Micro-assay Method for Rapid Enzymatic Optimisation

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All experiments were planned in collaboration with Dr M.P. García-Aparicio and Mr J. Swart and executed by myself. The results were analysed with the help of Dr M.P. García-Aparicio and Mr J. Swart. All co-authors contributed to draft the final manuscript which will be submitted for publication in the current format.

Keywords: Lignocellulose, Enzymatic hydrolysis, Micro-assay, Handsheets

Abstract

An increasing number of enzymes and process options for hydrolysis of lignocellulosic biomass are under development, with the requirement to optimise these combinations for specific biomass- and pretreatment-options. The increase in the number of experimental

runs required in such optimisation protocols, calls for a rapid and realistic method of evaluation of enzymatic hydrolysis, such as micro-assays considered here, to save on reagents, time and therefore costs.

The main challenge in developing micro-assays for enzymatic hydrolysis of lignocellulose derives from the heterogeneity of lignocellulose, which hampers the representativeness of samples and sample handling at milligram scale. Micro-assays therefore require the milling of pretreated materials, which alters the physical-chemical properties of these materials, potentially improving digestibility and masking possible differences in enzyme-feedstock-process combinations.

In this context, the purpose of the present study is to set up a micro-assay methodology based on handsheets to study the enzymatic hydrolysis of steam-exploded agricultural by-products. Triticale straw and sorghum bagasse were selected as agricultural by-products for the micro-assay, based on their response to steam-pretreatment. The solid fractions from pretreatment of these feedstocks were subjected to homogenisation steps in a disintegrator and liquidiser at different severities (measured in rotation speed in rpm), with subsequent measurement of hydrolysis properties in micro-assays from handsheets of the processed materials.

Control of homogenisation, quantified by fiber lengths of < 8 mm (representing the diameter of the handsheet-discs) enabled a significant reduction in particle size and handsheets homogeneity without increasing the digestibility significantly. In the case of steam-treated sorghum bagasse, the disintegrator treatment (37 500 rpm) was sufficient to obtain the required fiber lengths, while the combined treatment with liquidiser (6 250 rpm) and disintegrator (31 250 rpm) was needed for steam-treated triticale straw. The selected homogenisation steps were able to achieve the required fiber lengths (averages of 1.5 - 2.5 mm for sorghum and 2.5 – 4.5 mm for triticale), without significantly increasing the digestibility of the pretreated materials. Moreover, the enzymatic hydrolysis from handsheets generated after those treatments allowed for differentiation between different enzymes combinations. Therefore, the proposed homogenisation method enabled the development of a micro-assay for the optimisation of enzymatic hydrolysis of pretreated

lignocellulose, thereby allowing optimisation according to particular feedstocks/ pretreatment options.

7.1. Introduction

The growing demand for energy and depletion of fossil-derived fuels, coupled with environmental concerns towards the reduction of greenhouse gas (GHG) emissions, have led to the search for alternative renewable energy sources [1]. Liquid biofuels, derived from plant biomass, are one of the few alternatives for short-term diversification in the transportation sector, given that they can be easily integrated in the current distribution systems [2], [3].

The development and use of novel dedicated energy crops with improved agronomic traits has been suggested as a strategy to produce biofuels without impacting food security or the environment [4]. In this context, sweet sorghum (*Sorghum bicolor*) is a promising feedstock for ethanol production. It belongs to the C4 family (representing a specific group of plants in which photosynthesis takes place via the phosphoenol pyruvate carboxylase pathway) and therefore requires less water and nitrogen compared to other sugar plants [5]. Similarly, triticale (*x Triticosecale*), a hybrid of rye and wheat, is able to grow in marginal soils and adapt to stress, and it also requires low nitrogen input [6]. The biochemical conversion (hydrolysis fermentation) of the lignocellulosic residues from these crops (bagasse and straw of sorghum and triticale, respectively) provides feedstock to increase ethanol production from these energy crops, in addition to ethanol from the sugar or grain components.

Ethanol derived from lignocellulosic biomass residues, also termed as second generation (2G) bio-ethanol, not only does not compete with food, but also provides numerous advantages compared to sugar and starch-based ethanol [7]. Lignocellulosic biomass is widely distributed, they are generally cheaper and they have shown to be more effective in net GHG emissions reduction [8]. However, the technology is still in development in order to make cellulosic ethanol cost competitive with conventional fossil fuels. The success of this technology relies largely on the selection of appropriate feedstocks and efficient pretreatment, enzymatic hydrolysis and fermentation processes [9].

Improvements on pretreatment technologies and reduction on enzyme production costs through biotechnology have led to several-fold reduction of enzyme loading for lignocellulose hydrolysis. Nonetheless, enzyme production and its use are still considered one of the main cost contributors of the cellulosic ethanol production process [1]. Several strategies have been proposed to reduce the dosages and costs of enzymes required for hydrolysis. These strategies can be grouped in terms of enhancement of the enzyme systems and process development. The first group includes development of enzyme-producing strains with improved protein titres, development of enzymes with improved specific activity by protein engineering of known enzymes, and/or by bio-prospecting of new enzymes [10], [11].

Alternatively, there is a trend to construct tailor-made enzyme combinations adapted to specific feedstocks and pretreatment methods, to improve the efficiency of enzymes and thereby reduce the required dosages [12]. In this approach, combinations of different enzyme preparations (characterised as cellulase, xylanase, pectinase, etc.) or individual components and their ratios can be studied and optimised statistically for particular biomass-pretreatment combinations [12]. Process configurations such as simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) can further reduce the required enzyme dosages. The SSF process alleviates end-product inhibition and thermal inactivation of the enzymes [13], while in CBP the fermentative micro-organism displays cellulase activity, reducing partially or completely the amount of external cellulase required [14].

The increasing number of enzymes and process options involved in the hydrolysis of lignocellulosic biomass are under development, with the requirement to optimise these combinations for degradation, coupled with the number of factors to consider in order to optimise enzymatic hydrolysis of specific biomass- and pretreatment-options. It is generally accepted that enzyme activity assays in model substrates such as filter paper, cellobiose or Birchwood xylan do not represent the real hydrolytic potential of enzyme preparations on lignocelluloses [15]. Moreover, the enzyme requirements will differ depending on feedstock, selected pretreatment methods and process conditions.

The increase in the number of experimental runs required in such optimisation protocols calls for rapid and realistic methods of evaluation of enzymatic hydrolysis. A method, such as the micro-assays considered here to test lignocellulosic feedstocks could save on reagents, time and therefore costs, allowing the study of multiple factors [15]. The main challenge in developing micro-assays for enzymatic hydrolysis of lignocellulose such system derives from the heterogeneity of lignocellulose, which hampers the representativeness of samples and sample handling at milligram scale [16]. Several attempts have been conducted to develop high-throughput systems where the lignocellulosic biomass is ground or milled very fine (0.25 - 0.5 mm) to facilitate the distribution of the biomass [12], [16]–[18]. However, the milling alters structural properties of the initial substrate and can improve the digestibility, thereby masking possible differences in performance among enzyme combinations and/or feedstock-pretreatment combinations [16]. Berlin *et al.* (2005) proposed a micro-assay system to evaluate enzyme performance on real lignocellulose, a more realistic option compared to model substrates such as filter paper [15]. In this approach, the lignocellulose, yellow poplar, was subjected to ethanol organosolv pulping to obtain handsheets that can be easily cut and dispensed at micro-scale [15]. A more recent work used the same approach from the solid fraction of steam-exploded wheat straw [9]. For both studies, there were no significant differences in the enzymatic hydrolysis yield obtained from handsheets and the original pretreated material at laboratory scale.

In line with the work carried out by Alvira *et al.* (2010), the purpose of the present study is to set up a micro-assay methodology, based on handsheets, to optimise the enzymatic hydrolysis of steam-exploded agricultural by-products for specific feedstock-pretreatment-process combinations [19]. One variety of sorghum and triticale each was selected for the present study, based on the response of the lignocellulosic fraction (bagasse/straw) to pretreatment-hydrolysis processes performed at small scale [20], [21]. The solid fractions from pretreatment of these bagasse and straw samples were subjected to a homogenisation step in a liquidiser at different rpm prior to handsheets making, to provide representative samples of the pretreated materials for micro-scale hydrolysis assays. Those homogenisation conditions, quantified by fiber lengths of < 8 mm which represent the diameter of the handsheet-discs, were selected for handsheets making and feedstock preparation for micro-assays. This proposed homogenisation is expected to favour the representative sampling at

micro-scale, especially for those lignocellulosic materials that do not respond well to pretreatment and/or which have been pretreated under milder conditions.

7.2. Materials and Methods

7.2.1. Raw materials

Sorghum bagasse and triticale straw were provided by the University of KwaZulu-Natal (Ukhulinga Experimental Research Farm in Pietermaritzburg), Durban, South Africa and Department of Genetics at Stellenbosch University (Mariendahl Experimental Research Farm), Stellenbosch, South Africa, respectively.

The raw material was sieved and milled and the material, with a particle size between 0.38 and 10 mm, was collected and stored (23 ˚C, relative humidity of 50 %) for pretreatment and hydrolysis.

7.2.2. Pretreatment

Steam-pretreatment was carried out in a 19 L reactor at different conditions for each biomass: sorghum bagasse MSJH16 was treated at 200 ˚C for 5 minutes, while triticale straw M13 was treated at 190 ˚C for 5 minutes. These were the optimum pretreatment conditions for these feedstocks as indicated by previous studies [20], [21]. The “whole slurry” from pretreatment was filtered to obtain the liquid and solid fractions, which were characterised using the standard laboratory analytical procedures (LAPs) for biomass and liquid analysis [22]–[26]. The solid fraction was thoroughly washed with water prior to use as substrate in enzymatic hydrolysis and for the preparation of handsheets for micro-assays.

7.2.3. Enzymes preparations

Commercial enzyme preparations were used in this study. Cellic® CTec2, Cellic® HTec2 and Novozyme 188 were kindly supplied by Novozymes (Novozymes A/S, Denmark), whereas Spezyme CP was kindly supplied by Genencor (Genencor, Leiden, Netherlands). Cellic® CTec2 and Spezyme CP are mainly cellulase preparations whereas Cellic® HTec2 is a

hemicellulase (endo-xylanase (EX)) complex. Novozyme 188 was added to the Spezyme CP mixture to provide enough β-glucosidase (BG) activity during enzymatic hydrolysis.

7.2.4. Preparation of handsheets

The water-insoluble solids (WIS) from the pretreatment of lignocellulosic feedstocks were subjected to different treatments to ensure homogenisation by combining the use of two pieces of equipment: a liquidiser (Hamilton Beach Rio™ Stainless Steel Commercial Bar Blender Product Code: HBB 250S-RIO) and a disintegrator (British Pulp Evaluation Apparatus Manufactured by Mavis Engineering Ltd. London N.I. England No 7518 D) with a combined rpm of 37 500 rpm, as a measure of homogenisation severity. The different treatments are summarised in Table 30.

Table 30: The different homogenisation treatments of the WIS fraction of the materials with their respective disintegrator and liquidiser revolutions per minute (rpm).

Treatment	Disintegrator rpm	Liquidiser rpm
Untreated	-	-
1	37 500	0
2	31 250	6 250
3	25 000	12 500
4	18 750	18 750
5	12 500	25 000
6	6 250	31 250
7	0	37 500

Fiber length was determined using a digitiser (Summagraphics, MM1201 tablet) whereby linear and non-linear lengths of fibers can be measured with the help of a digital stylus as well as an image that is projected from a microscope onto an electronic tablet. Thereby the effect of the previous treatments on fiber length and distribution could be evaluated. To ensure that none of the previous treatments alter the digestibility, samples were used as substrate for enzymatic hydrolysis as described in section 7.2.5.2. Based on these results, treatment 2 was selected and applied prior to preparation of handsheets based on the TAPPI standard methodology T205 sp-02 [27].

The prescribed consistency of the fiber solution in the standardised TAPPI method for making handsheets is 1.2 % (dw.v^{-1}) [27]. This was, however, modified to 1.0 % (dw.v^{-1}) of WIS as reported in literature [19]. Also, in the standard handsheets method, the pretreated material is subjected to 3 000 rpm in a disintegrator (suggesting not to go higher than 50 000 rpm), with the main function of homogenising the material. Based on the resulting fiber lengths, determined with the digitiser, it was, however, observed that the resulting material was still not homogenous enough to be used for the preparation of handsheets (Addendum B, section 7.6.1). Therefore, the use of a liquidiser (6 250 rpm) along with the disintegrator (31 250 rpm) was brought in as modification to the method as a total of 37 500 rpm suggested by Berlin *et al.* [15]. The treated material, yielding a more homogenous distribution of the fibers was then subjected to handsheets preparation. Handsheets were dried overnight at 23 ˚C and at relative humidity of 50 %.

7.2.5. Enzymatic hydrolysis experiments

All experiments took place at a 2 % (dw.v^{-1}) solids loading. This solids loading was selected to minimise end-product inhibition on enzymatic hydrolysis, enabling improved capacity to distinguish between the different enzyme preparations. This was also the maximum solids loading sufficient for hydrolysis at micro-scale as a small glass bead already had to be added to each Eppendorf tube in order to favour mixing.

7.2.5.1. Micro-scale procedure

Enzymatic hydrolysis assays were performed in 2 mL Eppendorf tubes (0.5 mL working volume) with 0.05 M citrate buffer at pH 5.0, 50 °C and 800 rpm in a micro-plate incubator (Heidolph Incubator 1000). The buffer was supplemented with 2.0 % sodium azide to prevent microbial growth. Small discs obtained from handsheets of homogenised, pretreated materials with a paper perforator were used as substrate for the micro-assays. Some of the experiments were supplemented with different volumes of liquid fraction to mimic a 2 % (dw.v^{-1}) WIS loading for dilutions of the slurry or pressed-slurry. A small glass bead was included in each assay tube to improve mixing. At specific time points, samples were removed from the incubator and frozen for enzyme inactivation prior to high-performance liquid chromatography (HPLC) analysis.

7.2.5.2. Small scale procedure

Small scale hydrolysis was performed in the same manner as for micro-scale hydrolysis. The only differences were that this hydrolysis took place in 250 mL screw cap Erlenmeyer flasks (100 mL working volume) and at 150 rpm in an orbital shaker incubator (YIH DER LM-575D 414927). The same WIS fraction was used as substrate for the micro-scale and small scale hydrolysis; some samples were supplemented with different liquid fraction volumes to mimic pressed-slurry and whole slurry from pretreatment at 2 % ($dw.v^{-1}$) WIS. The digestibility tests to determine if the different homogenisation treatments (disintegrator plus liquidiser) alter the substrate properties were performed in the same way as small scale hydrolysis, but using only washed WIS as substrate.

7.2.6. Analytical methods

7.2.6.1. Enzyme activity determination

The control enzyme preparations (Spezyme CP and Novozyme 188) were subjected to standardised tests to determine the protein content and activities of the main enzymes relevant to the conversion of lignocellulose, namely cellulase and cellobiase. Cellulase (filter paper units (FPU), carboxy-methyl cellulose (CMC)) and β-glucosidase activities were measured according to methods described by Ghose (1987) [28]. Xylanase activity was determined by the method described by Bailey *et al.* (1991) [29]. The protein content was determined using a bicinchoninic acid [BCA]™ assay; (BCA-Compat-Able Protein Assay kit, ref. 23229, Pierce, Rockford, IL) using bovine serum albumin as protein standard.

7.2.6.2. Chemical analysis

The chemical composition of the WIS fraction as well as the handsheets material was analysed using the standard LAPs for biomass analysis that is provided by the National Renewable Energies Laboratory (CO, USA) [22]–[25]. The liquid fractions were also analysed for soluble sugars and the formation of by-products during pretreatment [26].

7.2.6.3. High-performance liquid chromatography (HPLC) analysis

Concentrations of monomeric sugars (glucose, xylose and arabinose), cellobiose, acetic acid and by-products such as formic acid, 5-hydroxymethyl-2-furaldehyde (5-HMF) and

furfural were analysed using an Aminex HPX-87H Ion Exclusion Column equipped with a Cation-H cartridge (Biorad, Johannesburg, RSA). Sugars were measured with a RI detector (Waters 2141, Microsep, Johannesburg, RSA), whereas by-products were analysed with a Ultra-Violet (UV) detector set at 215 and 280 nm (Waters 2487, Microsep, Johannesburg, RSA). The column was operated at 65 °C with a mobile phase of 5 mM sulphuric acid (H_2SO_4) and a flow rate of 0.6 $mL.min^{-1}$.

Mild acid hydrolysis was applied to determine the amount of sugars within the liquid fractions of both the WIS fraction and handsheets material that was present in oligomeric form [26]. This process entails the addition of a calculated amount of 72 % ($w.w^{-1}$) dilute H_2SO_4, based on the pH of the sample, to 5 mL of the liquid fraction that would reduce the concentration of the H_2SO_4 to 4 % ($w.w^{-1}$). This mixture is then autoclaved at 121 ˚C for 30 minutes during which all the sugars are hydrolysed into their monomeric form. The amount of oligomers could thereafter be calculated as the difference between the concentration total monomers after acid hydrolysis and the initial monomers.

Each sample was filtered through a 0.22 µm nylon membrane filter before being analysed by HPLC. All analytical determinations were performed in triplicate and average results are shown.

7.3. Results and Discussion

7.3.1. Substrate composition

Differences in the chemical composition of the WIS fractions of pretreated lignocelluloses, before and after handsheets preparations, were compared to see if possible modifications occurred during the formation of handsheets. These results are illustrated in Table 31.

Table 31: Composition of the solid fraction (washed WIS) from pretreatment and handsheet material (analysis done in duplicate).

Component	Pretreated Sorghum Bagasse MSJH16		Pretreated Triticale Straw M13	
	Washed WIS (% dry weight)	**Handsheets** (% dry weight)	**Washed WIS** (% dry weight)	**Handsheets** (% dry weight)
Cellulose	52.36	51.46	51.70	57.20
Hemicellulose	9.35	8.25	9.23	9.45
Lignin	24.98	27.40	22.10	25.14
Ash	1.77	1.28	0.80	0.10
Acetyl groups	0.04	0.03	0.05	0.04

Standard deviation less than 5 %

From the analysis of the chemical composition of the WIS fractions of both feedstocks, it was observed that most of the compounds were higher within sorghum, with cellulose being the main fraction. For the handsheets material, sorghum also contained large fractions of all the components, with triticale comprising a slightly higher cellulose, hemicellulose and acetyl group content.

Variations of approximately 3 % in the lignin were observed in both feedstocks between the WIS and homogenised material used for handsheets. There were also only slight differences in the ash content of both the WIS and handsheets material of sorghum and triticale.

Overall, no significant differences were observed in the composition of sorghum and triticale WIS and handsheets material. It could therefore be concluded that the chemical composition of the lignocellulosic feedstocks was not altered during handsheets preparation and therefore the treatment was effective in homogenising the pretreated material.

The liquid fraction, obtained after pretreatment, consists of inhibitors which formed during the pretreatment process. The composition of the sugars and by-products within this fraction is indicated in Table 32. Enzymatic hydrolysis can be largely affected by the presence of inhibitors that are produced during pretreatment. Overall, the sorghum feedstock had a larger concentration of inhibitors compared to triticale. This was possibly as a result of the hemicellulose component thereof that is more susceptible to degradation during

pretreatment and/or the more severe pretreatment that was required to maximise sugar recovery within this feedstock. The impact of the liquid fraction will thus depend on the substrate used and the solids loading during enzymatic hydrolysis.

Table 32: The composition of all the sugars and by-products present within the liquid fraction after steam-explosion pretreatment.

Sugar and By-product content	Chemical species in the liquid fraction from steam-explosion ($g.L^{-1}$)	
	Sorghum MSJH16	Triticale M13
Monomeric glucose	0.45	0.26
Oligomeric glucose	5.14	8.21
Monomeric xylose	6.46	1.31
Oligomeric xylose	39.36	23.72
Acetic acid	5.29	1.29
Formic acid	1.40	0.35
Furfural (215 nm)	0.59	0.11
5-HMF	0.19	0.06

Standard deviation less than 5 %

7.3.2. Enzyme preparations

The enzyme activities of the control enzyme preparations were tested on model substrates such as filter paper, CMC, cellobiose and Beechwood xylan. These activities along with the respective protein concentrations of the enzymes are summarised in Table 33.

Table 33: The protein concentration and enzyme activities of the respective commercial enzyme preparations.

Enzyme preparations	Protein concentration ($mg.mL^{-1}$)	Enzyme activities			
		FPA ($FPU.mL^{-1}$)	CMC ($U.mL^{-1}$)	β-glucosidase ($U.mL^{-1}$)	Xylanase ($U.mL^{-1}$)
Spezyme CP	116.2	58.7	13.3	40.2	10307.5
Novozyme 188	120.0	0.31	< 0.1	929.0	< 0.1
Cellic® CTec2 * [30]	161.2	120.5	-	2 731.0	-
Cellic® HTec2	-	-	-	-	-

* values obtained from literature
FPA - filter paper activity

The above mentioned enzymes were combined into three separate cocktails. The first was a conventional cocktail of 0.25 $mL.g^{-1}$ WIS Spezyme CP + 0.016 $mL.g^{-1}$ WIS Novozyme 188, which corresponds to 15 $U.g^{-1}$ WIS and at 2 % ($dw.v^{-1}$) of WIS this corresponds to 0.30 $FPU.mL^{-1}$. This cocktail was used in both feedstocks. The second and third cocktails were the optimised cocktails specific for each feedstock, obtained from cocktail optimisation presented in Chapter 6. These consisted of 0.15 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.32 $mL.g^{-1}$ WIS Cellic® HTec2 for sorghum and 0.1 $mL.g^{-1}$ WIS Cellic® CTec2 + 0.2 $mL.g^{-1}$ WIS Cellic® HTec2 for triticale.

7.3.3. Effects of different treatments of liquidiser-disintegrator on WIS

To ensure the homogenous distribution of fibers throughout the handsheets, the use of a liquidiser was implemented. This was done to ensure that the small discs of pretreated materials, obtained by perforation of handsheets and used as feedstock for enzymatic hydrolysis assays at micro-scale, were respresentative of the whole substrate.

The effects of the applied homogenisation treatments (Table 30) on the fiber length (mm) of steam-pretreated sorghum bagasse and triticale straw are illustrated in Figure 35A and 35B, respectively. According to Chambers *et al*. [31], if the notches of the 2 plots in a box-and-whiskers plot do not overlap, it is 'strong evidence' of a significant difference that exists between the two medians.

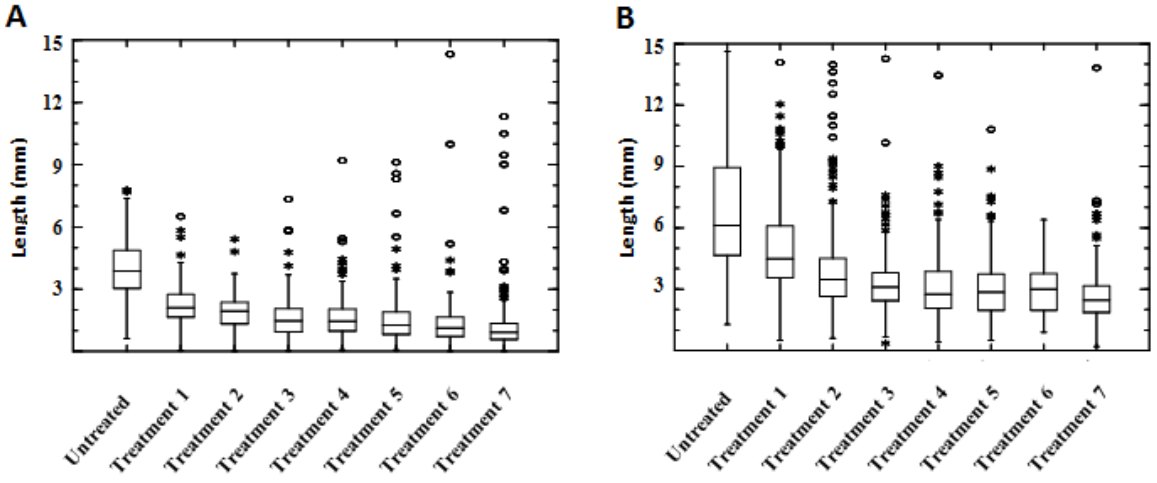

Figure 35: Box-and-Whiskers-plots of the fiber lengths of (A) Sorghum MSJH16 and (B) Triticale EliteM13, obtained after each treatment. The rpm provided by the liquidiser increased from left to right in the respective graphs.

All 7 of the treatments significantly reduced the length of the fibers in the pretreated sorghum bagasse samples (Figure 35A). This could be seen as the boxes of the treated fibers did not overlap with the one corresponding to the control (no treatment with disintegrator or liquidiser). It was clear that Treatment 1 (only disintegrator) was already sufficient enough to improve the homogeneity of the handsheets. In triticale (Figure 35B), all of the treated fiber lengths were shorter than that of the untreated fibers, but a combination of homogenisation by the disintegrator and liquidiser was required to obtain the desired reduction in fiber length and associated material homogeneity. In both materials a trend could also be seen as the fiber lengths decreased with increasing rotation speed (rpm) of the liquidiser, indicating that the latter was related to the severity of the homogenisation treatment. This was a clear indication that the use of the disintegrator, along with the liquidiser, could significantly reduce the fiber lengths of both materials, thereby making it more homogenous.

To make it easier to compare the effects of treatment, the same treatment (treatment 2) was chosen for the preparation of handsheets for both feedstocks. The average length of the sorghum and triticale fibers after treatment 2 was 1.5 – 2.5 mm and 2.5 – 4.5 mm, respectively. These represented a length reduction of approximately 50 % for both feedstocks compared to the control (untreated material).

To ensure that treatment 2 does not affect the digestibility of both materials and thereby change the amenability of the substrate to the enzymes, the handsheets generated from the WIS that underwent treatment 2 were subjected to small scale hydrolysis where the conventional Spezyme CP/Novozyme 188 cocktail was used. This took place after the subsequent drying of the material at 23 ˚C for 24 h to mimic the moisture content of the proposed handsheets. These results are shown in Figure 36.

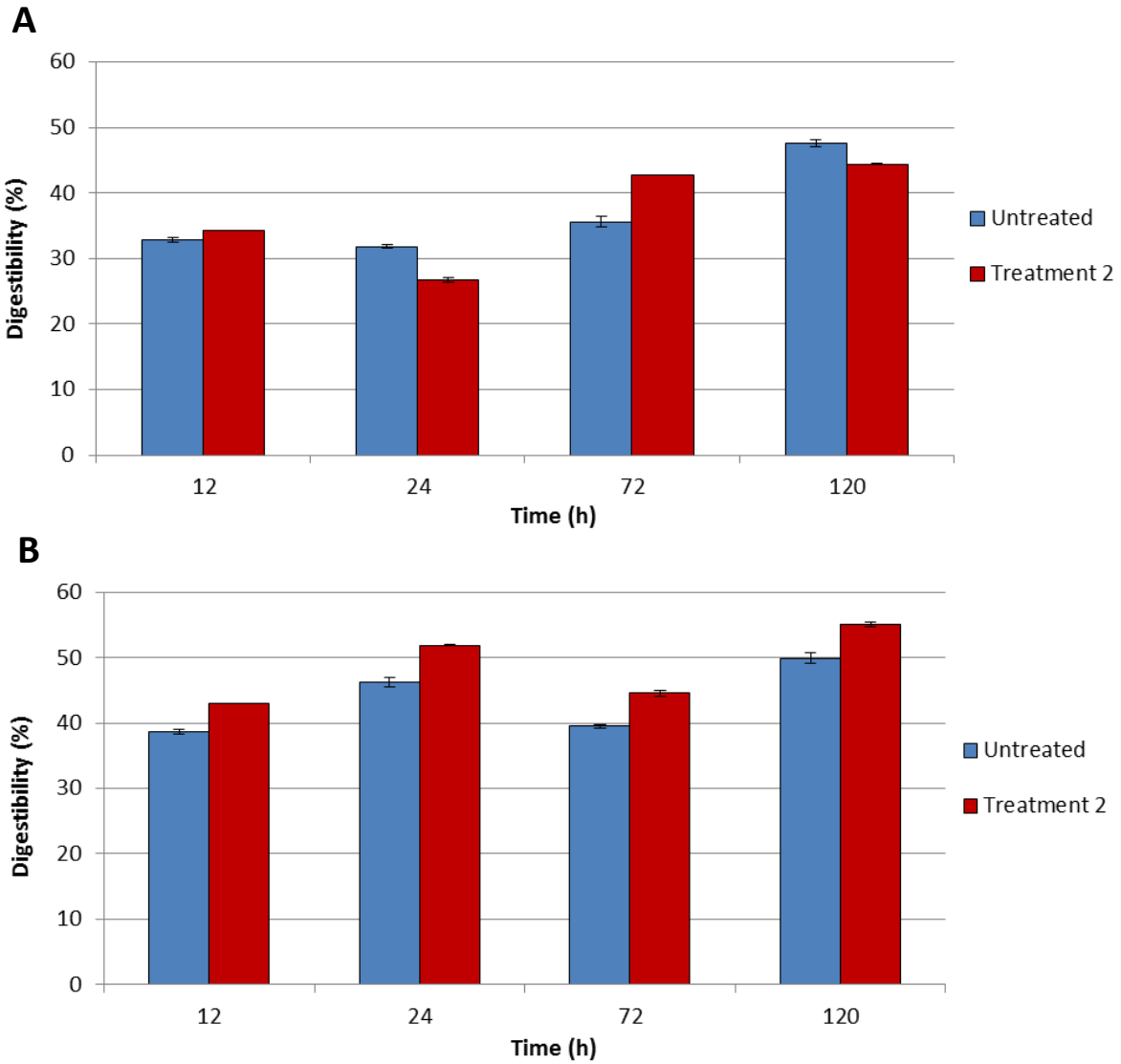

Figure 36: The effect of treatment 2 vs. untreated material on the digestibility of the WIS fraction of (A) Sorghum MSJH16 and (B) Triticale EliteM13, respectively at different time points.

Increases in the digestibility of the treated sorghum and triticale material were no more than 7.15 % and 5.62 %, respectively. The higher differences for sorghum could be due to the fact that treatment 2 was too harsh and that treatment 1 would have been sufficient for homogenising the material. These were, however, not seen as significant increases and the making of the handsheets were thus continued for its use in micro-assays.

7.3.4. Enzymatic hydrolysis using handsheets

The production of ethanol from lignocellulosic material can take place via three possible configurations, which involve using different substrates for hydrolysis and fermentation: WIS, pressed-slurry and slurry. The preferred substrate will be determined mainly by the feedstock itself and the potential of the fermentation micro-organisms to co-ferment hexoses together with pentoses as well as its ability to tolerate inhibitors. For example, using whole slurry would yield a higher concentration of sugars due to the liquid fraction being rich in hemicellulosic-derived sugars. The use of whole slurry also reduces operational costs as no pressing or filtration is needed. There are, however, formation and solubilisation of components which may be inhibitory during enzymatic hydrolysis and fermentation, depending on the specific feedstock and the pretreatment conditions that are applied.

Additionally, some hemicellulosic-derived sugars in their oligomeric form have been shown to inhibit cellulases at low concentrations [32]. Another option would be to separate the slurry into the solid (pressed-slurry) and liquid fractions by pressing and filtration, for the separate hydrolysis and fermentation (SHF) of hexoses and pentoses. In most studies, the pressed-slurry is washed to remove the majority of inhibitors which are soaked in the cellulose fibers. In this way, toxicity during the hydrolysis and fermentation processes is minimised. This step, however, contributes to the extra cost of the overall process while also generating additional waste streams.

Based on the previous considerations, selected enzyme combinations from previous studies were used during the enzymatic hydrolysis of slurry, pressed-slurry and WIS to study their performance both at micro- and small scale. Hydrolysis was subsequently performed in 2 mL Eppendorf tubes (0.5 mL working volume) and 250 mL screw cap Erlenmeyer flasks (100 mL working volume) to test the effect of handsheets. Both feedstocks were hydrolysed for 120 h with three possible substrates (washed WIS; pressed-slurry; whole slurry) and two enzyme combinations (the conventional Spezyme CP/Novozyme 188 cocktail; the new optimised Cellic® CTec2/Cellic® HTec2 cocktail). Samples were taken at 12, 24, 72 and 120 h. The glucose concentrations of the hydrolysis experiments performed at micro-scale and small scale for WIS, pressed-slurry and slurry are represented in Figure 37, 38 and 39, respectively.

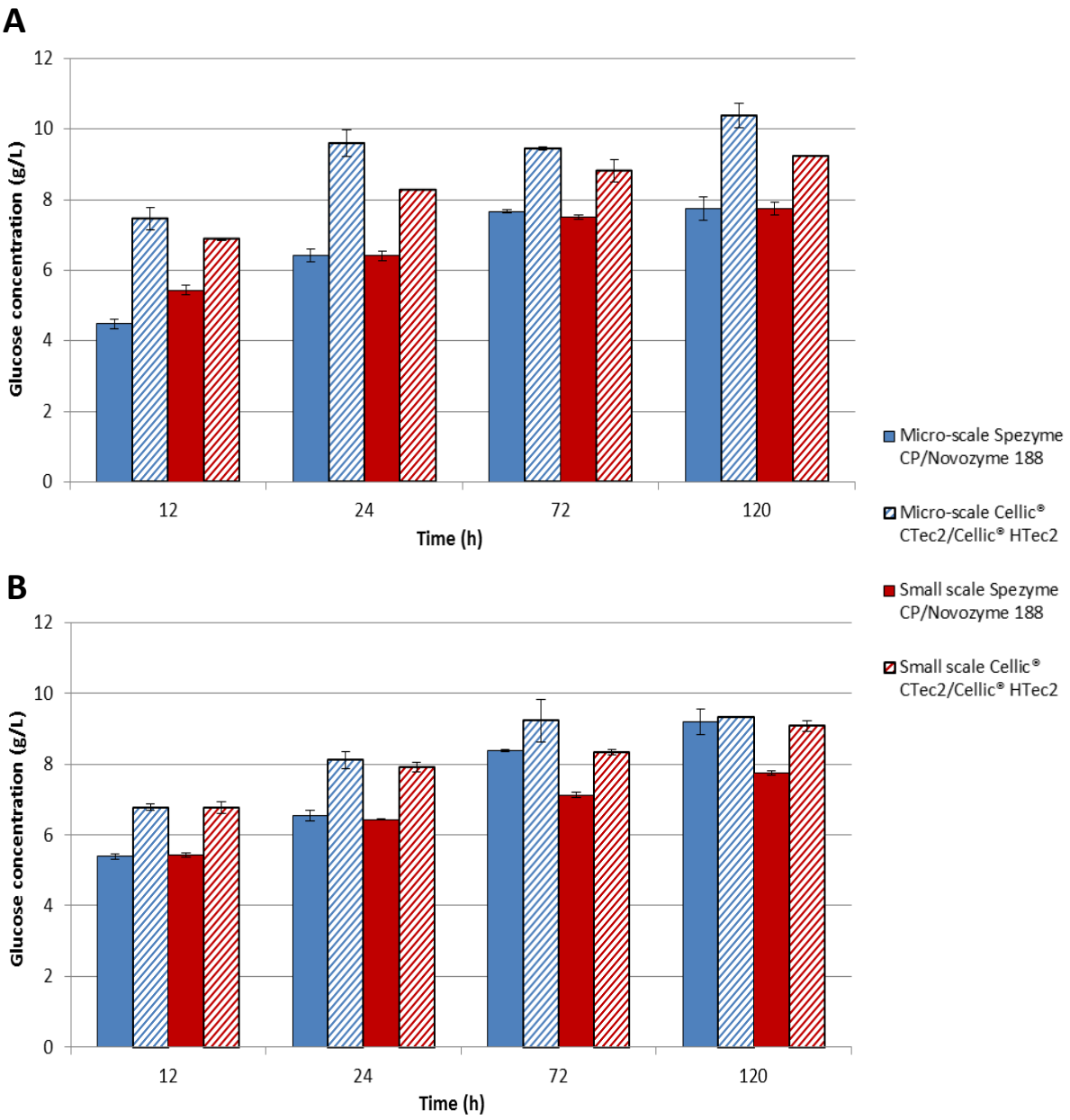

Figure 37: Glucose concentrations obtained during hydrolysis of the WIS fraction at both micro-scale and small scale of (A) Sorghum and (B) Triticale.

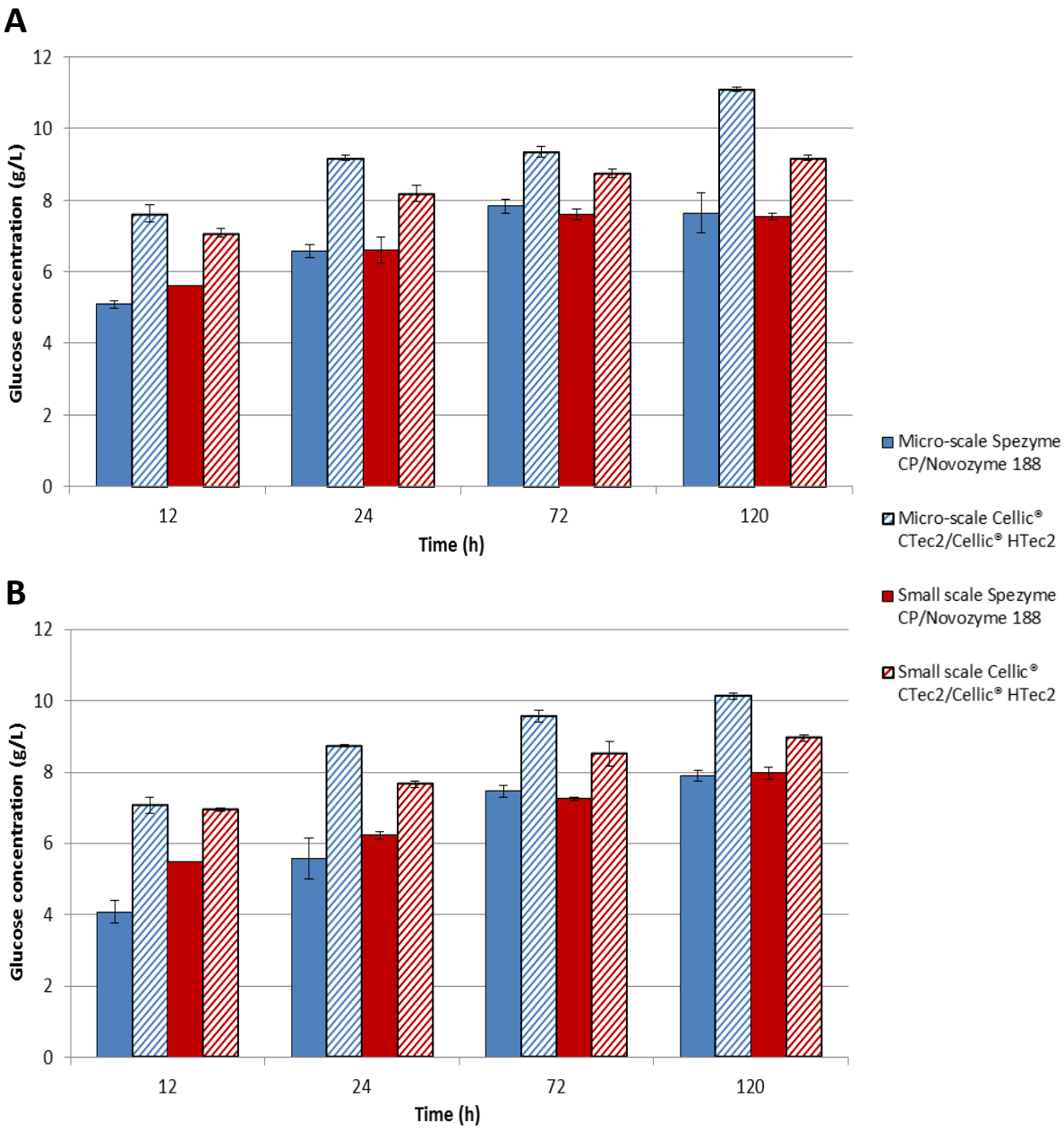

Figure 38: Glucose concentrations obtained during hydrolysis of the pressed-slurry fraction at both micro-scale and small scale of (A) Sorghum and (B) Triticale.

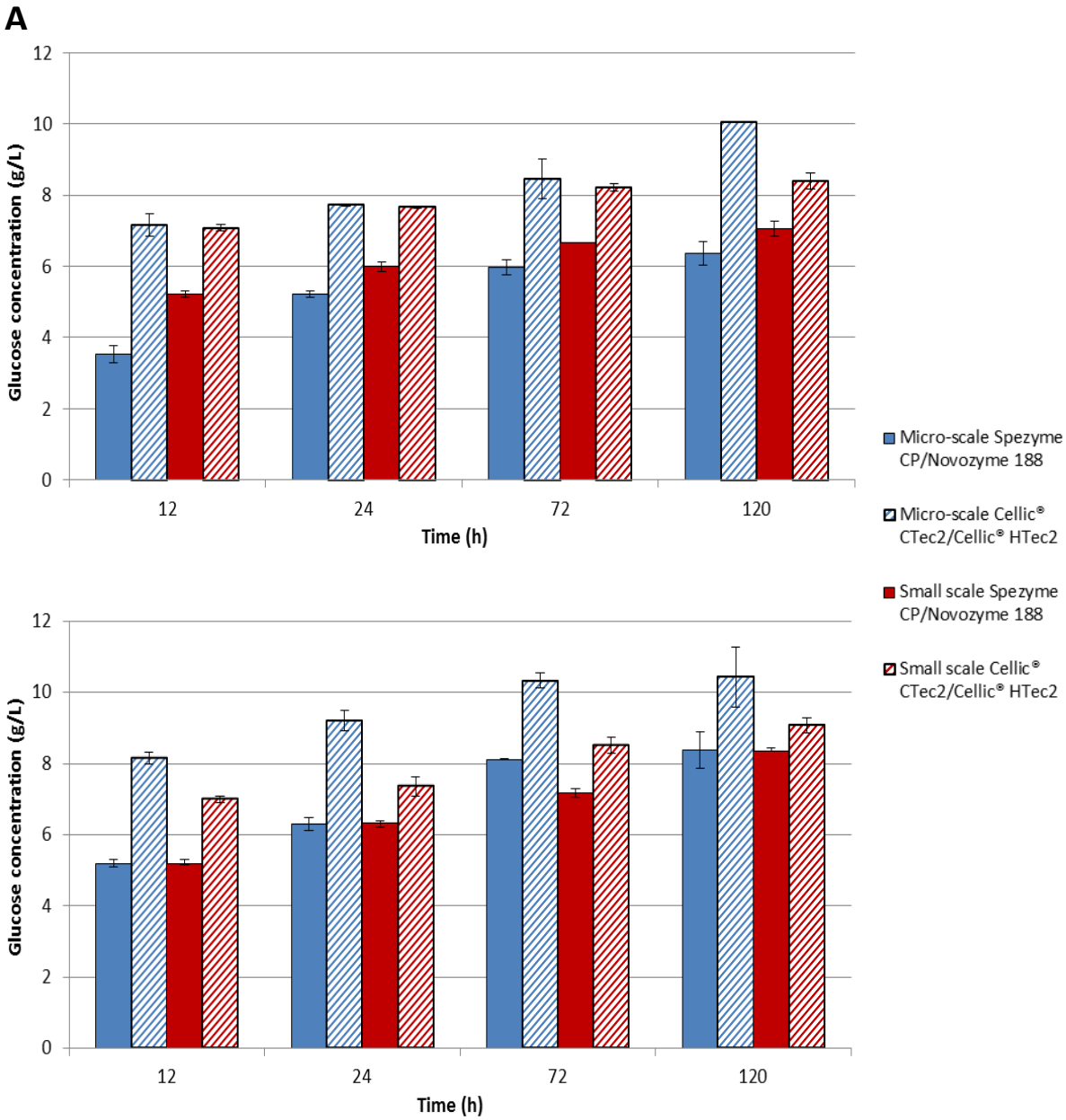

Figure 39: Glucose concentrations obtained during hydrolysis of the slurry fraction at both micro-scale and small scale of (A) Sorghum and (B) Triticale.

In the sorghum feedstock, comparable results were obtained with the Spezyme CP/Novozyme 188 control cocktail for both the micro-scale and small scale assays when WIS was used as substrate (Figure 37A). This was true for all the time points except 12 h, where the micro-assay provided a lower glucose concentration. This finding is in agreement with the study of Alvira *et al.* where they explained the lower conversion within the micro-assays as a result of the drying effect of the handsheets [19]. The same trend was observed when using pressed-slurry as substrate for hydrolysis as illustrated in Figure 38A. Results of the control cocktail for the two assays were also comparable at all data points but again, at 12 h, there was a slight decrease in the glucose concentration in the micro-assay which could be explained by substrate drying as mentioned above.

The use of whole slurry of sorghum bagasse pretreatment as substrate illustrated that glucose released during enzymatic hydrolysis was superior in the Erlenmeyer flasks compared to the handsheets, but only for the Spezyme CP/Novozyme 188 control enzyme combinations, as can be observed in Figure 39A. With the Cellic® CTec2/Cellic® HTec2 optimised cocktail, results were comparable at early hours, where after the micro-assay outperformed the hydrolysis at small scale. Overall, the micro-assay of sorghum with the control cocktail showed to be more affected than the small scale assay when using slurry as substrate. This aspect should be taken into account when using high-throughput systems where the slurry is directly mixed. The Cellic® CTec2/Cellic® HTec2 enzyme cocktail was less influenced by inhibitors, even when using the slurry of sorghum which presented a higher concentration of inhibitors.

For the sorghum feedstock, glucose yields obtained at small scale were larger than those of micro-scale when using the Spezyme CP/Novozyme 188 control cocktail. In contrast to the control cocktail, the use of the Cellic® CTec2/Cellic® HTec2 enzyme combination yielded higher glucose concentrations at micro-scale compared to small scale. A reason for this could be that treatment 2 might have been too harsh and favoured the latter enzyme cocktail more than the control. Enzymatic hydrolysis have also shown to be influenced by fiber length [33]. It is therefore possible that the optimised cocktail could take advantage of this occurrence in a better way than what is possible for the control cocktail. The results for WIS and pressed-slurry accentuated the differences between the two enzyme combinations for

both the micro-assay and small scale assay. Using only slurry as substrate, a similar trend between micro-scale and small scale was noted, except for the last data point which might be higher at micro-scale due to evaporation.

In the triticale feedstock, both assays were comparable at early hours for both enzyme cocktails and with the use of WIS as substrate (Figure 37B). With pressed-slurry and whole slurry as substrate, however, the application of the control Spezyme CP/Novozyme 188 enzyme combination yielded glucose concentrations which were either comparable only at early hours or only at late hours, rendering these results inconclusive. This can be observed in Figure 38B and Figure 39B, respectively.

In general, glucose was released more easily from triticale than sorghum during hydrolysis with both enzyme cocktails. This difference between feedstocks could be explained by several material properties such as cell wall structure, cellulose structure, the presence of inhibitors and the amount of cellulose and lignin that is present within the feedstock. Although the final sugar concentration of the sorghum feedstock was higher compared to triticale, the yield for sorghum was still lower. This could possibly be explained by its larger lignin and ash content compared to triticale. Inhibitors were also present in larger quantities, which might have influenced the enzymes in this feedstock. The liquid fraction had a significant influence on glucose concentration in both feedstocks using the control and optimised cocktail when comparing the WIS fraction to both pressed-slurry and whole slurry at all of the time points. The influence, however, seemed to be less prominent in triticale.

As it can be observed for both raw materials, the new cellulase preparations of Novozymes, Cellic® CTec2 and Cellic® HTec2 were superior to the conventional Spezyme CP/Novozyme 188 combination for all substrates tested as it yielded larger glucose concentrations with respect to the maximum possible for each feedstock. When looking only at the micro-assay and the difference between the enzymes, it is clear that the method is vulnerable to a very good performing cocktail because the yields are higher than what was expected.

The incorporation of a homogenisation step reduced the fiber length of both pretreated materials, which could enhance digestibility depending on the enzymes used. The material

that is used should thus be fine enough to decrease the amount of variation in dispensing due to heterogeneity, but not too fine that its digestibility will be affected [34], [35]. Some advantages to the reduction in size for digestibility assays are (1) great ease in the delivery of ground substrate to the micro-assay, (2) homogenisation of the variety of biomass fractions with their different composition and (3) adequate mixing of the biomass during the hydrolysis process within the micro-wells [36]. The ideal would, however, be to perform particle size reduction in a way that the physical and chemical state of the processed material is minimally altered [36]. It therefore seems that the handsheets treatment accentuated the differences between the two enzyme combinations, mainly for triticale, because it is more digestible. In this case, the Cellic® CTec2/Cellic® HTec2 enzymes were favoured above the Spezyme CP/Novozyme 188 combination. When analysing the micro-scale results, it seems that whatever damage was done during the preparation and subsequent drying of the handsheets was made up for by decreasing the particle size. This works for the Spezyme CP/Novozyme 188 control cocktail, but with a very potent cocktail like Cellic® CTec2/Cellic® HTec2 it is difficult, because this optimised cocktail can take advantage of that treatment in a way that the control Spezyme CP/Novozyme 188 cocktail cannot. It is therefore more difficult to screen different enzyme cocktails with micro-assays.

The increased glucose concentrations in the micro-assays might also be explained by the addition of a mixing aid, such as a small stead bead, at micro-scale. This has shown to improve glucan conversion primarily due to inadequate mixing that takes place at larger volumes [36]. The conversion of glucan can, however, only be improved up to the maximum possible for the specific feedstock.

7.4. Conclusions

There exists a great need for realistic methods by which the digestibility of lignocellulosic materials can be evaluated.

Firstly, it could be concluded that Cellic® CTec2 and Cellic® HTec2 is a very powerful cocktail as higher glucose concentrations were obtained with this optimised cocktail during hydrolysis compared to the conventional control cocktail of Spezyme CP/Novozyme 188.

Secondly, the micro-assay confirmed that the triticale feedstock is a "softer" and therefore more hydrolysable feedstock after pretreatment than sorghum, because it yielded much better results compared to the latter. A multitude of material properties such as cell wall structure, cellulose structure, surface area, concentration of inhibitors, as well as the amount of cellulose and lignin play into this phenomena.

Analysis of the micro-scale results accentuated the difficulty of screening different enzyme cocktails at micro-scale. This followed as the Cellic® CTec2/Cellic® HTec2 enzyme combination was able to take advantage of the manner in which the handsheets were prepared, in a way that is not possible for a normal control enzyme combination like Spezyme CP/Novozyme 188.

In conclusion, when comparing the results of the micro-assay hydrolysis to those of the hydrolysis performed on small scale in terms of both feedstocks, both pretreatment conditions and both enzyme cocktails, it becomes increasingly difficult. This difficulty is due to the way in which the material responds to a particular enzyme, as this will determine whether small scale or micro-scale provides the same answer or not. It might therefore have been too optimistic to expect the same results at micro-scale (where the material was treated) than at small scale under all possible conditions, as there will always be differences between the two. These differences should be kept in mind when using high-throughput systems. It was, however, shown that the micro-assay hydrolysis could distinguish between different feedstocks as well as between the different enzyme preparations.

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7.6. Addendum B

The following results are presented as screening experiments in order to determine a few baseline parameters for subsequent studies.

7.6.1. Testing micro-assays

Micro-assays represent a faster and cheaper way for optimisation. Current methods of micro-assay optimisation use milled material that are not representative of the 'true substrate'. Previous works have addressed this problem by elaborating handsheets from pretreated material, facilitating the homogenisation and distribution at smaller scale by subjecting the fibers in suspension to a disintegrator for 37 500 rpm.

In this part of the study, the homogeneity of a suspension of WIS from steam-pretreated triticale straw was studied with a digitiser. During the first experiments, very long fibers were observed. This was due to the heterogeneous nature of lignocellulosic biomass and because the pretreatment was less severe than those from literature [19]. To improve homogeneity, the effect of the incorporation of liquidiser treatment for different intervals on fiber length was also studied. This was done in order to improve the uniform handsheet thickness and favour the homogeneous distribution of all the various dimensions/sizes of the cell wall pieces. The different treatments of the original 37 500 rpm are illustrated in Table 34.

Table 34: The different treatments applied during handsheets preparation tests.

Sample	Revolutions in Disintegrator	Revolutions in Liquidiser
1	37 500	-
2	25 000	12 500
3	18 750	18 750
4	31 250	6 250

The effects of different speeds of the disintegrator and liquidiser on fiber length and distribution were evaluated with the use of a digitiser. These results are indicated in Figure 40. Treatment 2 showed to differ significantly from the untreated material (boxes do

not overlap). It was therefore decided that this treatment would be used in the subsequent preparation of handsheets.

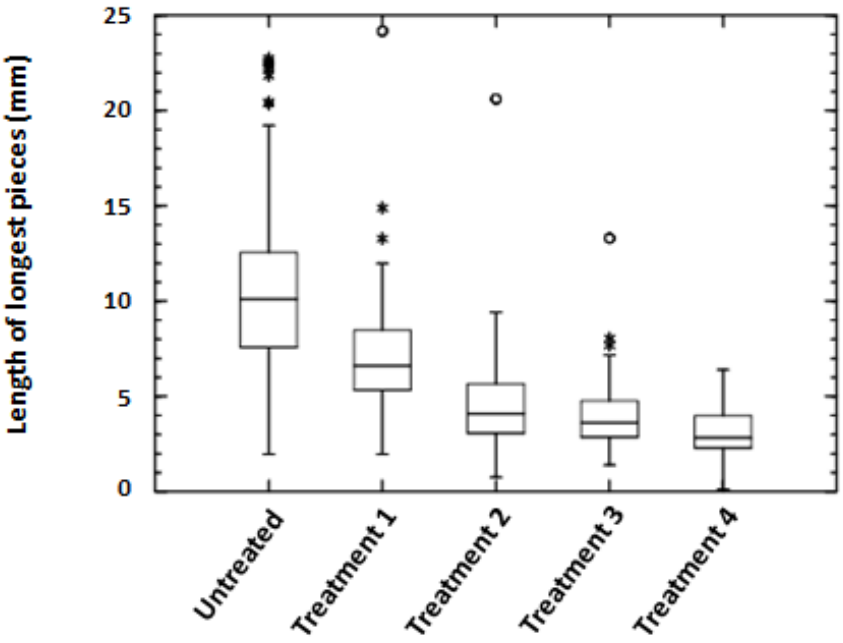

Figure 40: Box-and-Whiskers-plots of the fiber lengths of steam-treated Triticale EliteM13, obtained after each treatment. The rpm provided by the liquidiser increased from left to right in the respective graphs.

As a first trial, however, handsheets were prepared from material that was subjected to the disintegrator alone. The results of glucose release from small scale and micro-scale hydrolysis are illustrated in Figure 41. The values of glucose concentration were similar for both small scale and micro-assay hydrolysis. There were, however, lower cellulose conversions for the micro-assay tests at 12 h. Although the handsheets were subjected to a short drying at low temperature, these differences could be explained by the negative impact of the temperature on the fibers. However, this effect is overcome already at 24 h when using Cellic® CTec2 (Figure 41). As demonstrated in previous experiments, Cellic® CTec2 provides higher cellulose conversion compared to conventional cellulase preparations. This means that enzymes with lower hydrolytic potential may require longer incubation times to equal the yield obtained at small scale. It was therefore decided to extend the incubation period for further experiments.

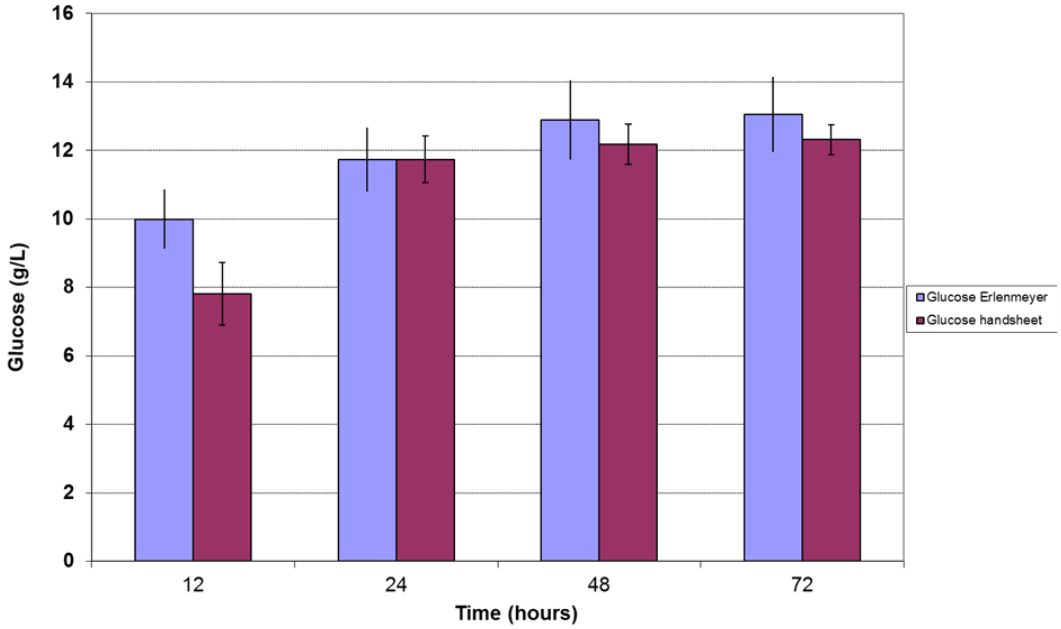

Figure 41: Bar graph showing the glucose concentrations from small scale (Erlenmeyer flasks) and micro-scale (Eppendorf tubes) hydrolysis of Triticale, respectively. Material from treatment 2 was used as substrate at micro-scale. Hydrolysis were performed at a 2 % (dw.v^{-1}) solids loading, 50 ˚C, a Cellic® CTec2 dosage of 0.25 mL.g^{-1} WIS and 90 rpm for the small scale and 800 rpm for the micro-scale assays.

These preliminary results looked promising and it was therefore decided to continue with the hydrolysis test at micro-scale and apply it to other pretreated materials such as sweet sorghum bagasse.

8. Concluding Remarks

As the current price of 1G bio-ethanol (e.g. corn and maize) keeps increasing due to energy costs and environmental problems, the only viable alternative to serve as renewable feedstock is lignocellulosic material. In this study, the agricultural wastes such as sweet sorghum bagasse and triticale straw were especially popular as they do not demand separate land, energy and water requirements and do not compete with the food market, as they have no food value. They are also abundant, cheap and of low commercial value, thereby representing the most prospective feedstocks to be used for bio-ethanol production.

During preliminary optimisation studies it was found that although these feedstocks were similar in composition, they required different optimum pretreatment conditions. After subsequent screening, optimisation and verification experiments, an optimised enzyme cocktail for each feedstock was constructed to maximise the total sugar yields. This cocktail consisted of the Cellic® CTec2 and Cellic® HTec2 enzymes supplied by Novozymes. Data indicated that similar feedstocks, which are pretreated under different conditions, require different optimum enzyme dosages for hydrolysis. These optimised cocktails also proved to be most effective during enzymatic hydrolysis in comparison with a control enzyme combination of Spezyme CP and Novozyme 188.

The micro-assay method that was developed during this study confirmed what was observed during the enzymatic hydrolysis optimisation; that the triticale feedstock is an easier material to hydrolyse as higher glucose yields were reached compared to sorghum. The optimised enzyme cocktail of Cellic® CTec2 and Cellic® HTec2 was again the most powerful, yielding the highest glucose concentrations during enzymatic hydrolysis in both sorghum and triticale.

Looking at the micro-assay and the different ways in which the enzymes behaved, it could be reasoned that this method is more vulnerable to a very good performing cocktail such as the Cellic® CTec2/Cellic® HTec2 enzyme combination. This follows as it is very difficult to compare the micro-assay with the assays done at small scale due to the fact that

a very potent cocktail can take advantage of the specific treatment which the material undergoes in a way that the control cocktail is not capable of doing. It thus becomes very difficult to determine how the material will respond to a particular enzyme, because that will determine whether the same value is obtained with the micro-assay as well as at small scale. Differences detected should be kept in mind during the application of high-throughput systems. It was, however, observed that the hydrolysis performed at micro-scale could distinguish between the different enzyme preparations as well as between different feedstocks.

9. Recommendations

The following recommendations are made based on the results and discussion as well as the conclusions that were drawn from this study:

9.1. Optimisation of Enzymatic Hydrolysis

During optimisation studies within a single feedstock, hydrolysis should be compared based on the following parameters: different pretreatment conditions, the use of different substrates during hydrolysis studies and the application of more than one optimised enzyme cocktail.

The optimised enzyme combinations should also be evaluated based on:

- Higher solid loadings;
- Feeding strategies of both enzyme and substrate;
- In a SSF process (to evaluate the formation of aldonic acids and the possible effects of the chosen enzymes on the micro-organism, since high enzyme concentrations (mL enzyme. mL^{-1} solution) have shown to affect the microbe and this should be a problem when using high solid loadings)

9.2. The Development and Validation of a Micro-assay

Some aspects of the method that should be developed or evaluated are:

- Treatments for homogenisation required for different feedstocks/pretreatment: Due to the heterogeneity of lignocellulosic biomass, it is very difficult to obtain a representative sample at milligram scale. Some studies milled the material as a way of homogenising the sample and making the distribution thereof in the assays easier. Milling has, however, shown to alter the structural properties of the feedstock, thereby increasing

the digestibility and masking the differences with regard to enzyme combinations and feedstock-pretreatment combinations used. During this study the aim was therefore to try to enhance the homogeneity of the samples without altering the structure of the feedstock. This treatment will, however, differ depending on the feedstock.

During treatment of the handsheets material, it was decided on treatment 2 for both feedstocks in order to compare them with greater ease. Treatment 1 (only disintegrator) was, however, sufficient to homogenise the sorghum material. It would therefore be good to prepare handsheets, using only the disintegrator, to see what the difference in the results would be.

- Agitation speed: Different agitation speeds should be tested and the optimum speed applied, as enzymatic hydrolysis at varying agitation speeds have shown a large variation in the overall conversion of glucan in some studies.
- Evaporation loss: As some data indicated that results were not comparable between the two assays, evaporation loss within the micro-assays should be evaluated. This could be done by adding a glucose sample of known concentration together with the samples during hydrolysis. HPLC analysis of the samples at the different time points will then indicate any changes in the glucose concentration and therefore if any evaporation occurred.

Additionally, in order to compare with a micro-assay based on distribution of milled material, further experiments that involve the milling without long drying periods, should be performed. This will reveal the real effect of milling on the structure of pretreated materials.

Micro-titre plates: The next step would be to down-scale the micro-assays (currently performed in Eppendorf tubes) further to micro-titre plates as a high-throughput screening approach to allow for even faster hydrolysis studies of an even greater variety of feedstocks.