Integrated Optimization of Pretreatment Conditions for Bioethanol Production from Steam Treated Triticale Straw

by

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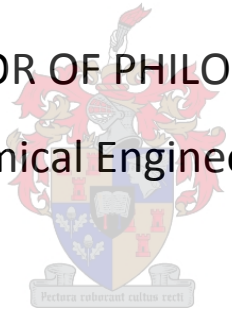
Dissertation presented for the Degree

of

DOCTOR OF PHILOSOPHY

(Chemical Engineering)



in the Faculty of Engineering

at Stellenbosch University

Supervisor

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March 2016

Declaration

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This dissertation includes 1 original paper published in peer reviewed journals and 4 unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

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Abstract

Cultivar/environmental variabilities in the production of triticale straw, and its impact on pretreatment-hydrolysis processes for conversion to bioethanol, were addressed in this study. Twenty triticale cultivars, grown in three geographical areas in the Western Cape of South Africa, were screened to select those cultivars with the largest combined ethanol output per hectare from grain and straw.

A four-stage systematic approach was applied to screen and identify preferred cultivars: **I.** Triticale cultivars were screened to identify samples with better agronomic traits and estimate experimental combined ethanol yields per hectare from straw and grain **II.** Preferred cultivars were subsequently screened at bench-scale to maximise sugars from dilute-acid pretreatment followed by enzymatic hydrolysis and to estimate maximum potential for production of fermentable sugars from straw samples. **III.** Straw samples with higher processability at bench-scale and availability for further study were selected. Selected samples were subjected to maximisation of combined sugars yield (CSY) at pilot-scale steam explosion (SE) by two types of impregnation, and **IV.** Fermentability of treated materials from optimised pretreatment (optimum conditions) was evaluated using Simultaneous Saccharification and Fermentation (SSF).

After the first selection, straws from cultivars grown in Mariendahl resulted in significant lowest ash and higher yields of xylose from pretreatment (~62% of theoretical maximum) and glucose from enzymatic hydrolysis (>10%), compared to straws from Swartland and Overberg. Cultivars 9, 13 and 14 (Mariendahl-site) displayed higher CSY values (43-45 g/100 g dry material) and were thus selected for pilot-plant pretreatment optimization. The set of SE conditions with temperatures between 190 and 205°C, together with times that resulted in severities between 3.35 and 3.79 and temperatures between 173 and 187°C combined with times that will give severities (Log (*Ro*')) between 3.30 and 3.41 were found to maximise CSY from the preferred straws by uncatalysed and SO_2-SE, respectively. Pretreatment optimisations led to improvement in CSY by up to 11%. Catalysed SE was the preferred method of pretreatment since more CSY was obtained from all the feedstocks (8-16%) and there were less differences in pretreatment requirements among straws.

Estimated lignocellulosic ethanol (2G) yield based on measured sugars from optimized pretreatment-enzymatic hydrolysis was 434 $L.ha^{-1}$, representing an overall improvement of ~28% in lignocellulosic ethanol yield estimate per hectare. Maximum ethanol yields of 171 $L.ton^{-1}$ were

estimated after SSF at 13% solid loading for pressed-WIS from uncatalyzed-SE, whilst ethanol yield per hectare using WIS intensively washed from SO_2-SE of straw 14 was estimated above 200 $L.ha^{-1}$. Thus, the final ethanol concentration was close to the benchmark of 4% (v/v). This study showed that cultivar selection based of feedstock quality, processability and further pretreatment optimisation impacted positively on the 2G ethanol yield per hectare. Such improvements in ethanol yield from straw are of relevance for the sustainability of triticale straw as potential bioethanol feedstock in South Africa. Besides, this study showed that higher 2G ethanol yield per hectare could be achieved without compromising the grain yield or ethanol yield from grain per hectare and thus providing a foundation for future selection of triticale by local farmers to better manage their farming economy.

At the time of submitting the present thesis dissertation the findings in chapter 6 (Screening of steam explosion pretreatment conditions for realizing areas of maximal sugars release and improved digestibility from triticale straw) were published in *New Biotechnology* 33 (2016) 153 – 163.

Opsomming

Kultivar- of omgewingsverwante veranderlikheid in die produksie van triticale (koringrog) strooi, en die impak daarvan op voorbehandeling hidrolise vir die omsetting na bioetanol, is ondersoek in hierdie studie. Twintig triticale kultivars, verbou in drie geografiese areas in die Wes-Kaap provinsie van Suid-Afrika, is geevalueer om dié kultivars te selekteer wat die hoogste gekombineerde etanol opbrengs lewer per hektaar graan en strooi.

'n Vier-fase sistematiese benadering is toegepas om die geskikte kultivars te evalueer en identifiseer: **I.** Triticale kultivars is geevalueer om monsters te identifiseer met beter agronomiese kenmerke asook om die gekombineerde eksperimentele etanol opbrengste per hektaar strooi en graan te peil. **II.** Kultivars geïdentifiseer in fase I is verder geevalueer op laboratorium-skaal om suiker opbrengs te maksimaliseer vanuit verdunde suur voorbehandeling gevolg deur ensiematiese hidrolise. Sodoende kon die maksimum potensiaal vir die produksie van fermenteerbare suiker vanuit strooi gepeil word. **III.** Strooi monsters met hoër proseseerbaarheid op laboratorium-skaal en met groter beskikbaarheid vir verdere studie is geselekteer. Geselekteerde monsters is onderwerp aan eksperimente vir die maksimalisering van gekombineerde suiker opbrengs (CSY) vanuit loodsaanleg-skaal stoomploffing (SE) met die gebruik van twee tipes deurwekingstegnieke, en **IV.** Fermenteerbaarheid van die behandelde materiaal, verkry uit die geoptimiseerde voorbehandeling, is geevalueer deur gebruik te maak van Gesamentlike Versuikering en Fermentasie (SSF).

Gedurende die eerste stel evaluerings, het strooi monsters van kultivars wat gegroei is in Mariendahl noemenswaardig verskil van strooi monsters verkry uit die Swartland en Overberg areas. Dié Mariendahl monsters het naamlik die laagste asinhoud getoon asook hoër opbrengs van xilose uit voorbehandeling (~62% van die teoretiese maksimum) asook hoër glukose uit ensiematiese hidrolise (>10%) in vergelyking met strooi monsters uit die ander areas. Kultivars 9, 13 en 14 (Mariendahl area) het hoër CSY waardes (43-45 g/100 g droeë material) getoon en is dus geselekteer vir loodsaanleg voorbehandeling optimisering. SE kondisies met temperature tussen 190 en 205°C, tesame met tye wat gelei het tot felheidsgrade van tussen 3.35 en 3.79 het die CSY uit voorkeurkultivars gemaksimaliseer vir ongekataliseerde SE. SE kondisies met temperature tussen 173 en 187°C, tesame met tye wat gelei het tot felheidsgrade (Log (*Ro*')) van tussen 3.30 en 3.41 het die CSY uit voorkeurkultivars gemaksimaliseer vir SO_2-SE. Voorbehandeling optimiserings het gelei tot 'n verbetering in CSY van tot 11%. Gekataliseerde SE is geïdentifiseer as die voorkeur metode vir voorbehandeling omdat meer CSY daardeur verkry is vir al die voermateriale (8-16%) en omdat daar

minder verskille in voorbehandeling vereistes was tussen strooi monsters vir hierdie voorbehandeling.

Geskatte etanol (2G) opbrengs vanuit lignosellulose, gebasseer op analities bepaalde suikers verkry uit geoptimiseerde voorbehandeling ensiematiese hidrolise, was 434 $L.ha^{-1}$. Dit verteenwoordig ‘n totale verbetering van ~28% in geskatte lignosellulose etanol opbrengs per hektaar. Maksimum geskatte etanol opbrengs was 171 $L.ton^{-1}$ na SSF by 13% soliede materiaal lading vir gepersde-WIS (water onoplosbare soliede materiaal) van ongekataliseerde SE. Etanol opbrengs per hektaar vir intensief gewaste WIS vanuit SO_2-SE van strooi 14 is geskat om bo 200 $L.ha^{-1}$ te wees. Dus is finale etanol konsentrasies naby die bedryfstandaard van 4% (v/v) bereik. Hierdie studie het aangetoon dat kultivar seleksie gebasseer op toevoermateriaal kwaliteit en proseseerbaarheid asook verdere voorbehandeling optimisering ‘n positiewe invloed het op die 2G etanol opbrengs per hektaar. Sulke verbeteringe in etanol opbrengs uit strooi is van belang vir die volhoubaarheid van triticale strooi as potensiële bioetanol grondstofmateriaal in Suid-Afrika. Verder het hierdie studie ook aangetoon dat hoër 2G etanol opbrengs per hektaar verkry kan word sonder verlies in graan opbrengs of etanol opbrengs per hektaar graan. Sodoende is ‘n goeie fondasie geskep vir die toekomstige seleksie van triticale deur plaaslike boere om hul boerdery ekonomie optimaal te bestuur.

Dedication

To my mom's memory and my supporting and extraordinary family, Alberto, Antonio, Marlen and Doris; my greatest treasures.

Acknowledgement

First and foremost I wish to thank my Ph.D supervisor Professor Johann F. Gorgens for giving me the exceptional opportunity to pursue my doctorate in his research group and constant support and guidance during my studies.

I will forever be thankful to Dr. Maria del Prado Garcia-Aparicio “Maria”, for her invaluable and continuous support regarding the successful completion of my research. She has been helpful in providing guiadance, advice and critical reading many times during my hopeless times. Thanks also Maria for becoming a very close friend who was there for me not only in my happiest moments but also in toughest ones.

Thanks to Department of Genetics (Stellenbosch University) and especially to Dr. Willem Botes for performing the field trials and data collection of agronomic parameters that served as basis for this study and made it possible.

James Batt (Department of Process Engineering) is acknowledged for the extensive chemical and pretreatment analyses at the early stage of the study.

My sincere and grateful acknowledgement to The Technology Innovation Agency (TIA), an entity of the Department of Science and Technology (DST) of the Republic of South Africa for funding support.

Many thanks Paul Machintosch for sharing with me the knowledge on steam explosion and technicalities, and the exceptional friendship I keep deep in my heart. I extend my gratitude to Manda Rossouw for not only giving priority to my “tons” of samples for HPLC analysis during my experimental work but also for becoming a very close friend who always was and hopefully be there for me. Thank you lot for the trips to many wonderful places you took me with and help and voices of encouragement in hard times. I wish the best in your new career at Distell, Stellenbosch.

Many thanks to technicians and laboratory assistants at Department of Process Engineering (Stellenbosch University) and specially Alvin Petersen, Levine Simmers, Edward Hendricks, Solly Solomon and Melony Adams for all the assistance and good-will during my experimental work.

My sincere thanks to Lynette Bresler, I never felt alone thousands of miles away from home.

Thanks to the awesome people I met along the way to distract myself from academia and now they are part of my life.

Thanks a lot to my brothers Alberto and Antonio and sister Marlen, and my sister-in-law Doris. I really need to thank all of you in Spanish: *Muchas gracias por ser lo más preciado que tengo en mi vida, sin ustedes no hubiera logrado dar otro paso importante en mi vida. Ustedes hicieron de mis estudios un inolvidable tiempo. Los adoro!*

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List of Abbreviations

1G	First generation
2G	Second generation
ANOVA	Analysis of variance
AOS	Arabinose oligosaccharides
ASL	Acid-soluble lignin
C5	Pentoses (sugars)
C6	Hexoses (sugars)
CCD	Central composite design
CCFD	Central Composite Face-centred Design
CSY	Combined sugars yield
DA	Dilute acid
DOE	Design of experiments
DRM	Dry raw material
EH	Enzymatic hydrolysis
FPU	Filter paper unit
GHG	Greenhouse gas
GOS	Glucose oligosaccharides
HMF	Hydroxymethyl-furfural
HPLC	High-Performance Liquid Chromatography
IU	International unit
MC	Moisture content
RSM	Response surface methodology
SE	Steam explosion
SF	Severity factor
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TFS	Total fermentable sugars
WIS	Water-insoluble solids
WSS	Water-soluble solids

Chapter 1

1 Introduction

1.1 Background

Concerns over dependence on oil-based fuel imports and energy security in developed countries such as United States (US), together with the need to mitigate climate change and achieve lower greenhouse gas (GHG) emissions such as CO_2 associated with the use of fossil fuels, have in recent years driven the development of liquid biofuels such as bio-ethanol [1]. Currently, a well-established bioethanol industry, commonly referred to as first generation (1G) ethanol, is based on food crops (e.g. maize and sugar cane) through direct starch and simple sugars to ethanol fermentation. Analyses on 1G ethanol have shown some CO_2 benefits and improvements in domestic energy security [2] although serious concerns have been raised: 1G ethanol production is water and energy intensive and the escalating demand for bio-ethanol has boosted the sourcing of feedstocks and land competition for feed and food production [3], which may lead to increase food prices and deforestation [4]**.** Thus sustainability of 1G ethanol is questionable.

New generations of bio-ethanol technologies have been developed to address many of the constraints associated with 1G ethanol. Choice of feedstock is vital to provide sustainability to bio-ethanol, where lignocellulose or second generation (2G) feedstocks are intended to address 1G feedstocks' drawbacks [5]. Lignocellulosic biomass is basically comprised by cellulose, hemicellulose and lignin as major fractions and it is estimated that 1.4 billion tons of such biomass is available annually for energy production, only considering US [6]. Lignocellulosic feedstocks include materials such as agricultural residues, short rotation woody crops, herbaceous crops, forestry residues, waste paper and other wastes containing fibrous plant biomass, which have been widely investigated [7]. New challenges for 2G feedstocks have arisen recently: monoculture crops with high input demands (e.g. fertilizers) and crops grown on fertile soil would result in displacement of food production or loss of biodiversity [3]. Hence at present it is recognized the importance of crops with low input demands grown on agriculturally degraded lands and specially their waste biomass such as straw [3].

The present thesis focuses on the study of triticale lignocellulosic biomass to realize its untapped potential as bioethanol feedstock. Triticale is a non-food, low-input and high straw yielding grain crop with demonstrated agronomic robustness to sustain grain and straw production when cultivated in marginal lands [8-10]; these are preferred features sought in crops for sustained bio-ethanol production at low cost feedstock, by combining 1G ethanol from grain together with 2G ethanol from straw. Triticale has proven suited agronomic performance under Africa-specific agroclimatological conditions [11] where about 55% of the land is unsuitable for food agriculture [12]. Good agricultural performance of triticale has also been shown in arid and semi-arid regions in South Africa [13; 14]. Thus, several societal and economic benefits such as food security, reductions in the reliance on imported fuel and improvements in African farmers' economy [15; 16] can be derived from bioenergy production from triticale grown on African marginal lands.

A significant number of triticale cultivars are now commercially available in South Africa [17], although these are exclusively used for animal feed purposes, sometimes also cultivated as ground cover in between other crops. The potential use of triticale straw for efficient ethanol production requires the following technical solutions that have not been addressed yet: **1**) Intrinsic biological/cultivar/environmental variabilities that result in significant variations in straw quality, providing opportunity to select cultivars with preferred straw properties, generally referred to as desirable processibility attributes [6]. **2**) Requirement for development of pretreatment processes according to the unique properties of triticale straws to realize maximal yields of sugars for cost-competitive ethanol outputs, and **3**) Assessment of an integrated process configuration for the pretreatment, enzymatic hydrolysis and fermentation of straws that is performed at high solid loadings, in excess of 20% [18; 19]. High solids loadings are essential to achieve a final ethanol concentration in the fermentation broth of 40 gram of ethanol per liter, consider as the industrial standard for distillation processes [20]. A brief introduction of the main themes developed in this thesis is given in the next subsections.

1.1.1 Cultivar/environmental variabilities of triticale lignocellulose for improved 2G ethanol

Efficient bio-ethanol production from triticale lignocellulose relies on agronomic characteristics of high yields of superior quality (high processibility as reduced recalcitrance) straw, but without compromising on the grain yield [21]. Studies on triticale straw quality and processibility for 2G ethanol production has not yet been reported, while there are limited examples for wheat straw (a crop that is genetic-related to triticale) in literature. Positive cultivar correlation between carbohydrate content and sugar release from pretreatment/hydrolysis has been found for wheat

straw [22], while other components, such lignin and ash have been found to negatively impact its processibility to sugars and ethanol.

Variability in grain yield of triticale cultivars has been widely addressed in studies, although not associated with 2G ethanol production or under Africa-specific agro-climatological conditions [23-26]. Straw yield is an important agronomic property for assessing the potential yields of cellulosic ethanol per hectare. Straw yield has been found highly influenced on specie-type, location and growing seasons on wheat and triticale cultivars under Denmark agro-climatological conditions [27] and cultivar × locations interaction variation in triticale grown in Canada [28]. Additionally, the selection of cultivars with the highest straw yields in South Africa must be weighed sensibly to avoid compromises with grain yield, since most of the current triticale cultivars with high grain yields tend to have low straw yields and vice-versa [29].

1.1.2 Pretreatment development with industrial relevance for realizing maximal sugars yield from straw

The recalcitrance of lignocellulosic biomass to biological processing (e.g. hydrolysis-fermentation) is one of the primary barriers to realizing industrial 2G ethanol [30]. Different pretreatment technologies can be applied to effectively overcome lignocellulose recalcitrance and provide high yields of monosaccharides for the subsequent fermentation. However, pretreatment conditions may impact biomass differently [30; 31]. Dilute acid (DA) and steam explosion (SE) are promising pretreatment technologies from both technical and economical points of view, and currently in early commercialization [32; 33].

DA at high temperatures (>160°C) and acid concentrations below 4% (w/w) has been shown to effectively hydrolyze hemicellulose to xylose and enhance the enzymatic digestibility of cellulose in the residual solids [34-40]. Approaches aimed at maximization of CSY (as total of hemicellulose and cellulose sugars released from pretreatment and subsequent enzymatic hydrolysis) from DA pretreatment have been reported for corn stover [41], switchgrass [42] and sugarcane bagasse [43], but no study has been done on triticale straw. DA performed at bench-scale in tubular reactors is ideal to perform large number of experiments at low cost due to its flexibility [44]. Thus, these advantages were considered for DA pretreatment optimization of triticale straw from top cultivars to assess the impact of cultivar variability in feedstock quality.

On the other hand, pretreatment studies performed on leading technologies at pilot-plant as in the present study are of industrial significance. SE pretreatment exhibits great versatility: It can be performed uncatalyzed (water-soaked material) or acid-catalyzed (commonly SO_2) to improve hemicellulose-sugars recovery and cellulose digestibility [45; 46]. However, conditions that maximize hemicellulose sugars recovery (mild severities) often do not match those for maximizing digestibility (more harsh severities). Thus optimal conditions for maximal combined sugars yield will be a compromise between both sugar streams. Hence optimization of the CSY for maximized sugars is necessary [41]. Regardless of the advantages displayed by SE, some limitations such as partial degradation of hemicellulose-derived sugars and consequent sugar losses should be taken into consideration to avoid negative impacts on the ethanol yield per ton of straw due to sugar losses and hydrolysis and fermentation inhibition [47]. Hence a realistic approach of pretreatment optimization, to maximize combined sugar yields for ethanol production, should also impose constraints on inhibitors formation, below the thresholds of toxicity for enzymes and yeast [48].

1.1.3 Assessment of an integrated sugars-to-ethanol process configuration for reaching benchmark ethanol concentration

Bioethanol production from lignocellulosic biomass entails three main processes: pretreatment, saccharification, and fermentation. Saccharification is important to convert the pretreated solids rich in cellulose to fermentable sugars. After saccharification, fermentation will convert the fermentable sugars to ethanol, by using an ethanologenic microorganism such a *S. cerevisiae*. Different technological approaches can be followed to accomplish ethanol as final product [49]: Separate Hydrolysis and Fermentation (SHF) or alternatively Simultaneous Saccharification and Fermentation (SSF) processes. The former is carried out in two separated steps, one for enzymatic hydrolysis for fermentable sugars production and the second for sugar fermentation to ethanol; however, the glucose produced during saccharification has been seen to strongly inhibit the cellulase activity and additional production of cellobiose also inhibits cellulase activity [50].

SSF is an integrated approach where saccharification and fermentation take place simultaneously in a single reaction vessel [51]. As the enzymatic product inhibition is overcome during SSF, this configuration is ideal to use higher substrate loading compared to SHF, and thus higher final ethanol concentrations in the fermentation broth are achieved [52]. In operational terms, SSF is limited to work under suboptimal temperatures for enzymes to able to preserve yeast under certain working limits, which has to be compensated with higher enzyme dosages [53;54]. Based on the displayed

advantages, SSF was selected for experimental testing in the present dissertation, aimed at reaching final ethanol concentrations close to benchmark of 4% (v/v), by using higher solid loadings and pretreatment at optimal conditions. However, the use of SSF in this study is limited to the effects of other factors that possibly would affect in different extents the course of ethanol production: Reduction in cellulose conversion in a linear fashion with linear increments of solid loading [53; 55] and accumulation of inhibitors during pretreatment [56].

1.2 General objective

On basis of the above background, the overall goal of this original study was to establish an experimental and conceptual process development and analysis to maximize bioethanol yield from triticale lignocellulose, without compromising ethanol yields per hectare from grain, and thus to contribute to its inclusion as a feasible feedstock into the bio-ethanol industry in South Africa. The development of a methodological approach was based on selection of preferred triticale cultivars considering 1G and 2G ethanol yields as determined by agronomic performances, preferred locations and the use of leading pretreatment technologies at bench- and pilot-plant scale to optimize sugars from straw, to realize improved total (1G+2G) ethanol output per hectare of triticale cultivated.

1.3 Thesis outline

This research aimed at contributing in the development of triticale biomass in the bio-ethanol industry in South Africa as part of the project —**Pretreatment, hydrolysis and fermentation of triticale lignocellulosic biomass for cellulosic bio-ethanol production**. This thesis is divided into 9 chapters.

Chapter 1 comprises the conceptual framework for supporting the study, general objective and thesis outline. **Chapter 2** examines generalities and the state of art of ethanol production from lignocellulosic biomass, energy crops and triticale and its lignocellulosic biomass. The processes and leading technologies for conversion of lignocellulosic biomass to fuel ethanol are also discussed. **Chapter 3** explains how the objectives of the study attempt to address the gaps within the literature review, shows the research framework and the methodological considerations followed in the study and the scientific contribution to knowledge made by this research work. **Chapter 4** presents the approach followed for the screening and selection of cultivars performed in the study and shows the results of the impact of cultivar selection on fermentable sugars yield from triticale straw. The

additional criteria applied on the results of this chapter to narrow the screening stage to finally three top performer straws are also given. **Chapter 5** describes the methodology applied on the pretreatment optimization at bench-scale of the triticale straws selected in chapter 4 and presents the experimental results. **Chapter 6** presents the pretreatment approach followed on triticale straw to establish optimal steam explosion pretreatment ranges for maximal hemicellulose-derived sugars release, combined sugars yield, highest cellulose digestibility and inhibitors production levels required in the experimental optimization. Results obtained with SE at pilot-scale and implications of the study are provided in this chapter. **Chapter 7** Pretreatment optimizations (uncatalysed and SO_2-catalysed) performed on the selected straws for maximum combined sugars yield constrained to low inhibitors production are presented in this chapter. **Chapter 8** shows the different strategies of solids loading in the SSF configuration for integrated sugars-to-ethanol conversion, to evaluate the ethanol yield from optimized pretreatment conditions for preferred straw samples. **Chapter 9** provides the synopsis of the most important findings of the study, summarizes the main conclusions of the thesis, states the limitations of the experimental work and presents an outlook for future work.

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Chapter 2

2 Literature Review

The present chapter outlines concepts related to different types of lignocellulosic materials and the current status concerning advances in 2G ethanol production. Emphasis is given to dedicated energy crops and especially straws. Generalities on structure and chemical composition typical to lignocellulosic materials, including triticale biomass, are presented. Recalcitrance and the modes of action of pretreatments to overcome it are also discussed. Dilute acid and steam explosion pretreatments as leading technologies are reviewed. Concepts of enzymatic hydrolysis and fermentation, as important downstream steps after pretreatment of lignocellulose, are also introduced.

2.1 Lignocellulosic biomass

Lignocellulosic biomass refers to organic matter that is primarily made up of cell walls with varied composition of cellulose, hemicelluloses and lignin resulting from the photosynthesis of light. Other minor components in cell walls are soluble sugars, extractives, pectin and minerals (e.g. ash) [1]. Cellulose fibrils and hemicellulose polymers are generally layered together and hydrogen bonded to the microfibril surface, as shown in Figure 2-1. Hemicellulose, a class of polysaccharide with variable composition and structures, is usually branched. This structure of hemicellulose and its non-covalent cross links with cellulose may help to prevent the aggregation of cellulose microfibrils in the lignocellulose structure [1].

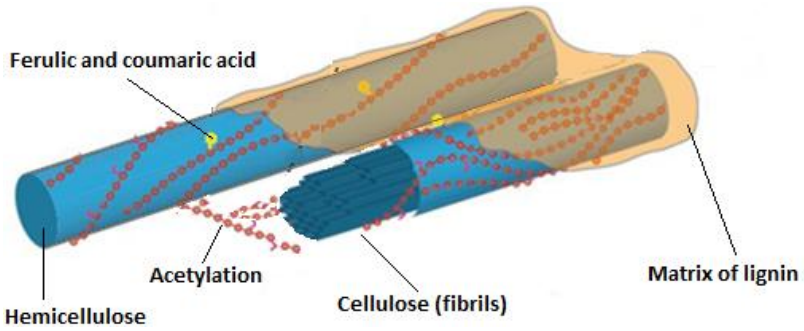

Figure 2-1: Typical structure of plant cell walls in lignocellulosic biomass (adapted from Sorek *et al.*, 2014).

As shown in Figure 2-1, the polysaccharide matrix formed by cellulose and hemicellulose is sheltered by a random structure of lignin. Lignin is a very rigid, amorphous and irregular polymer that allows formation of new tissue, provides support to vertical growth and gives resistance to microbial attack and oxidative stress [2].

Variation in cell wall composition is widely recognised among species, but may also occur within species or even individual plants. This variation is dependent on the cell wall type or due to the response to environmental conditions or their interaction [3; 4]. Other sources of variation may be caused by differences in harvest time, agricultural practices and breeding technologies [5; 6]. Lignocellulosic biomass includes short rotation woody crops, agricultural residues such as bagasse, crop straws and corn stover, herbaceous crops such as switchgrass, alfalfa, forestry residues, waste paper and other municipal and industrial wastes [7]. Lignocellulosic biomass is one of the most plentiful renewable resources on earth with estimates of 1.4 billion tons available annually, when considering the US only [1]. Table 2-1 shows the ranges of composition of four different categories of lignocellulose (softwoods, hardwoods, grasses and crop and industrial residues), which illustrate the typical differences in composition of the most representative lignocellulosic biomasses. A detailed discussion of the functions of the main components in lignocellulosic biomass and their relevance to bioethanol production follow.

Table 2-1: The lignocellulosic and ash composition of selected feedstocks.

Category	Biomass	Biomass composition (% in dry weight)				Reference
		Cellulose	Hemicelluloses	Lignin	Ash	
Softwoods	Pine	25-42	21-30	18-35	0.3-2	[3;8;9]
	Spruce	39.5	30	27.5	~0.3	[10; 11]
Hardwoods	Poplar	44-55	24-40	18-25	1-4	[3]
	Aspen	~53	~27	~19	~0.9	[12]
Grasses	Miscanthus giganteus	37-45	19-25	17-21	1-3	[13]
Industrial residues	Sugarcane bagasse	35-45	23-35	16-24	2.4-9	[14-16]
Crop residues	Wheat straw	32-47	20-30	5-24	4.3-12.4	[1;15;16]
	Triticale straw	36.3	24.8	19.9	7.5	[17;18]
Crop by-products	Cornstalks	39-47	26-31	3-5	12-16	[4]
Municipal solid wastes	Newspaper	40-55	25-40	18-30	1.8-8.8	[8]

2.1.1 Cellulose

Cellulose is a linear chain homo-polymer consisting of (1-4)-β-D-glucopyranosyl units with degrees of polymerization up to ~15,000 glucose molecules. It is the primary carbohydrate comprising the cell walls of plant material, representing around 30–60% of total feedstock dry matter [4]. Cellulose forms long microfibrils in the cell wall by molecular hydrogen bonds of hydroxyl groups on its glucose units, which leads to a crystalline and amorphous molecular structure [19]. Deconstruction of cellulose (depolymerisation) to cellobiose and glucose (shown in Figure 2-2. Simplified structure of Cellulose) can occur by enzymatic action of endo- and exoglucanases, glucosidases, and polysaccharide monooxygenases (PMOs), each of which are believed to act on only one part of the microfibril [3].

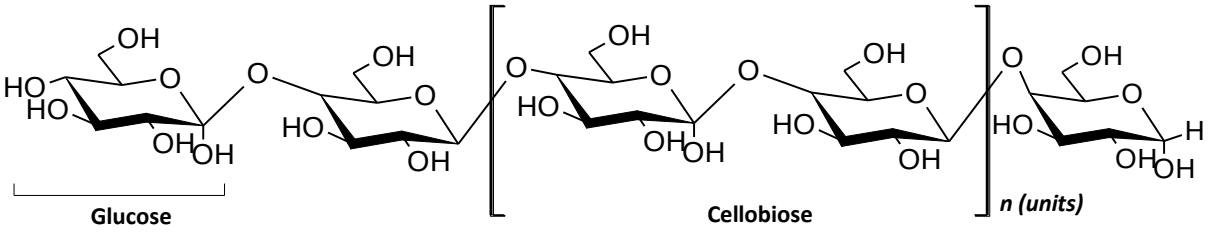

Figure 2-2: Simplified structure of cellulose. Monomer of glucose and repeating disaccharide units of cellobiose are shown. Adapted from http://free-stock-illustration.com/chemical+structure+hemicellulose.

Cellulose depolymerisation is a process also known as saccharification where the polysaccharide is broken down to free sugar molecules in the presence of water, to yield glucose, a six-carbon sugar, as the primary product. This is a slow process in which the outermost chains of glucan on one face are broken-down by endo-glucanases or PMOs and then gradually depolymerised by the action of reducing-end and non-reducing-end exoglucanases. Consequently, due to this relatively slow process of cellulose depolymerisation, significantly long residence times are required for cellulose hydrolysis compared to, for example, the depolymerisation of similar amounts of starch (source of 1G bioethanol) [3].

2.1.2 Hemicelluloses

Hemicelluloses are heterogeneous types of polymers representing around 15–35% of lignocellulosic biomass. Hemicelluloses function as load bearing, cross-linking agents in the cell wall of the plant, binding bundles of cellulose, non-structural polysaccharides, lignin, cell wall proteins and pectins by different covalent and noncovalent interactions [3]. Most hemicelluloses have a β-(1,

4)-linked glucan, xylan, galactan, mannan, or glucomannan backbone (as pictured in Figure 2-3). Thus, sugar units such as pentoses (β-D-xylose, α-L-arabinose) and hexoses (β-D-mannose, β-D-glucose, α-D-galactose) may be present in the structure. Others such as uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids) link the branched structure with single or longer glycosyl residues [20]. Minor sugars such as α-L-rhamnose and α-L-fucose may also be part of the hemicellulose, while acetyl groups can be present by partial substitution of the hydroxyl groups of sugars [20]. Hemicelluloses of the secondary cell wall are of importance for bioethanol production as they make up roughly half of the carbohydrates and one-third of the entire biomass in the woody tissues and stems, often considered as feedstock. From a bioethanol point of view, process performance, i.e. bioethanol yield from lignocellulosic biomass, is directly related to hemicellulose but also to cellulose and individual sugar concentration in the feedstock [21]. Hemicelluloses can be grouped roughly into four major classes, based on their backbone structures: xylans, mannans, galactans, and non-cellulose glucans.

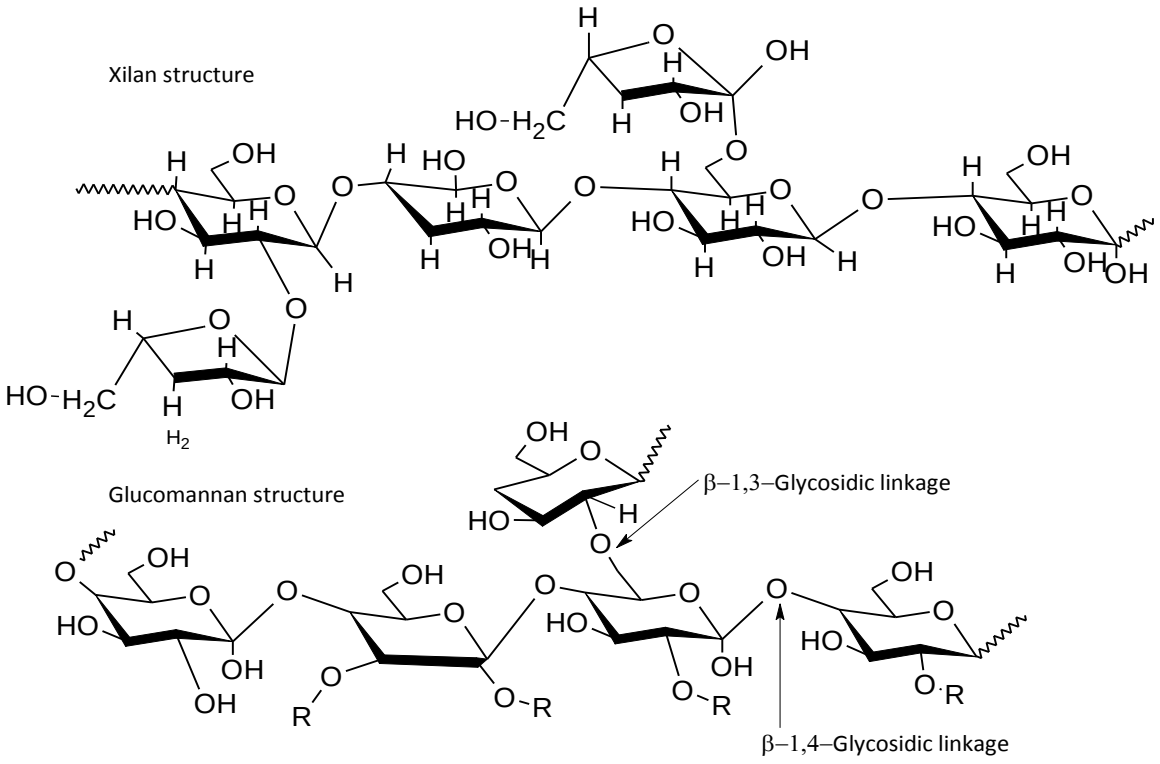

Figure 2-3: Simplified structure of hemicellulose. Adapted from http://free-stock-illustration.com/chemical+structure+hemicellulose.

2.1.2.1 *Xylans*

Linear backbones of β-(1-4)-linked xylosyl units are characteristic in xylan, which most frequently occurs with xylosyl, arabinosyl, or glucuronic acid substituents [22]. Further side chains of the arabinosyl may be linked to glucuronic acid with common *O*-methyl and *O*-acetyl group substitutions. Other side chain modifications are also possible, including ferulic acid and *p*-coumaric acid in covalent cross links with each other or with the lignin polymers [3;22]. Xylan substitution is highly variable in the amount and pattern among species. Hemicelluloses in grasses (e.g. Miscanthus and switchgrass) are primarily xylans in the form of arabinoglucuronoxylans which constitute up to 50% of the cell wall. Lower xylan (20-30%) is present in hardwoods (e.g. aspen) although composed of methylglucuronoxylans, while the types of hemicellulose in softwoods are comprised of not higher than 15% methylglucuronoxylan (e.g. pine) [3].

2.1.2.2 *Mannans and glucomannans*

Mannans are structured by a β-(1-4)-linked backbone of mannosyl units (mannose). Mannans are found as the major proportion of hemicelluloses in some gymnosperm softwood species, and in minor amount in some hardwoods [3]. Crop residues such as straw contain very small amounts of mannans and glucomannans and its contribution to the total sugars content of these lignocelluloses is not substantial. Glucomannans form up to 50% of the linear backbone [22] and in some cases *O*-acetyl groups and galactosyl side chains are also included in the backbone, in some softwood species. The structure of mannans and glucomannans are often acetylated in these species [23].

2.1.2.3 *Galactans*

Galactans are branched, soluble polysaccharides principally constituted by β-(1-3) or β-(1-4) galactosyl units (galactise), generally present in minor amounts in the secondary cell walls [3; 24]. Materials such as heartwood of larch have significant β-(1-3) galactan contents, with 10%–15% substitution with arabinose. Galactans, arabinans and arabinogalactans have usually been considered part of pectin structures and not as substructures of hemicellulose backbones [22]. However, in the present study, arabinan and xylan were grouped in the form of arabinoxylan as typically occurs in cereal endosperms (e.g. wheat and barley) and which can readily be removed [22].

In summary, the key polysaccharides in lignocellulosic biomass, cellulose and hemicellulose, typically make up two thirds of cell wall dry matter. They can be hydrolysed to sugars and then fermented to bioethanol. Hydrolysis of cellulose (saccharification) to simple glucose units

(fermentable sugar) is achieved by endo- and exo-glucanases by a slow depolymerisation process. Hemicelluloses, on the other hand, are more heterogeneous in structural sugars with xylans, arabinans, galactans, mannans and glucomannans in different proportions forming part of the structure, depending on the species. Xylose is the prevailing sugar in the hemicelluloses present in hardwoods and agricultural residues such as straw, while mannose is dominant in softwood hemicelluloses [25]. In addition, due to its amorphous and branched nature, hemicelluloses are more readily hydrolysed compared to cellulose.

2.1.3 Lignin

Lignin is a generic name for a large group of complex phenolic biopolymers of no regularity or repeating multi-unit structures. These biopolymers are derived principally from three hydroxycinnamyl alcohols (or monolignols), namely *p*-coumaryl alcohol (MH), coniferyl alcohol (MG), and sinapyl alcohol (MS) and synthesised by all plants [26]. Lignin comprises between 15 and 25% of the total dry weight of woody plants (lignocellulose), and is the second most abundant organic material on earth, exceeded by cellulose and hemicellulose [27]. Lignin is deposited predominantly in the secondary wall cells. Its functions in plants are related mainly to development of the structural support and rigidity of the plant, and biological adaptation in response to different biotic/abiotic stress conditions, such as pathogen infection, metabolic stress, wounding and perturbations in cell wall structure [28]. As lignin protects cell wall polysaccharides from microbial degradation, it is always associated with the carbohydrate fraction and mainly with hemicelluloses. Thus, the recalcitrance of lignocellulosic biomass to enzymatic hydrolysis has been highly associated with lignin, through mechanisms of single lignin effects or coupled lignin-carbohydrate complexes [30]. Lignin structures and compositions vary depending on their origins, but in general three main lignin groups can be distinguished: (1) gymnosperm lignins (lignins of softwoods), (2) angiosperm lignins (lignins of hardwoods) and (3) non-woody or herbaceous crop lignins (lignins of grasses) [29]. Figure 2-4 illustrates the structural linkage of the lignin-carbohydrate complexes formed in herbaceous crops such as wheat and triticale straws.

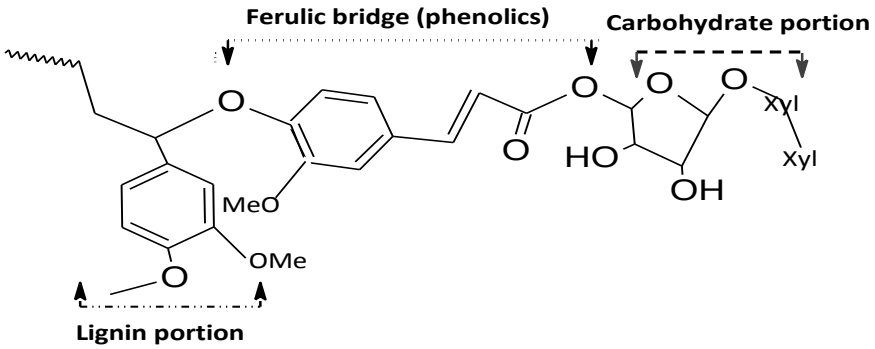

Figure 2-4: Structure of lignin-carbohydrate complex in straw (wheat). Adapted from Buranov and Mazza, 2008; [29].

Lignins in straw contain up to 5% ferulic acid, with a significant portion of these ferulic acid residues linked to polysaccharides (mostly arabinoxylan) via ester bonds [29]. These covalent links can also form a bridge (complex) between cell wall polysaccharides and lignins through ether bonds with phenylpropane, as shown in Figure 2-4, thus also reducing the carbohydrate availability [29]. Additionally and most importantly, the presence of lignin has been shown to negatively affect the three-step process of lignocellulose conversion to ethanol: pretreatment, polysaccharide hydrolysis and sugar fermentation to ethanol [31]. Thus, success in bioethanol production from lignocellulosic biomass highly depends on effective removal of substrate specific barriers to cellulases, such as lignin, by well-designed pretreatment regimes to improve cellulose digestion [32-34].

Different strategies have been applied to improve digestibility in various lignocellulosic materials. Genetic manipulation to down-regulate lignin expression in the plant material and well-designed pretreatment regimes to improve cellulose digestion are of significance. Fu *et al*., 2011 [35] showed that the production of phenotypically normal switchgrass by gene down-regulation of the caffeic acid 3-O-methyltransferase (COMT) in the lignin pathway led to reduced lignin content in the crop. This resulted in reduced recalcitrance demonstrated by less severe pretreatment requirements and lower cellulase dosages (between 300 - 400%) for comparable ethanol yields, using simultaneous saccharification and fermentation (SSF). An increase of 166% in sugar production was also achieved by down-regulation of lignin synthesis in alfalfa biomass [36;37]. Genetic modification leading to alteration of lignin properties also led to improvements of ~30% in glucose yield (saccharification) from liquid hot water pretreatment of *Arabidopsis* (flowering plant) at reduced cellulase and β-glucosidase dosage (40%), in comparison to the wild-type material [38]. Bagasse from engineered sugarcane clones with reduced lignin content have shown a ~5.5 fold improvement in total sugars yield (pretreatment and enzymatic hydrolysis) and a ~14-fold increase in enzymatic digestibility, reaching nearly 3 $g.L^{-1}$ of ethanol concentration more than wild clones [39]. Thus, techno-economic

benefits of lignocellulose feedstocks genetically modified in lignin expression, rely on potential cost reductions in pretreatment requirements, higher enzymatic saccharification yields and/or lower required enzyme dosages. Lower or altered lignin content can thus significantly improve the processability of lignocelluloses for conversion to ethanol.

An overview of the most common pretreatment technologies applied to different lignocellulose feedstocks, is given below under the subsection 2.2. Pretreatment in the bioethanol production scheme. This is a strategy to overcome recalcitrance, improve cellulose digestion by enzymes, and determine the impact of biomass quality/processability on pretreatment methods.

2.1.4 Ash

Ash represents the inorganic, incombustible fraction of the biomass left after complete combustion and is formed by the bulk of the minerals contained in biomass [7]. Major elements in ash are O, K, Ca, Mg, Si, Al, S, P, Fe, Na, Cl, Mn, and Ti, while ash content in biomass composition is strongly dependent on biomass species [7]. Particularly, high variation in ash content (4.3-12.4%) in straw from herbaceous materials (as shown in table 2-1) should be seriously considered in straw quality. Ash content has been directly associated with the neutralising capacity of switchgrass, corn stover and poplar [40], thus limiting the efficiency of acidic pretreatments such as DA and SE. The neutralising capacity of a material is its ability to uphold the pH (acidity) in aqueous acid solutions almost invariable. The higher the neutralising capacity (higher ash content) the less effective the catalytic effects of acids commonly used in pretreatment to release sugars and improve cellulose digestibility.

Cultivar variability in terms of ash content has adverse effects on straw processability and conversion to sugar [41]. It has been demonstrated that variations in the ash content occurs not only between different species of lignocellulosic biomass, but also among cultivars within the same species. Possibly similar negative effects on sugar conversion from straw may be expected in triticale cultivars. Ash content is associated with the mineral uptake during growing stages of the plant. Thus, local environmental conditions, such as type of soil and climate during the growing season, could also have a major impact on cultivars and their physiological function of mineral uptake [42].

2.2 Pretreatment in the bioethanol production scheme

Pretreatment is a prerequisite step in the second-generation bioethanol production process (see Figure 2-5), which aims to disrupt the rigid matrix of polysaccharides and lignin. Thereby the

cellulose in the material is made more accessible to the enzymes for subsequent enzymatic hydrolysis. In this way high monosaccharide yield for the subsequent fermentation is achieved [43]. During pretreatment processes the lignin and/or hemicellulose are removed and/or modified, depending on the pretreatment technology applied. Thereby the cellulose is exposed for effective enzymatic hydrolysis. Removal of either lignin or hemicellulose can significantly increase the cellulose digestibility of lignocellulose [44; 45], as is typically observed with acidic pretreatments.

The design of an efficient pretreatment strategy should be characterised by [43]:

- No particle size reduction of biomass needed.
- Preservation of the pentose (hemicellulose) fractions
- Limited formation of degradation products that inhibit growth of fermentative microorganisms.
- Minimum energy demands and cost

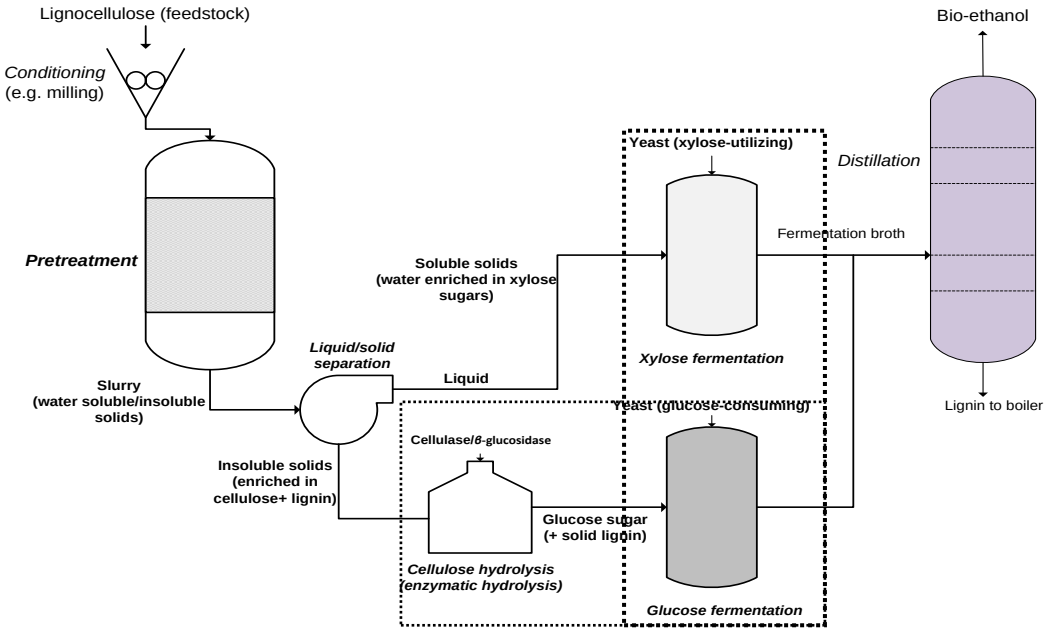

Figure 2-5: Simplified schematic steps process of lignocellulosic biomass to bio-ethanol conversion. Dotted lines enclose possible process integration opportunities. Adapted from [46].

It has been well documented that feedstock physico-chemical properties such as plant architecture, degree of polymerisation, cellulose crystallinity and components such as lignin have significant influence on the enzymatic reactivity of lignocellulosic biomass [47-49]. The role that the degree of crystallinity of cellulose plays in the digestibility of biomass has also been recognised. The highly ordered structure of cellulose is between 50 and 90% crystalline [50]. Studies on cellulose

reactivity have demonstrated that hydrolysis of amorphous (less ordered) cellulose proceeds at higher reaction rates compared to more ordered crystalline cellulose [51;52], and better accessibility of the enzymes to cellulose is achieved when crystallinity is reduced [53-55]. Studies on enzymatic digestibility of cellulose have shown lignin to be a factor influencing the reactivity of cellulose to enzymes to a greater extent than acetyl content or crystallinity [56]. Additionally, differences in lignin content among lignocellulosic materials (as shown in Table 2-1), may result in variations in recalcitrance. For instance, as observed in Table 2-1 the higher amount of lignin found in softwoods (18-35%) compared to hardwoods (18-25%), industrial by-products and crop residues such as sugarcane bagasse and straws (up to 24%), makes softwoods more recalcitrant to enzymatic digestion [57]. However, it is not only the variations in lignin content that are decisive in the design of a pretreatment strategy for the feedstock.

In addition to the diversity and complexity of the biomass cell architecture, as stated above, and natural occurring factors such as tissue layers and vascular bundles at microscopic level, other factors relating to composition of hemicellulose and neutralising ash have also been seen to contribute to recalcitrance [58]. Some studies have suggested that the composition and structural network formed by hemicelluloses, cellulose fibrils and other cross-linked polysaccharides, such as pectins, in the architecture of the plant biomass are related to recalcitrance [58;59] and thus influence pretreatment performance. As stated above (subsection Hemicelluloses), hemicelluloses vary in proportion and structure (degree of substitution) among different lignocellulosics. Hemicelluloses, described as shields around glucan chains in biomass, would hamper pretreatment severity for effective cell wall deconstruction. A relationship between hemicellulose removal and cellulose digestibility has been found for corn stover, oak [60], and wheat straw [61]. The degree of arabinose substitution in xylan was shown to be correlated to lignocellulose enzymatic digestion after pretreatment in Miscanthus [62]. However, other studies on woods and stover solids have shown no correlation between hemicellulose removal and cellulose conversion [63], suggesting that other possible factors, such as disruption of the lignin-carbohydrate complex, could play a greater key role in improving cellulose digestibility [61; 64].

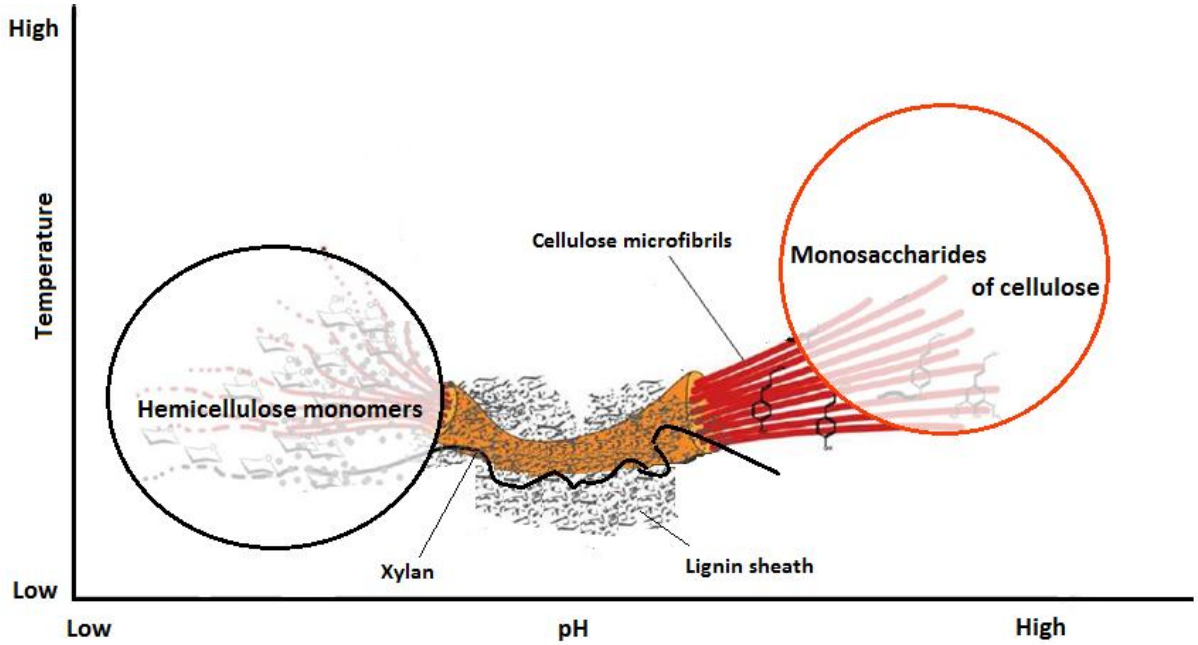

Figure 2-6: Schematic representation of main effects of pretreatment conditions (Temperature and pH) on lignocellulosic biomass during pretreatment (adapted from Pedersen and Meyer; 2010).

Current pretreatment methods can be generally divided into different categories: **1**) Physical (milling and grinding). **2**) Physico-chemical (steam pretreatment or autohydrolysis and wet oxidation). **3**) Chemical (dilute acid, strong acid hydrolysis, alkali, liquid hot water, and organic solvents). **4**) Biological, electrical, or a combination of these [65]. The different pretreatment strategies vary with respect to pH, temperature and holding time, and consequently the severity of the pretreatment and the resulting biomass composition after pretreatment [66]. A brief description of the different categories of pretreatment technologies and their recognised mode of action on lignocellulosic biomass is summarised in Table 2-2.

In general, the structures of herbaceous crops such as triticale (and triticale straw) have particular features that differentiate them from other plant species such as hardwoods and softwoods. These features in composition of herbaceous materials have been found affect differently pretreatment and thus selection of a pretreatment strategy must be based on these particularities of the feedstock [66]. Triticale straw composition is lower in lignin content whereas softwoods have the highest lignin contents (see Table 2-1). This fact makes triticale straw less recalcitrant to enzymatic hydrolysis [66]. Besides, cellulose content in triticale straw is often lower than levels of cellulose in softwoods (ranging between 34 and 50 of the total dry matter). Hemicellulose content in triticale straw can also vary; however its type of xylan substitution in galactogluco-mannan is lower than that

for softwoods [66]. As a result, herbaceous such as triticale straw have hemicellulose with a xylan backbone highly acetylated.

In this dissertation work two different pretreatment technologies and configurations were preferred to be evaluated on triticale straw: dilute-acid at bench-scale and steam explosion at pilot-plant scale. Hemicellulose is highly chemically and thermally susceptible to solubilisation and temperatures higher than 150°C typically promote hemicellulose solubilisation into xylan which is easily recovered. Dilute acid and steam explosion are two of the most cost-effective and efficient pretreatments currently at early stage of commercialisation in some of the second generation bioethanol plants. Both types of pretreatment are generally performed in the range of temperatures 160-230°C [43], and it is well-recognized that improvement in cellulose digestibility is achieved by a larger extent of hemicellulose solubilisation while lignin modification also takes place during both pretreatments [109]. Dilute acid pretreatment at bench-scale in tubular reactors is a low-cost and easy-to-use option for pretreatment optimisation and screening due to the large number of samples that can be processed. The outcomes of performance of lignocellulose pretreated by dilute-acid at small-scale offer a close outlook of what could be expected from pretreatment with steam explosion a larger scale due to those referred similarities. Pretreatment of triticale straw by dilute-acid for improved total sugars yield from the combined process pretreatment-enzymatic hydrolysis has not been reported in literature, thus knowledge on how triticale straw performs under this pretreatment technology is of importance for novel pretreatment development.

Considering triticale straw composition high in hemicellulose, low in lignin and high content of acetyl groups, steam explosion is an excellent pretreatment option that may considerably favour high digestible fibres through high extent of hemicellulose-derived sugars solubilisation. This can be facilitated by acids released from acetyl groups in the material during pretreatment which catalyse further hemicellulose solubilisation. Little is known about the performance of triticale straw during steam explosion pretreatment for improvement in cellulose digestibility and additionally high hemicellulose-derived sugars recovery. In this dissertation work, this gap in knowledge is addressed by subjecting triticale straw to pretreatment optimisation to identify optimum pretreatment conditions (severities) for maximum sugars recovery at limited inhibitors formation. Pretreatment of triticale straw through leading technologies with demonstrated cost-effectiveness at large scale is vital to provide sustainability to triticale straw as potential bioethanol feedstock.

In this section, special attention will be given to dilute-acid pretreatment and steam explosion based on the stated features of both pretreatments as leading technologies of recognised feasibility.

2.2.1 Dilute-acid pretreatment

Dilute sulfuric acid at low concentrations effectively removes and recovers most of the hemicellulose as dissolved sugars [43], achieving high reaction rates and significantly improving cellulose hydrolysis to almost 100%, for complete hemicellulose hydrolysis [40]. Likewise, cellulose hydrolysis is favoured by high temperatures in dilute acid (Table 2-2) [56; 57].

2.2.1.1 *Process description and mode of action*

The lignocellulose substrate is loaded into a stainless-steel reactor after being pre-soaked in the desired aqueous solution of sulfuric acid at concentrations usually < 4 wt. %. The mixture of biomass and acid is then heated to the target pretreatment temperature [48]. Stainless-steel reactors resistant to corrosion are consequently required. The mixture is rapidly heated up and pretreatment commences upon reaching the required pretreatment temperature. Alternatively, preheated acid solution can be used with the biomass and the time to commence pretreatment corresponds to the time of acid addition [48]. DA is usually performed at temperatures ranging from 120 to 210°C [72]. The residence time ranges from a few seconds to minutes, depending on the temperature of the pretreatment. However, variations of DA according to the reactor configuration (e.g. batch or continuous) can lead to other ranges of conditions, to achieve greater effectiveness [65].

The presence of sulfuric acid causes effective hydrolysis of hemicellulose into its component sugars (e.g. xylose, arabinose and galactose) that leads to enhanced digestibility of cellulose in the pretreated solids [72]. On the other hand, high temperatures in DA promote high reaction rates and significantly improve cellulose hydrolysis and yet no significant changes on lignin [73;74]. Almost complete hemicellulose removal is achieved by DA and recovered in the aqueous phase as dissolved sugars (monomers and oligomers) if pretreatment severity is adequately selected. Conversely, further reactions involving released sugars can proceed to sugar degradation products such as furfural and HMF if severity of the pretreatment is particularly severe [72].

Table 2-2: Leading technologies for pretreatment of lignocellulosic biomass.

Pretreatment	Catalyst	Temp (°C)	Time (min)	Severity	Mode of action	Advantages	Drawbacks	Reference
Lime	$Ca(OH)_2$ NaOH	50-150	60-4800	2.1-3.9	Lignin & hemicellulose removal	Low temperatures; low inhibitors production	Very slow process	[65;66]
Ammonia fiber explosion (AFEX)	Aqueous Ammonia	160-180	5-30	0.4-3.5	Lignin depolymerization	Lowers enzyme requirements	High sugar degradation at high Temperatures	[56;67]
Wet oxidation	Na_2CO_3	175-200	175-195	3.2-4.0	Disruption of the lignin-cellulose association	Reduce cellulose crystallinity	Expensive	[56;66;68]
Dilute acid (DA)	Sulfuric acid	160-230	Seconds-mins	(CSF) 0.1-3.4	Hemicellulose removal increases porosity/improves enzymatic digestibility	Effective remotion and recovery of hemicellulose	Neutralization of pH is necessary for downstream enzymatic hydrolysis	[56;61;69;70]
Uncatalyzed Steam explosion (SE)	Water	160-230	5-10	2.8-4.5	Alter the structure of cellulosic biomass	High yield of glucose, Cost-effective	Generation of inhibitory compounds incomplete disruption of the lignin-carbohydrate matrix	[65;66]
Acid catalyzed Steam explosion (ASE)	SO_2	180-210	2-12	3.0-4.3	Explosive decompression	Superior yield of pretreated material and better substrate for hydrolysis	Generation of inhibitory compounds	[65;71]
Two-step (acid-alkaline)	HCl-NaOH	~140	10 min	(CSF) 1-8	Monosaccharides release in acidic treatment while releasing lignin and glucose from the solid fraction in alkaline treatment.	Minor loss of fermentable monosaccharides	Energy intensive compared to single-step processes	[66]

For convenience of modelling kinetics, hemicellulose hydrolysis during DA has been described as a biphasic (solid-aqueous) mechanism from solid xylan to xylose in aqueous phase; xylan is considered as a two-fraction component where each fraction is classified according to its reaction rate: easy-to-hydrolyse and hard-to-hydrolyse [75]. Different schemes to describe the hydrolysis of hemicellulose in batch reactors during DA (as performed in the present dissertation work) have been proposed and first-order reaction kinetics have been assumed [76]. More refined kinetic models account for product formation of compounds from sugar decomposition such as furfural (from further degradation reactions of xylose and arabinose) [76].

Pretreatment of biomass by dilute acid requires neutralisation of the sugars streams for downstream enzymatic hydrolysis (one of the disadvantages of dilute acid shown in Table 2-2). DA displays significant advantages over other technologies: high reaction rates are achieved and consequently significant improvements in hemicellulose and cellulose hydrolysis. This can be done by sensibly varying the severity of the pretreatment [77]. So, the concept of combined severity is of utility to achieve high sugar release. In order to maximise the pretreatment yield of sugars, which are solubilised hemicellulose derived sugars, and also to produce highly digestible solids, the severity parameters must be carefully optimised for the specific feedstock under study. As stated above, high reaction rates lead also to formation of hemicellulose degradation products such as furfurals and hydroxymethyl furfurals [72], compounds of toxicity in biological downstream processes such as enzymatic hydrolysis and fermentation [78]. Sugar degradation products and fermentation inhibitors are discussed in detail in subsection 2.2.2.5. Pretreatment inhibitors. It is commonly accepted that pretreatment severity conditions usually engage a compromise if the aim targeted is maximising sugar recovery, usually referred to as “combined sugars yield” [16;72;79;]. However, the influence of combined severity on sugars productivity is not only specific to DA but also other technologies such as steam explosion (uncatalysed and acid-catalysed steam explosion). These pretreatments are discussed in the next subsection Steam explosion pretreatment. Thus, maximisation of the combined sugars yield (as target for the pretreatments applied to triticale straw in this study) would also depend upon optimisation of the type of pretreatment method used [72]. Specifically, the maximisation of the combined sugar yield would attempt to preserve the integrity of the hemicellulose sugars and favour its release to a great extent after pretreatment. Hemicellulose sugars are generally released in the form of solubilised hemicellulose derived sugars (xylose and arabinose) in the liquid fraction, commonly referred to as pretreatment liquor. The solid treated fraction is similarly of importance in the pretreatment optimisation due to the fact that glucose potential in cellulosic biomass represents the major fraction of sugars for recovery (Table 2-1). Thus,

fibres enriched in highly digestible cellulose are desired as a combination of both solid and liquid fractions [80].

Dilute acid pretreatment has been investigated for herbaceous materials such as rye straw and Bermuda grass [81], corn stover [82] and wheat straw (83; 84] for high conversion of total reducing sugars from pretreatment and enhancement of enzymatic digestibility. DA pretreatment of corn stover [79], switchgrass [85] and sugarcane bagasse [86] for improved combined yield of sugars from pretreatment and enzymatic hydrolysis has also been studied. Even though extensive work has been done on DA of a variety of feedstocks, no work has been reported for the maximisation of sugars yield from DA of triticale straw up to date. The use of less severe conditions has been found to achieve high xylan to xylose conversion yields [65]. Achieving high xylan to xylose conversion yields is necessary to realize favourable overall process economics because xylan accounts for up to one third of the total carbohydrate in many lignocellulosic materials, including triticale straw (Table 2-1).

2.2.2 Steam explosion pretreatment

Steam explosion pretreatment (SE) is one of the most promising pretreatment technologies that have reached commercialisation status in some of the large scale ethanol production plants currently in operation [87;88].

2.2.2.1 *Process description and mode of action*

SE pretreatment is a physico-chemical process which refers to the use of saturated steam at temperatures typically between 160 and 230°C (Table 2-2) and pressures between 0.7 and 4.8 MPa, injected into a batch or continuous reactor filled with biomass [66]. Steam is held in contact with biomass at different temperatures for short periods of time (normally 5-10 min. [66]), resulting in rapid heat-up of the biomass and then a sudden decompression to atmospheric pressure, commonly known as explosion, to terminate the process. The abrupt depressurisation taking place in SE causes separation of the fibres or defibration of the lignocellulosic biomass [57] as a result of the expansion and freeing of the embedded steam inside the treated material [89]. Steam injection and explosion are then convenient ways of rapid heat-up and cool-down of lignocellulose, allowing for more accurate fine-tuning of the variables of the process [89]. During pretreatment formation of acids, mainly acetic acid from acetyl groups in the biomass is promoted by the high temperatures causing hemicellulose hydrolysis and solubilisation [65;77;90]. Other acids, such as levulinic and formic acids resulting from further reactions of hydroxymethylfurfural (HMF) and furfural, are also formed and contribute to the referred hydrolytic reactions [71]. Coupled to hemicellulose hydrolysis and

solubilisation, condensation reactions involving carbohydrate derived by-products and lignin that cause lignin redistribution, are the major physico-chemical modifications of biomass undergoing SE [44;91]. Lignin in the biomass is generally redistributed during SE and to some extent removed from the material together with the partial hydrolysis and solubilisation of hemicellulose after pretreatment [92]. Carbohydrate and lignin by-products have been associated with the formation of pseudolignins. This is a term broadly used to define the higher insoluble lignin content measured in the pretreated material compared to the original content in the biomass [93]. Hemicellulose removal, favoured by higher temperatures and assisted by acetic acid released during pretreatment, leads to exposure of the cellulose fibres which results in improvement in the enzyme reactivity of the pretreated solids [40;94]. Released acids during SE also favour further reactions where pentoses and hexoses are degraded to furfural, HMF and other compounds such as formic acid. These compounds, along with lignin condensation products and possibly also solubilised extractives, are of high toxicity to downstream biological processes [95].

SE can be performed with or without the addition of a catalyst. Uncatalysed SE, commonly referred to as autohydrolysis, is usually performed on water impregnated biomass, where water at high temperatures can perform as a weak acid catalyst [57]. Autohydrolysis occurs when the acids released from biomass in combination with the acidic environment caused by water at high temperature, catalyse the hydrolysis of hemicellulose without the addition of an external acid catalyst [72]. Thus, a combination of mechanical forces (shear effects), chemical effects (hydrolysis) and heat (as steam) take place during pretreatment. Hemicellulose is the predominant fraction of the carbohydrates solubilised in the liquid phase during pretreatment, which makes cellulose in the solid fraction more accessible and consequently increases the digestibility of the material [44].

SE can alternatively be performed with the addition of an acid catalyst, commonly SO_2, H_2SO_4 or alternatively CO_2. The addition of a catalyst improves hemicellulose hydrolysis even more and consequently the release of sugars derived from hemicellulose, while improving the cellulose digestibility of the pretreated material [43]. Additionally, a reduction in inhibitory compounds production is also expected as the lignocellulosic biomass can then effectively be pretreated at pretreatment conditions of lower severity than those used during uncatalysed SE [72]. As stated, SE pretreatment is highly reliant on pretreatment temperature, residence time and inclusion of a catalyst, but other factors such as moisture content and particle size of the biomass also have an impact on the performance of this pretreatment type [77;96].

Water trapped in excess in the pores of biomass (as moisture) may result in inefficient heat transfer between the steam and the whole biomass as a result of low steam access into the biomass structure. Conduction is consequently the dominant regime for transport of heat during SE in these conditions [96]. Particle size of biomass also influences pretreatment outcome. It has been observed that very large particle sizes impede efficient heat transfer across the biomass particles during SE and results in non-homogenous pretreatment of the biomass and 'over-pretreatment' of the exterior portion of the material [97]. Therefore, when performing SE pretreatment under fast processing conditions, it is important to reduce the size of the biomass particles to a size that favours simultaneous mass and heat transfer [98]. Studies on the effect of particle size of herbaceous waste on the recovery of sugars from SE have found sizes between 8 and 12 mm suitable for high recovery of cellulose, while smaller particles did not represent advantages for recoveries or favour the economy of the pretreatment process [97].

Pretreatment temperature (T) and residence time (t) can be correlated in just one single factor, namely severity factor. The expression and definition of as well as theoretical concepts pertaining to the severity factor are given in subsection 2.2.2.2 Severity factor – uncatalysed pretreatment. The severity factor was used in this study to define the pretreatment severities and their influence on sugar release for uncatalysed steam explosion pretreatment. This is shown in Chapter 7, section 7.2.5. Calculations, which illustrate the utility of this concept in the framework of this dissertation.

Uncatalysed SE pretreatment features advantages such as high hemicellulose recoveries, substantial increase in the amenability of the cellulose to enzymatic action and low energy requirements compared to other pretreatment technologies. Another advantage of this pretreatment is that it avoids the additional chemical and environmental costs incurred by the use of chemicals and the resulting enhanced equipment corrosion [65;99]. Regardless of these advantages, limitations of SE include the generation of sugar degradation products (Table 2-2. Leading technologies for pretreatment) such as HMF and furfural, similar to dilute acid pretreatment, which may inhibit downstream processes [95].

Catalysed SE is a variation of steam explosion pretreatment that entails impregnation of the lignocellulosic material with acid (generally water-SO_2) at low acid concentrations (normally 3% w/w) prior to the pretreatment [100]. The addition of acid as catalyst has been shown to improve hemicellulose hydrolysis and biomass accessibility, lessen the energetic (temperature) requirements of the process and, depending on the process temperature, to reduce the production of degradation

products [44; 77]. Regardless of its high efficiency, the main drawbacks of catalysed SE are related to the high production of inhibitor compounds compared to uncatalysed SE, equipment corrosion [43; 78] and partial hemicellulose loss by degradation [101]. It is therefore necessary to optimise pretreatment on specific materials in order to balance these combined effects of the pretreatment conditions to obtain maximum effectiveness. The type and extent of inhibitors generated during catalysed SE depend on the biomass and the severity of the pretreatment. The major inhibitors produced are furan derivatives (mainly furfural and 5-hydroxymethyl furfural), weak acids and phenolic compounds [44]. Sugar degradation products and inhibitors formation from pretreatment are discussed in subsection 2.2.2.5. Pretreatment inhibitors. Pretreatment temperature, residence time and acidity of the aqueous environment during pretreatment can be combined into a single process parameter named combined severity factor. The combined severity factor expression is given in subsection 2.2.2.3. Combined severity factor - Catalyzed pretreatment.

Steam explosion under uncatalysed conditions has previously been shown to be very effective for the pretreatment of a wide variety of lignocellulosic materials such as poplar [102], sugarcane bagasse [103-105], eucalyptus globulus [106] sunflower stalks [107], wheat straw [108; 109], alder and triticale brand [110], barley husks [111] and rapeseed straw [112]. Strategies of individual sugar recovery, with hemicellulose derived sugars and/or glucose from subsequent enzymatic hydrolysis in separate streams, have been studied on a variety of biomass types treated with steam explosion. High yields of 86% xylose from pretreatment and 92% glucose after the subsequent enzymatic hydrolysis have been achieved for acid-catalyzed SE of Salix [113].

2.2.2.2 *Severity factor -uncatalyzed pretreatment*

Overend *et al.*, (1987) developed an expression (equation 2-1), namely reaction ordinate or severity factor (Log (*Ro'*), which correlates temperature (T) and residence time (t) in steam-aqueous pretreatments by just one single parameter [114]. This reaction ordinate is applied to compare different pretreatments without the addition of organic or inorganic catalysts (such as uncatalysed steam explosion pretreatment). The importance of the severity factor concept is that temperature and residence time can be varied while still achieving the same process severity, often leading to similar pretreatment results [114].

$$\mathrm{Log}\,(R'_0) = \mathrm{Log}\left(t \cdot \exp\left(\frac{T-100}{14.75}\right)\right) \qquad (2\text{-}1)$$

Where, t is the residence time (min), T is the process temperature (°C), T_R is the reference temperature which is assumed to be 100°C and the constant 14.75 corresponds to the activation energy value for processes of first order kinetics following Arrhenius law.

The concept of severity factor is of relevant utility to correlate pretreatment efficiency in sugars release and digestibility improvements. However, the lack of association with feedstock moisture and particle size, have been seen as limitations of this concept, since feedstock moisture and particle size are amongst some of the parameters that strongly influence the kinetics of steam explosion [115]. Moisture content, particularly at high levels, was shown to negatively affect the kinetics (chemical and physical changes of biomass) of SE, since the empty spaces in the lignocellulose (voids) are often packed with condensate water before the steam temperature is reached [116].

2.2.2.3 *Combined severity factor -Catalyzed pretreatment*

Similarly to uncatalysed pretreatment, the effects of the temperature and residence time on catalysed steam explosion pretreatment can be correlated in one single expression. However, changes in the acidity due to the inclusion of an acid as catalyst are important and thus the severity factor expression (Equation 1) is extended to acid-catalysed conditions by equation 2-2, according to Chum *et al*. (1990).

$$\mathrm{CSF} = \mathrm{Log}\, R'_0 - \mathrm{pH} \qquad (2\text{-}2)$$

Where Log R'_0 is defined by the equation 2-1 and pH corresponds to the pH of the aqueous environment in which the pretreatment takes place [117], often measured in the whole slurry resulting from pretreatment.

2.2.2.4 *Combined sugars yield for maximum ethanol yield*

With cellulosic biomass typically containing up to 35% hemicellulose as a sugar heteropolymer [118], the recovery of hemicellulose derived sugars from pretreatment is a benefit that may significantly contribute to achieving high sugar yields, as demanded at industrial levels. Although high hemicellulose sugar yield is favoured by mild SE pretreatment conditions/severities, these conditions will not result in digestible fibres. On the other hand, while highly severe conditions would result in a highly digestible fibre, such conditions also result in hemicellulose sugar degradation and low reduced solids recovery. Thus, a compromise is necessary between highly

severe conditions that result in highly digestible fibres, and low severity conditions providing good hemicellulose derived sugars recovery from pretreatment. Such a compromise is best achieved by maximisation of both the hemicellulose sugars yield from pretreatment and the glucose/xylose yield from subsequent enzymatic hydrolysis as a single output commonly referred to as *combined sugars yield* (CSY) [79; 86].

It has been observed that the severity factor concept performs according to the aim of the SE pretreatment. For example, biomass deconstruction starts at severities of approximately 2, while at severities greater than 4, sugar degradation will become extensive, due to dehydration and condensation reactions taking place during pretreatment [115]. Thus, SE severities between 2 and 4 should be selected for conversion of lignocellulose to sugars, which should not incur extensive sugar loss. Therefore, the "optimal" pretreatment conditions will be a compromise between hemicellulose sugar solubilisation and recovery in pretreatment liquor, cellulose digestibility and solids recovery – thus requiring optimisation of the CSY. If the substrate of choice for fermentation is the slurry or pressed-slurry, further consideration should be given to the inhibitors concentration in relation to the combined sugar concentrations, for each specific fermentative microorganism.

Determination of the range of pretreatment conditions that provide the maximum sugar yield, through statistically significant models, is relevant for industrial 2G plants. Several studies have been done on various approaches to the pretreatment of triticale straw. These include hydrothermal pretreatment with low polarity water (PLPW) [17; 18], phosphoric acid catalysed fractionation [119], Feedstock Impregnation Rapid and Sequential Steam Treatment (FIRSST) [120], ensiling [121] and microwave extraction [122]. However, the former pretreatments focused only on one fraction of the straw: hemicelluloses recovery, digestibility of cellulose or lignin extraction. To the author's knowledge there is no described work either on steam explosion pretreatment of triticale straw or based on maximisation of the combined sugar yield.

2.2.2.5 *Pretreatment inhibitors*

Pretreatment of lignocellulosic biomass with acidic methods such as DA and SE pretreatments, typically produces a range of degradation products. These products inhibit the enzymes and organisms used for downstream enzymatic hydrolysis and fermentation, thus reducing the ethanol yield and productivity [44; 88]. In addition, fermenting microorganisms may become gradually resistant or adapted to the inhibitors present in the broth. However, other fermentation variables such as cell physiological conditions, pH and concentration of the dissolved oxygen of the medium

also have effects on toxicity level [88]. Type of biomass (softwood, hardwood or herbaceous plants), pretreatment conditions and presence of an acid as catalyst have been shown to influence the nature and concentration of the inhibitors produced during SE [95]. The production of inhibitors during steam explosion pretreatment can be originated from different sources as shown in Figure 2-7, although major sources are sugar degradation products, deacetylation reaction products and lignin degradation products (as discussed in the subsection Lignin degradation products) below.

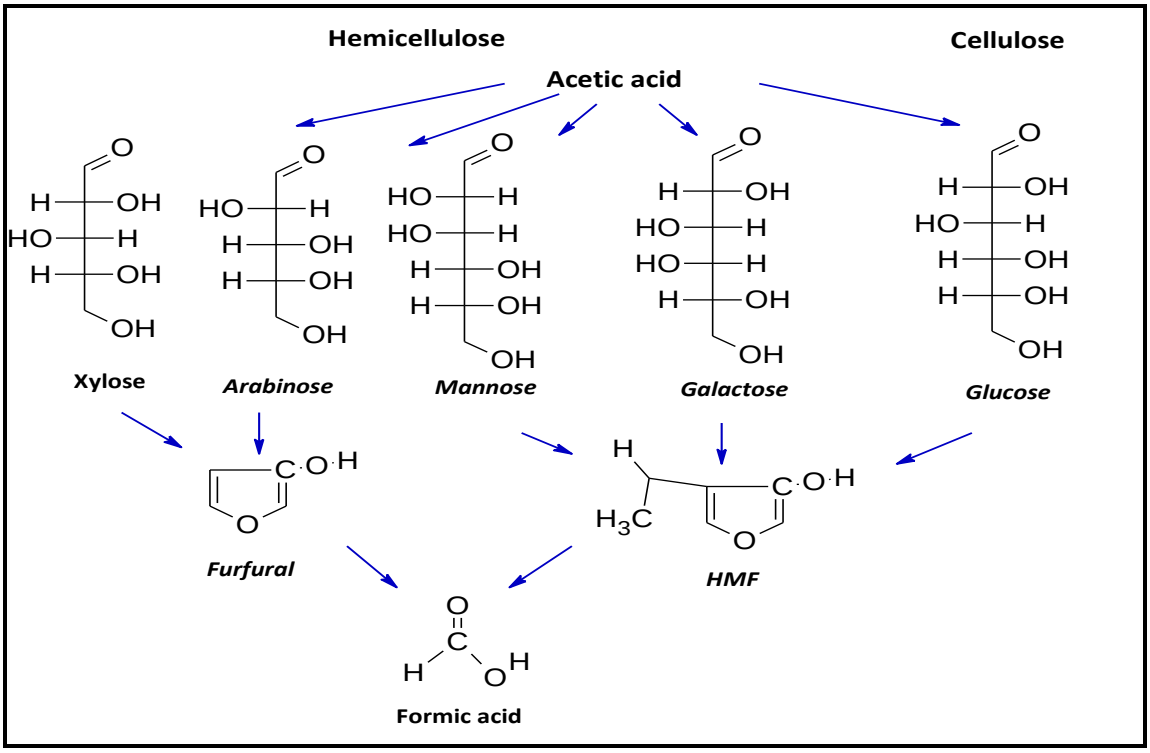

Figure 2-7: Inhibitors compounds and their sources from sugars and deacetylation reactionproducts of relevance considered in this study. Adapted from Palmqvist et al., 2000 [78].

Sugar degradation products

Hexose sugars, in the form of mainly glucose released during pretreatment, may degrade to hydroxymethyl-furfural (HMF) [95]. In addition, hydrolysis of hemicellulose produces pentose sugar monomers (arabinose and xylose) that may dehydrate to furfural due to pretreatment severity (Fig 2-9). Furfural is generally considered more inhibiting to cell growth, although the presence of furfural and HMF in the fermentation broth also has a negative impact on yeast respiration [88]. Toxicity levels of both inhibitors are given in Table 2-3. Significant production of furfural has been found to be highly associated with higher temperatures or longer residence times [88].

Deacetylation reaction products

Hydrolysis of hemicellulose during pretreatment promotes deacetylation reactions that result in acetic acid formation derived from the acetyl groups in hemicellulose (Figure 2-7). In this sense, acetic acid production during pretreatment cannot be prevented, since its formation is intrinsic to the pretreatment nature of hemicellulose hydrolysis. However, depending on the chosen downstream processes for the use of the sugars streams, pretreatment conditions can be balanced to result in major acetic acid release together with hemicellulose sugars in the hydrolysate, or alternatively be contained in the pretreated solids and further released during enzymatic hydrolysis. The inhibitory effect of acetic acid (and other inhibitors) in SSF can be largely avoided by removal of the majority of these inhibiting compounds through solid-liquid separation. Different process flow alternatives, such as pressed slurry and washed-WIS SSF, can be performed where the inhibitory effect of acetic acid and other inhibitors in SSF is largely avoided by removal of the majority of them through solid-liquid separation. Therefore the former process may be more tolerant of acetic acid inhibition than the latter [123].

The inhibitory effect of acetic acid is pH dependent and is responsible for cell activity inhibition due to the capacity of acetic acid to lower intracellular pH in its dissociated form after diffusing into the yeast cells [88; 124]. Thus, acetic acid toxicity varies according to the fermentation conditions and can be reduced by performing fermentation at higher pH values or through additional neutralisation of the acid before fermentation is initiated [88].

Lignin degradation products

Effects of pretreatment on the lignin fraction of the material may result in the release of a variety of aromatic, phenolic, polyaromatic and aldehydic compounds such as catechol and guaiacol. These compounds have significant inhibitory effects on cell and membrane partitioning in fermenting microorganisms, inhibiting both cell growth and sugar assimilation during fermentation [88]. Formation of phenolic compounds during pretreatment is highly related to pretreatment severity (temperature-time). Steam explosion pretreatment, at the conditions studied in this dissertation (uncatalysed and no strong acid (SO_2 at 3% w/w)), is expected to cause insignificant lignin degradation [88]. Therefore, lignin degradation products were not considered for evaluation as inhibitory compounds in this study.

Production of inhibiting compounds from uncatalysed SE pretreatment has been well documented. Cantarella *et al*. found that a formic acid concentration of 11.5 $g.L^{-1}$ was released from

SE of poplar wood biomass at a severity of 4.13. This had a strong inactivation effect on cellulolytic enzymes in subsequent enzymatic hydrolysis [125]. Acetic acid at concentrations around 2.1 $g.L^{-1}$ and comparative formic acid and furfural concentrations of 0.7-0.8 $g.L^{-1}$ have been reported for uncatalysed SE of barley straw [95], with strong negative effects on the hydrolysis step (up to 25% reduction in cellulose conversion). Similar enzyme inhibitory effects by the furan derivatives, furfural and HMF, were found for acid catalysed SE of wheat straw [126]. Table 2-3 shows the inhibiting concentration and corresponding extent of inhibition of the main sugar degradation products and deacetylation reaction products on the fermentative microorganism *S. cerevisiae* and cellulolytic enzymes (in the case of formic acid).

Table 2-3: Inhibition concentration levels of main inhibitors compounds on ethanol fermentation from steam explosion pretreatment

Compound	*S. cerevisiae*		Reference
	Conc. ($g.L^{-1}$)	Inhibition (%)	
Furfural	4.0	79	[126]
5-hydroxymethyl-furfural (HMF)	8.0	50	[127]
Acetic acid	6.0	74	[128]
Formic acid	4.0	100*	[129]

*Refers to the hemicellulolytic enzyme activity inactivation on cellulases from *Trichoderma reesei*

In this dissertation work uncatalysed and acid catalysed SE pretreatments on triticale straw were studied and differences in the levels of inhibitors produced by the various pretreatments would be expected. Although the addition of catalyst implies harshest conditions, it has been found that acid-catalysed SE results in higher inhibitors production compared to uncatalysed SE only if both pretreatments are performed at the same pretreatment conditions of temperature and residence time [130]. Thus, more sugar yield is obtained from acid catalysed SE compared to uncatalysed SE. Reduction in pretreatment temperature and/or residence time will be required for acid SE to match sugar productivity from uncatalysed SE, and consequently less inhibitors production would be expected [130].

2.3 Sugars-to-ethanol conversion by SSF

One of the most promising process configurations according to which enzymatic hydrolysis and fermentation can be performed is the Simultaneous Saccharification and Fermentation (SSF) process. This configuration minimizes the end-product inhibition of the enzymes by the continuous removal of the sugars by the microorganism, which allows the use of higher substrate loadings. Additionally, SSF avoids loss of sugar by not requiring glucose to be separated from the lignin

fraction [131]. Economic analysis has indicated that the high solid SSF process will significantly reduce the operating cost of ethanol production [132]. However, the hydrolysis and fermentation stages have different optimum conditions. In a SSF process the enzymes will act throughout the hydrolysis at suboptimal temperatures which vary depending on the microorganism [133; 134]. Additional operational limitations, related to mixing problems and mass transfer derived from solid loadings higher than 15% (w/w), are commonly found during SSF [135]. Performing fed-batch SSF is an alternative to improve mixing problems and allows the process to reach gradually higher solid loading [136] and to keep the concentration of inhibitors low [109]. In this way the process is enabled to reach concentrations of 4% (v/v) ethanol, considered as a benchmark for an economically viable distillation [137]. Other factors that have been observed to have a high influence on SSF performance is the quality of the cellulosic biomass, the quality of the cellulase enzyme, cellulase-substrate interaction and the interaction between microorganism and substrate [138].

Inhibitors such as furans, which are degradation products of pentoses and hexoses released after steam explosion, have been shown to cause a long lag phase during fermentation [95]. Negative effects on yeast growth and biomass formation have been associated with acetic acid which originated from solubilisation of acetyl groups of the hemicelluloses during steam explosion. Thus, strategies for conditioning of the pretreated material, such as pressing and washing, are also of importance to be evaluated in this study.

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Chapter 3

3 Objectives

Triticale, a genetic hybrid between wheat and rye, is a non-food and low-cost cereal crop displaying broad adaptability to poor-quality soils and exhibits high grain yields and abundant straw yields [1]. Current application of triticale grain is primarily for animal feed and ground cover, while straw is usually not utilized for value-adding, but rather left in the field after harvesting [2]. The selection of the most suitable feedstocks for bioethanol production has been focused on non-food crops with high biomass potential, adaptability to varied soil and climate conditions, low input requirements (fertilizers, agrochemicals and fuel used for farm operations) and positive environmental impacts [3]. Triticale thus has potential as a robust bio-energy crop for 1G and 2G ethanol production, due to suitability for cultivation on marginal lands, thus avoiding competition with food production. It also has low input demands, superior agroclimatological adaptability, and high cereal and lignocellulosic biomass potentials. Additionally, triticale has proven good performance in Africa with sustained productivity at extreme agroclimatological conditions in North Africa [4] and on about 35 000 ha in the drylands, winter rainfall region in South Africa [5], where triticale is locally cultivated.

Government's figures on grain production in the Western Cape of South Africa (2006) reported above 43 000 tons of triticale annually [6] which represents a gross potential production of 25800 tons of triticale straw (if considered the average grain-to-straw ratio of local cultivars found in this study). Recent studies have estimated a small grain production (wheat, barley and triticale) of approximately 200 000 metric tons annually if marginal lands available in the Western Cape are exploited [7]. Thus comparable production of triticale grain would be estimated and straw would run over 120 000 metric tons annually in those marginal areas if triticale is the crop of choice. Residue management for triticale is important to protect the soil from erosion and thus remaining stubble and straw should be left on the ground after the grain is harvested; an amount of 40% of the total straw produced is typically recommended to be harvested. In view of this, an estimated of 48 000 metric tons of surplus straw would be available for bioethanol production in the Western Cape of South Africa. Even though bioethanol production in South African is still small, based on sugarcane molasses and mostly for industrial applications, a National Industrial Biofuels Strategy was established by the government to promote biofuels production with target of 400 million litres

annually without risk of fuel/food competition [7]**.** Thus studies on triticale are needed to stimulate farmers' preference.

Potential use of triticale for 1G bioethanol production from grain would be based on the well-established process technology for ethanol from starchy materials, whilst 2G ethanol from triticale straw would require new technology development to incorporate the biomass into the lignocellulosic bioethanol scheme. There are presently at least 6 commercial scale facilities for ethanol production from lignocelluloses such as triticale straw, indicating that the technology has reached early commercialization. Pretreatment is vital to maximize the yields of sugars and polysaccharides from lignocelluloses, and is required for efficient straw processing in subsequent steps of enzymatic hydrolysis and ethanol fermentation. A variety of pretreatment technologies displaying advantages and disadvantages are currently available. With the aim of selecting pretreatment technologies that are already in large-scale application, dilute-acid and steam explosion pretreatments were selected for application to triticale lignocellulose, representing a novel application of these methods. In addition, pretreatment differently impacts on lignocellulosic biomass. Therefore, optimum conditions that suit triticale straw quality features, aiming at reaching high sugar productivity at low toxicity levels, need to be sought for feasible technical and economic application.

Pretreatment optimisation at pilot-scale is essential to reach the set targets for feasibility, and it will certainly impact the efficiency of subsequent processing steps. Similarities in the modes of action between pretreatments are likely to enable scale-up from small-scale dilute acid pretreatment to pilot-scale steam explosion pretreatment. Additionally, process integration such as the SSF processing of the slurry (pretreated material without further processing) at high solids loading [8] represent an extra tool to simplify processes and reduce operational costs and waste water. This opportunity for integration represents an additional novel target for triticale straw processing to reach a minimum of 40 $g.L^{-1}$ of ethanol, the established benchmark for economic viability in the distillation of the fermentation broth [9].

A fundamental challenge to overcome or manage in order to achieve success with triticale straw as a viable bioethanol feedstock in Africa, and particularly South Africa, has been identified: *Cultivar variability*. The large number of triticale cultivars available, with expected agronomic variability as observed in other foreign triticale cultivars [1; 10; 11], would lead to low predictability in performance of sugars-to-ethanol conversion. Despite the fact that good adaptability to different

agroclimatological conditions is generally recognized in triticale, genotypic and environmental factors may also influence agricultural performance and straw quality of locally grown triticale cultivars, as documented for straw from wheat cultivars [12-15].

In order to maximize the yield of ethanol per hectare of triticale cultivated in South Africa, cultivars with high yields in grain and straw, and preferred locations for cultivation, need to be identified. Quality attributes relating to high net biomass production and carbohydrates content (i.e. starch in the grain; cellulose and hemicellulose in the straw) are highly preferred. Other features, such as reduced recalcitrance of the lignocellulose fraction for processing to ethanol, also pose as potential beneficial characteristics that need to be investigated, due to a lack of available information on triticale straw in literature. Thus, evaluation of locations and the impact of their specific agroclimatological conditions (i.e. type of soil and climate) on bioethanol production from lignocellulosic biomass and agronomic yields will necessarily contribute to improved total area related ethanol yield and assure profitability for farmers.

3.1 General objective

On the basis of the above background, the overall goal of this original study was to establish an experimental and conceptual process development and analysis to maximize total bioethanol output per hectare of triticale cultivated. This was done through novel contributions in both cultivar performance screening and process development with straw, to advance its implementation as a feasible bioethanol feedstock in South Africa.

In essence the optimisation strategy, including cultivar comparison, cultivar selection, process development and process scale-up, was aimed at maximizing ethanol yield per hectare from straw, without compromising on the best observed performance in ethanol yield per hectare from grain. Agronomic yields, grain and straw qualities, areal ethanol yield from grain, as well as pretreatment and saccharification processability of the straw were considered for the screening of different cultivars. Pretreatment optimisation at bench-scale was followed to refine the selection of preferred varieties with improved combined sugars yields to finally three straws according to superiority in response to pretreatment. Subsequent pilot-plant scale optimisation sought the optimal pretreatment conditions for maximal yields of sugars at low toxicity levels for following enzymatic hydrolysis and fermentation steps. Thus, improved estimate ethanol yield potential per area of triticale was achieved, providing opportunity for development and commercialization.

The specific objectives of this study are as follow:

3.2 Specific objectives

Objective 1: Perform a systematic selection of triticale cultivars with superior agronomic properties, chemical composition of grain and straw (quality), and processability of the straw. Additional criteria of farmers' preferences for commercialization of cultivars, inclusion of the best performer cultivar per location of trial and availability for further pretreatment optimisation study were used to refine the selection.

This objective was accomplished by the experimental compilation of a complete data base for 60 straws from 20 triticale cultivars, obtained from field trials performed in 3 different locations in South Africa. The database comprised of agronomic information of grain yield and grain quality (starch and moisture contents), areal ethanol yield from grain as well as straw yield and straw quality regarding the whole lignocellulosic composition and response to pretreatment of triticale straw. Differentiation in response to pretreatment (recalcitrance) within straws was obtained by dilute-sulfuric acid pretreatment at bench-scale, performed at a typical pretreatment condition. The release of sugars, both from pretreatment itself and the subsequent enzymatic hydrolysis of pretreated solids using a conventional enzymatic cocktail at standard enzyme dosage, was taken as indicative of the relative processability of straw samples from different triticale cultivars and locations. A screening selection was then applied to the compiled experimental data enabling selection of the top 10 cultivars. This screening was based on superior performance according to area related ethanol yield from grain, and processability of the straw regarded as higher glucose and xylose release from pretreatment and enzymatic hydrolysis. The preferred 10 cultivars were thus identified based on the total 1G and 2G ethanol yield potential per hectare. Subsequent screening of these 10 cultivars applied additional criteria to include straws from each location of study, farmers' preferences and straw availability for subsequent optimisation steps, and led to the final selection of the top 5 preferred cultivars. The detailed methodology performed to address this objective is shown in **Chapter 4**.

Objective 2: Dilute acid pretreatment optimisation of the top 5 straws from preferred cultivars to maximize the combined sugars yields; thus, accentuation of the differentiation between the straw samples of preferred cultivars. Thereby, final selection of straws from the preferred three cultivars was facilitated.

Enzymatic saccharification stands as a bottleneck in lignocellulosic ethanol production due to the recalcitrance of the materials. Pretreatment is designed to improve substrate hydrolysis by

overcoming the recalcitrance. This normally requires the use of severe pretreatment conditions that may lead to sugar degradation and consequently sugar loss [16]. During pretreatment the yield of the hemicellulose sugars would be favored by mild pretreatment conditions/severities. However, such pretreatment conditions will not result in digestible fibres. Instead, very severe conditions would result not only in highly digestible fibres but also in sugar degradation and reduced solids recovery.

Pretreatment optimisation is an effective approach for improved conversion of cellulose to monomeric glucose by the search for "optimal" pretreatment conditions that typically requires a compromise between high enzymatic hydrolysis yields for the cellulose, while achieving good recovery of the hemicellulose sugars (low sugar loss) in the form of monomeric sugars. Thus, optimisation of pretreatment for the preferred cultivars will disclose the maximum potential yield of combined sugars realizable from pretreatment and enzymatic hydrolysis as a differentiating factor between straws.

In order to accomplish this specific objective, a Central Composite Face-centred Design (CCFD) was applied to design a set of experiments for dilute-acid pretreatment optimisation for each of the 5 selected straw samples, combined with enzymatic hydrolysis of the pretreated solids, to maximize the yield of combined monomeric sugars. The yields of monomeric xylose from pretreatment and glucose/xylose from hydrolysis, as major sugar fractions from the whole process, were considered as responses. The range of conditions into the design were 170 - 190°C for temperature, 0 – 0.6% (w/w) for sulfuric acid concentration and 6 – 18 min for residence time, which covers conditions typically categorized as mild and harsh for dilute acid pretreatment of straws. The methodological approach applied to accomplish this objective, and criteria applied on the results for final selection of three top straws, are presented in **Chapter 5**.

Objective 3: Develop predictive models with statistical significance for improved combined sugars yield constrained to tolerable concentrations of inhibiting compounds from steam explosion pretreatment at pilot-plant scale and enzymatic hydrolysis of the top three (preferred) triticale straws.

The models for prediction of sugars yields may be of industrial relevance for application of the triticale lignocellulose as bioethanol feedstock, as the levels of inhibitors and toxicity after pretreatment will be constrained to acceptable levels. In addition, the identification of a range of operational conditions for SE pretreatment, common for the preferred straw samples, was pursued,

thus allowing application of this pretreatment process to a range of triticale straws with similar properties. Thereby, the necessity for individual pretreatment optimisations for each straw sample will be reduced. This objective was achieved by performing optimisations of steam explosion pretreatment at pilot-plant scale with the top three preferred straws. This was done to establish the range of pretreatment conditions that provide the maximum combined sugars yield for each straw, while limiting inhibitors concentrations to acceptable levels for SSF processing of steam treated triticale straw. Uncatalyzed and SO_2-catalyzed steam explosion pretreatments were considered for optimisations based on preliminary experimentation with triticale straw that resulted in improved responses to pretreatment. Optimisations were done by using Design of Experiments (DOE) under Central Composite Design (CCD) which studied the influence of steam explosion pretreatment temperature and residence time on the combined sugars yield from triticale samples under the constraints of inhibitors formation.

Different technologies have been applied for pretreatment of triticale straws, such as hydrothermal pretreatment with low polarity water (PLPW) [17; 18], Feedstock Impregnation Rapid and Sequential Steam Treatment (FIRSST) [19], ensiling [20], phosphoric acid catalysed fractionation [21], microwave extraction [22] and ionic liquids fractionation [23]. However, the referred pretreatments were focused on one fraction of the straw: hemicelluloses recovery, digestibility of cellulose or lignin. DA and SE pretreatments display several similarities as advantages over other technologies. Both types of pretreatment are commonly performed at low acid concentrations below 4% (w/w) [24]. Hydrolytic reactions due to acid environments during pretreatment take place in both types of pretreatments and high yields of hemicellulose-derived sugars can be achieved if pretreatment conditions are suitably selected. High temperatures in combination with short residence times or alternatively low temperatures for a long residence time will result in high yields of fermentable hemicellulose sugars and subsequently improvements in cellulose digestibility from DA [24]. Likewise, temperature and time can be traded accordingly such that similar outcomes are achieved by SE. In this dissertation work, DA pretreatment optimisation was carried out on preferred straw samples and subsequently SE pretreatment optimisation was followed with straws that displayed superior performance. Thus, better pretreatment performance of preferred straw samples under DA would be also featured by SE.

According to the literature review performed to constitute the background of this study, steam explosion pretreatment of triticale straw has not been studied regarding the maximization of the combined sugar yield. Neither have broader studies been done on the release of sugars after

pretreatment followed by enzymatic hydrolysis. This aspect adds significance to the academic contribution of the present study. The effects of acid catalyst on the pretreatment conditions as pretreatment requirements, as well as sugar yields and levels of toxicity generated, were assessed to identify a preferred method. The predictive equations generated for each mode of impregnation were used to establish the range of conditions providing maximum combined sugar yield for each preferred straw variety. Further analysis led to the obtainment of contour plots where the predicted response of combined sugars yield overlapped into an area in common with pretreatment of the feedstocks for the range of conditions evaluated. Thereby, a range of pretreatment conditions that will be suitable for most (preferred) triticale straws was defined. The methodological approach followed to achieve this objective is shown in **Chapter 6**.

Objective 4: Assess the effects of cultivar selection and subsequent pretreatment optimisation on the experimental ethanol yield of the preferred straw cultivars in the integrated SSF configuration.

The screening of cultivars pursued in objectives 1 to 2, as starting point of the present study, sought the selection of the most promising triticale straws in terms of agronomic yields, pretreatment processability and combined sugar outputs. Subsequent optimisations, to accomplish objective 3, assessed the impact of cultivar selection on combined sugars yield maximization by pretreatment at pilot-plant scale. This enabled the disclosure of the optimum conditions for maximum realizable sugars yield under constraints of inhibitors formation. Top straws assessed at those optimal pretreatment conditions underwent the final assessment regarding sugar-to-ethanol convertibility in order to determine other important factors, such as fermentability and ethanol yield efficiency from optimal pretreatment optimisation.

To achieve this objective, the simultaneous saccharification and fermentation (SSF) process was selected for sugar-to–ethanol conversion based on the advantages of minimizing the end-product inhibition on enzyme activity, by performing both EH and fermentation in the same vessel. This simultaneous process facilitates the immediate conversion of the glucose released by the action of cellulases into ethanol (by the microorganism). This approach avoids exposing the enzyme component to high glucose concentrations, which could result in enzyme inhibition that is commonly observed in separate hydrolysis approaches [24]. Thus, common limitations found in large scale cellulosic ethanol production are studied. These limitations are due to high solids loading during enzymatic hydrolysis and/or fermentation to reach ethanol concentration at benchmark level (not lower than 4% [v/v]) [9].

The properties of the whole pretreated material, namely slurry, as important parameters directly impacting on the process performance and the fermentability were firstly evaluated. The pressed-slurry approach was selected to provide substrate for subsequent fed-batch SSF, as a compromise approach between the use of the highly inhibitory whole slurry and the washed WIS, which consumes extensive amounts of water. Fermentation testing was performed with the industrial strain of *S. cerevisiae* MH1000 as fermentative yeast.

Fed-batch SSF at 20% solid loading of pressed slurry was conducted to establish the maximum solid loading that the yeast is eventually able to ferment under the experimental conditions. Fed-batch SSF of the different varieties pretreated under optimum conditions were carried out for 150 h once the solid loading was defined. The concentration of fermentable sugars, ethanol, glycerol and weak acids were monitored to evaluate possible differences among the selected varieties and type of impregnation for pretreatment. The ethanol yield ($L.ton^{-1}$ of straw) obtained from the experimental conditions was determined and compared with the theoretical ethanol yield. This theoretical ethanol yield was based on the assumption of 0.51 grams of ethanol produced per gram of glucose or per gram of sugars (glucose + xylose) from pretreatment and enzymatic hydrolysis found in the pretreatment optimisation. Finally, comparisons were made of all the potential ethanol yields that could be calculated with the experimental data gathered in all stages of the study in order to be able to evaluate the accomplishment of the objectives of the study. Theoretical ethanol yield from grain (1G ethanol) based on starch content in grain and theoretical ethanol yield from straw (2G ethanol) based on total carbohydrate content in straw on average per location, as well as the predicted yields from optimum sugar released from steam pretreated straws by two impregnations were determined as a final remark. The approach followed to address this objective is detailed in **Chapter 7**.

3.3 Research framework

This dissertation was accomplished by the establishment of a systematic and integrated approach by optimising each step in order to positively influence subsequent steps. Economic conversion of lignocellulosic materials such as triticale straw into bioethanol relies not only in the efficiency of the steps namely pretreatment, enzymatic saccharification, fermentation and distillation, but also on feedstocks properties. In this context, the first part of the study was focused on the evaluation of different cultivars of triticale for commercial production of ethanol. Specifically, the core of this study was the production of 2G ethanol with the ultimate goal of determining the impact of cultivar selection on fermentable sugar yield for improved biofuel feedstock from triticale

straw. Different cultivars were selected for further optimization based on agronomic criteria and experimental results.

The potential ethanol yield for each cultivar was estimated considering agronomic parameters (biomass yield, grain yields, straw yields) as well as carbohydrates content (starch in grain, cellulose and hemicellulose in straw). Straw from 60 cultivars were collected from three different regions of South Africa. Dilute acid pretreatment followed by standard enzymatic hydrolysis tests was applied to the 60 straws to determine experimentally the sugar yield at bench-scale. The estimated and measured values were given different weight according to established criteria in order to select 3 suitable candidates for further optimisation of pretreatment conditions. Development of a pretreatment that maximise the yield of combined sugars as a pre-requisite for maximum 2G ethanol yield from straw is vital to provide feasibility for preferred triticale cultivars. Understanding pretreatment performance of straw from preferred cultivars will lead to better process predictability and positive economic reliability of the whole process. However, pretreatment conditions namely temperature and residence time (mainly) has shown to differently impact lignocellulosic biomass. These effects can be amplified due to biological variability of the feedstock as natural occurrence in plants. In the present study, pretreatment conditions (as manipulated variables) were optimised for dilute-acid pretreatment and steam explosion to able to identify their incidence on maximum sugars yield as major pretreatment response. Ranges of pretreatment conditions applied for optimisation study were based on preliminary experimentation.

A general outline of the research methodology followed in this study is shown in Figure 3-1. The design and execution of this methodology required expertise and knowledge in the areas of agronomy, biochemistry, microbiology and chemical engineering.

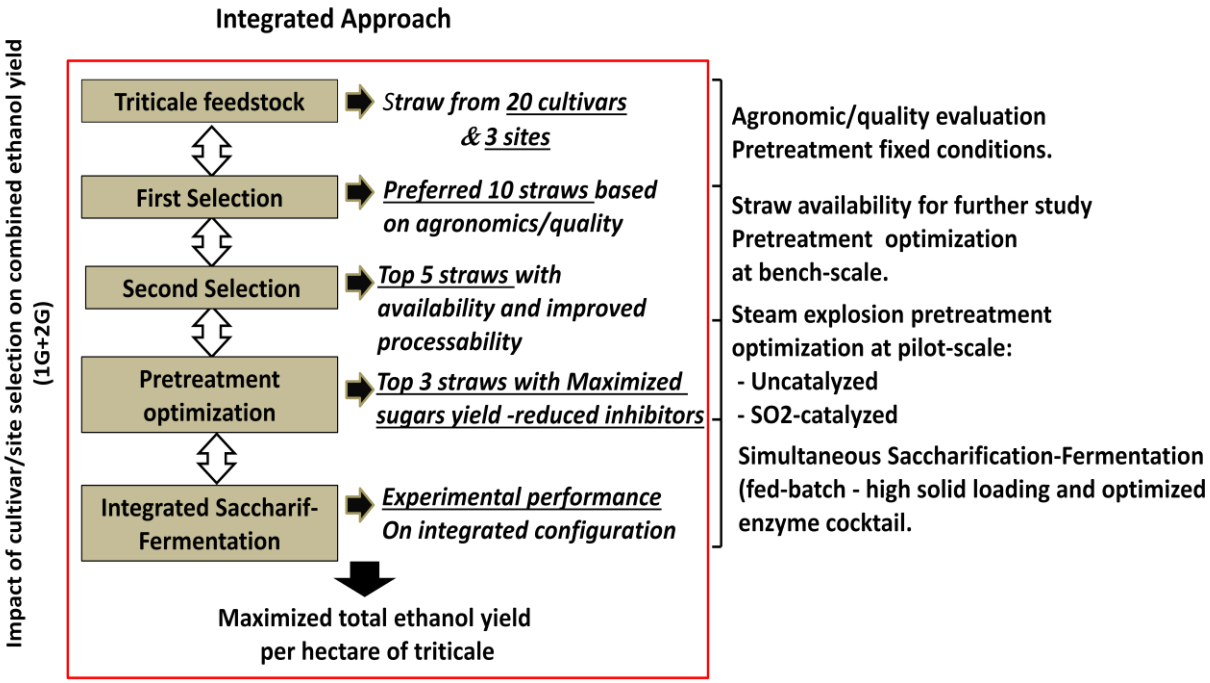

Figure 3-1: Research framework for the maximization of total ethanol yield per hectare of triticale cultivated in South Africa.

3.3.1 Methodological considerations

A group of twenty triticale cultivars subjected to field trials in the 2009 season at the sites Mariendahl, Overberg and Swartland was the subject of this study. The geographical locations of the field trials in South Africa are given in Figure 3.2. Technical entries of cultivars and site identifiers are given in Chapter 4 under Section 4.2 (Table 4-1). The trials generated a total of sixty straw samples (20 cultivars × 3 sites) in sufficient quantities to perform triplicate analysis of chemical composition of grain and straw, as well as to perform pretreatment during the screening selection of cultivars. Screening for cultivars with superior traits for improved total ethanol output successfully led to the selection of the top performer straws based on the criteria given in Chapter 4. Further studies on pretreatment optimisation at bench- and pilot-plant scale for improved sugar yield from straw from preferred cultivars required larger amounts of straw material. Therefore, straw availability was a limiting factor that was also decisive in the final selection of top 5 straws to undergo subsequent optimisations. The criteria applied and the results of the second selection of cultivars under straw availability constraints for successive pretreatment optimisation are given in **Chapter 4.**

Straw material from preferred cultivars after the first and second selection (Figure 3-1) corresponded to cultivars grown in the same location of origin and under exposure to similar agricultural practices (crop management) grown in season 2010.

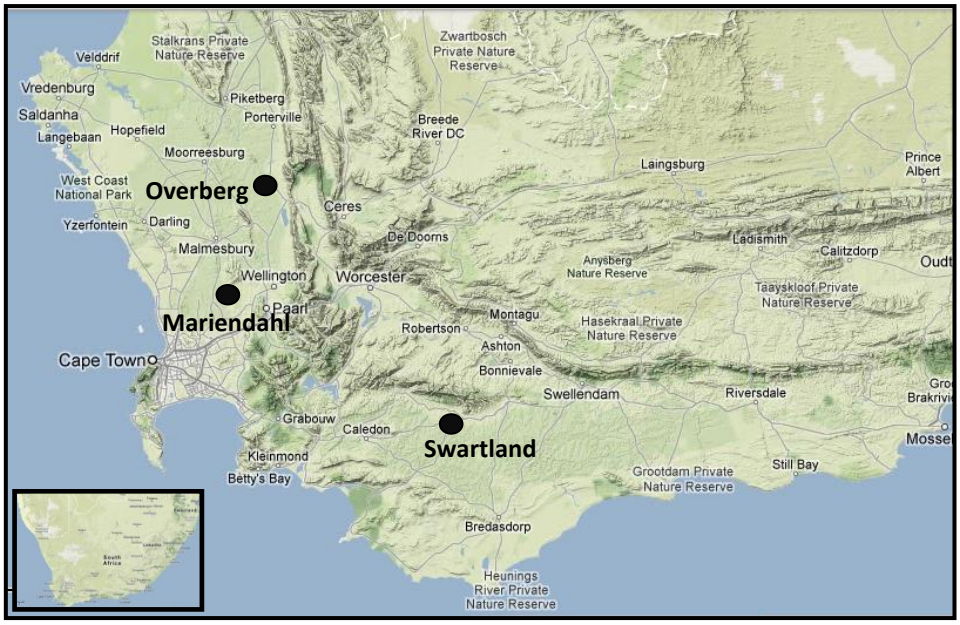

Figure 3-2: Geographical location of the sites of the study Mariendahl, Overberg and Swartland (black dots) in the Western Cape Province in South Africa [25].

3.4 Scientific contribution to knowledge

A. Generation of a large experimental data set of agronomic parameters (i.e. grain and straw yields), grain and straw quality, and pretreatment response from straw of triticale cultivars grown in three sites in South Africa. Data synthesis also yielded a tool for selection of cultivars and sites with improved yields of combined sugars and estimated total ethanol per hectare (**Chapter 4**). The Department of Genetics, Stellenbosch University, participated in the execution of the field trials which were under coordination of Dr. Willem Botes. The first stage of the study, comprising compositional analysis of grain and straw, and pretreatment of straw material at a single pretreatment condition was kindly performed under coordination of James Batt (Department of Process Engineering).

The scientific information experimentally gathered in the study is essential to establish criteria for selection of triticale cultivars with superior traits for improved ethanol yield per hectare of triticale under cultivar or site-specific variabilities. Biological variations of attributes in feedstock for bioethanol production among triticale cultivars, cultivar stability/instability across sites and the influence of site agroclimatological conditions on estimated ethanol productivity in South Africa have also been revealed in this study.

B. Development of statistically significant predictive models for the maximization of combined sugar yield under constrained inhibitors production from steam explosion pretreatment of preferred triticale straw at pilot-plant scale followed by enzymatic hydrolysis (**Chapters 5** and **6**). Steam explosion pretreatment is a leading technology that has already achieved commercialization status for bioethanol production. Pretreatment optimisation of preferred triticale straws to maximize combined sugars yield by uncatalyzed and SO_2 catalyzed steam explosion pretreatments at pilot-plant scale, provides statistically significant models with industrial relevance for feasible application.

C. Establishment of an experimentally validated range of uncatalyzed and SO_2-catalyzed steam explosion conditions that result in the maximization of the combined sugars yield from straw from triticale cultivars of Mariendahl origin (**Chapter 6**). Site-specific influences on processability of straw from triticale cultivars in South Africa were particularly notorious at the first stage of the screening selection in this study. Thus, the South African site at Mariendahl conferred particular attributes of higher straw processability on triticale cultivars that can potentially be advanced for the maximization of ethanol productivity per hectare. Thus, as a novel outcome, a statistically significant range of severities for uncatalyzed and acid-catalyzed steam explosion is proposed for maximization of the combined sugars yield for straw from triticale cultivars originated in Mariendahl. This range of conditions is applicable without the need for a previous pretreatment optimisation step and can also be applied to cultivars in other sites in South Africa.

3.5 Sustainability of the findings of this study

Currently, it is generally accepted that biofuels and specially bioethanol face environmental, social, and economic challenges. Although second generation bioethanol is intended to address many of the negative effects observed after the development of the first generation bioethanol, concerns still remain due to possible undesirable impacts as its predecessor. *Feedstock of choice* is essentially impacting the sustainability of second generation bioethanol. Several cellulosic crops (feedstocks) may require substantial chemical inputs and irrigation to sustain productivity which is finally reflected in increased costs in crop management, the potential for water pollution, and demand for more fertile and arable soils for cultivation. Crops such as triticale with very little input demands and good performance on degraded lands abandoned from agriculture use may be considered for a proper selection of feedstock with additional environmental benefits: cultivation of triticale on degraded lands could increase biodiversity in the area of influence, increase carbon sequestration in soils and improve quality of water. Negative social implications can also be minimised or even eliminated by promoting triticale as source of straw as feedstock; the use of the straw from triticale

grown on non fertile lands minimises land competition with food crops and consequently food production and its availability are not potentially affected. Some of the economic challenges derived from potential industrial production of lignocellulosic bioethanol are also addressed by the present dissertation. Firstly, improvement in performance predictability in field of triticale and its straw is issential for farmers' choice of crop. Better predictability in agronomic yields of cultivars provides triticale with high potential to be a preferred crop by local farmers. Secondly, the development of statistical models for reliable prediction of sugars from pilot plant pretreatment-enzymatic hydrolysis, as developed in this study, is of relevance from a process point of view. Both predictabilities of performance of triticale in field and of the straw during processing have positive impacts on economics of the whole process by reducing uncertainties.

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Chapter 4

4 Screening of triticale cultivars and selection

The entire content presented in Chapter 4 was structured as a manuscript for submission in Industrial Crops and Products.

Title: "Cultivar selection for improved ethanol yield from triticale straw"

Authors: Roberto A. Agudelo, Maria del Prado Garcia-Aparicio, Willem Botes and Johann Görgens

Objective of dissertation and summary of findings in present chapter

Chapter 4 addresses **Objective 1** of the study and represents the foundation for addressing **Objective 2.** The latter assesses the optimisation of pretreatment conditions for straw samples from the preferred 5 cultivars selected in the present chapter. Thus, **Objective 1** is a preliminary screening of cultivars to identify preferred straw samples for subsequent process optimisation.

Abstract

This chapter aimed at evaluating the impacts of cultivar and site-related variabilities in agronomic yields, grain and straw quality as well as straw processability, on sugars and ethanol yields of twenty triticale cultivars in three sites in South Africa. Field trials were performed by the Department of Genetics at Stellenbosch University. This department provided both straw samples for experimental testing as well as data on agronomic yields and ethanol yields for grains and straws. This data was used as inputs to the cultivar selection process performed by the candidate. Processability was assessed by measuring the total monomeric sugars released from dilute acid pretreatment performed at a single condition, combined with sugar production from subsequent enzymatic hydrolysis, also under standard conditions. Chemical composition analysis and processability of the sixty straw samples were carried out with the assistance of James Batt from the Department of Process Engineering at Stellenbosch University. Agronomic data on grain and straw yields, obtained from field trials performed with these cultivars, was used in combination with grain and straw quality and data on the sugars released after pretreatment and enzymatic hydrolysis, to determine the total sugars

yield per hectare of triticale cultivation. In this way the total (1G+2G) ethanol yield potential for all of the cultivars between sites could be estimated. Both cultivars with the highest sugars yields (2G ethanol) and those with the highest total ethanol yield were selected. These yields were subsequently subjected to analysis to quantify any compromise of the selection with grain yield. Thus, final selection of cultivars ensured little or no compromise between high cellulosic ethanol yield and grain yield. The final 5 best performers were selected based on availability of the straw for further pretreatment optimisation, farmers' preferences and commercial status. An additional decisive factor in the final selection was to include straws from all three sites of study to representatively generate experimental data with applicability to the main areas of triticale plantations in South Africa.

Variability in straw yield between cultivars (0.4-3.8 $Mg.ha^{-1}$) was higher than the observed variability for grain yields (2.8-3.4 $Mg.ha^{-1}$). In general, significant cultivar- or site-related variations in straw quality were attributed to location and mostly to ash content, whilst no significant variation in grain quality was observed. A direct association between low ash content straws from the Mariendahl site (as location displaying straws with significant lower ash content) and amenability (processability) to pretreatment-enzymatic hydrolysis was found. Xylose and glucose recoveries of up to 62 and 73% from 73 and 80% low-ash cultivars were observed. However, the estimated 2G ethanol yields between 217 and 388 $L.ha^{-1}$ for top cultivars from Swartland and Overberg were higher than the maximum estimates for Mariendahl (203 $L.ha^{-1}$), primarily due to low straw yields at Mariendahl. Locations for triticale cultivation therefore differed significantly: Mariendahl provided high straw processability, although at low straw yield, while Swartland resulted in the highest estimates for total ethanol yields per hectare, although providing relatively low grain yields. Overberg site gave the highest yields of grain even though at the largest variability between sites. On the other hand, straw yield in Overberg was only greater than Mariendahl although at the lowest variability across sites and with comparable processability to Swartland site. Yields of grain and straw differed significantly between cultivars and across locations and thus correlations between both yields could not be established. Despite the fact that the lack of any correlation between yields of grain and straw has negative impact on predictability of straw resource, especially when the latter is estimated based on grain-to-straw ratio, the selection of triticale cultivars displaying both high straw and grain yields is possible in South Africa. Thus high 2G ethanol yield can be realized collectively with high grain yield for farmer's benefits.

Keywords: Bioethanol; Cultivar selection; Straw quality; Sugars; Triticale straw.

Candidate declaration

With regard to chapter 4 page numbers 68−107 of this dissertation, the nature and scope of my contribution were as follows

Name of contribution	Extent of contribution (%)
Interpretation of results	70
Writing the chapter	100

The following co-authors have contributed to chapter 4 page numbers 68−107 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Maria del Prado Garcia-Aparicio	Garcia@sun.ac.za	- Experimental planning - Providing inputs - Interpretation of results - Reviewing the chapter	25 40 10 70
2. Johann Görgens	jgorgens@sun.ac.za	- Experimental planning - Interpretation of results - Providing inputs - Reviewing the chapter	35 10 30 30
3. Willem Botes	WCB@sun.ac.za	- Experimental planning - Experimental field trials - Agronomic measurements -Interpretation of results - Providing inputs	40 100 100 10 30

Signature of candidate……

Date……

Declaration by co-authors

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 4 page numbers 68−107 in the dissertation,
2. No other authors contributed to chapter 4 page numbers 68−107 in the dissertation besides those specified above, and

3. Potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material into chapter 4 page numbers 68−107 of this dissertation.

Department of Genetics (Stellenbosch University) under the coordination of Dr. Willem Botes made a major contribution to this study by executing the field trials and supplying derived agronomic measurements of grain and straw yields. These contributions are highly acknowledged.

4.1 Background

Cellulosic ethanol [second generation (2G) bioethanol] has recently progressed from demonstration phase to become a commercial and industrial reality at large scale [1]. A total capacity of over 430 million litres of bioethanol per year is currently operational in six commercial plants located in Europe and the United States, whilst other large scale plants are under construction with plans to open in the next few years [1; 2]. Corn stover, wheat straw, switchgrass, sugarcane bagasse and crop residues from oat/barley are among the dominant feedstocks of choice in the existing ethanol facilities [3].

The utilisation of the whole crop (e.g. leaves, straws and cobs) for ethanol production has been seen to be beneficial since it minimises the land space required for crop cultivation and contributes to sustained grain availability [2;4]. Other residues that are generated in large amounts, such as straw from cereals, also display additional advantages of sustainability and immediate availability [5]. Crop choice has been pointed out as a crucial factor affecting the sustainability of bioethanol production [6]. Triticale, a non-food cereal and close relative to wheat and rye, possesses favourable traits for bioethanol production compared to wheat classes [7]. These include high grain yield and straw potential and good disease resistance. Others traits, such as higher productivity than other cereals on marginal agricultural lands and low input requirements, have also highlighted triticale as a widely adapted and robust crop [8;9]. Triticale is suitable for cultivation on marginal lands, which are too low-yielding to be economically viable for production of wheat and maize as primary food crops in South Africa.

Triticale is gaining importance around the world with 14.6 million metric tonnes of grain produced from 3.9 million hectares in 2013 [10]. The total area under triticale cultivation almost doubled over the last 10 years in Europe, where it is considered as a food crop [11]. Triticale cultivation in South Africa is dedicated exclusively to the generation of animal feed and ground cover (e.g. planting open areas in vineyards to avoid overgrowth by weeds). Therefore, any potential competition with local cereal production is thereby excluded. South Africa has opportunity to produce bioethanol in the long run through exploitation of energy crops, such as triticale and their residues, thereby reducing its reliance on imported fuel. A large number of triticale cultivars from local breeding programmes are now commercially available in South Africa [12]. Triticale has proven suitability in agronomic performance in arid and semi-arid regions of South Africa [13]. Triticale can be cultivated without irrigation on marginal soils in dry land areas of South Africa – areas that are not suitable for wheat production due to low grain yields, yet are close to main wheat production areas. Triticale will give

better yields than wheat on these lands, albeit with lower input requirements, thereby allowing cost-effective crop production without competition with food production. Thus, there is an opportunity for grain growing farmers to cultivate triticale on available or marginal lands for bioethanol production in South Africa [14].

Triticale has been farmed extensively in the Western Cape in Mediterranean (drylands without irrigation) climate conditions and diverse types of soil. Estimates of approximately 200 000 metric tonnes per annum of grain could be potentially produced if cultivated on marginal lands available for bioenergy crops production [15]. The production of 200 000 tons of triticale grain per year would be associated with the production of up to 490 000 metric tonnes of straw on an annual basis, when considering typical straw:grain ratios of triticale cultivation [16;17]. Considering that there are limited amounts of marginal lands available for triticale production (e.g. 80 000 hectares and 200 000 tons of grain available annually in the Western Cape), it is imperative to consider triticale straw as a feedstock for ethanol production. Ethanol production of 80 million litres per year from available triticale grains has been estimated [14]. Estimated straw resourced per ton of triticale, as found in the present dissertation work, was on average 0.56 tons per ton of triticale grain (between cultivars and across sites), which is in agreement with reported estimates of 0.52 tons of straw per ton of triticale [17]. If it is hypothetically assumed that around 30% of the available straw is left on the field and/or used for steam and electricity generation as process energy for the combined 1G-2G production plant, available straw will run over 78 000 tons annually. Thus, nearly 18 million litres of ethanol produced per year from available straws can be potentially added to ethanol from grain to significantly intensify ethanol production per hectare.

Straw quality, measured here as the processability of straw with regards to pretreatment hydrolysis to isolate sugars for fermentation, is determined by a wide range of characteristics. These include chemical composition and multiple layers of physical-chemical structures in straw, similar to other types of lignocellulose. The processability/amenability of straws for ethanol production is of particular importance for cultivar selection for bioenergy crops and has not been addressed for triticale. Straws with higher fractions of fermentable sugars and with reduced recalcitrance to biological processing (natural resistance of the cell wall structure to hydrolysis processes represented by pretreatment and subsequent enzymatic hydrolysis) are preferred for 2G bioethanol production [18]. The combined effect of such improved processability/amenability for 2G ethanol production, based on the properties of straw samples from different cultivars and cultivation locations, is measured in terms of the sugar yields from pretreatment hydrolysis processes. In this regard, a

correlation between carbohydrate content and sugar release from pretreatment/hydrolysis has been reported for wheat straw [19], while other chemical components, such lignin and ash, have been found to negatively impact its processability.

Although some similarities between straw from triticale and wheat would be expected, as these are genetically related crops, little is known on cultivar- and site-related variabilities in straw quality of triticale, specifically with regards to 2G ethanol production. However, any assumption of similarities with wheat straw is yet to be proven with the local cultivars in light of the fact that variations in biomass composition may occur according to genotypic characteristics, environmental factors, harvest practices and possibly their interactions [20].

Studies on cultivar variability of triticale have been widely addressed regarding grain yield [21-23], whereas studies on straw yield variability aimed at 2G ethanol production are limited in literature. Straw yield stands as one of the most important agronomic properties for assessing the available biomass obtainable from cereals and thus is a real estimate of sugars and 2G ethanol that can potentially be sourced. Estimates of sugars potential in straw, the release of sugars from pretreatment/hydrolysis and ethanol output per hectare of triticale cultivated are consequently highly impacted by variations in straw yield. Straw yield is highly influenced by the type of species, location even across years for wheat and some triticale cultivars. Considerable differences of up to 57% more straw yield were determined between the highest and the lowest yielding cultivars across years [17].

Cultivar and location variations in agronomics, such as grain and straw yields, sugars content and recalcitrance/processability of the straw, are attributes of importance to be assessed in triticale cultivars grown in South African agro-ecological conditions. Assessment of such attributes will facilitate the search for top quality, high performing and more stable cultivars with superior traits. Expected variability in straw from triticale cultivars, as found in straw from other cereals such as wheat, may be weighted sensibly to meet high grain productivity or sustained 1G ethanol yield potential, as most of the current high yielding grain triticale cultivars tend to be poor straw yielding or vice-versa [24]. Site and season related grain yield variations [25] and cultivar × locations interaction variation in grain and specifically straw yield have been found for triticale cultivars [9]. Thus, suitable selection of the preferred cultivars may be based on little or even no compromise between high straw yield, straw quality and grain yield for improved 2G ethanol production per hectare. This will be to the benefit of growers and will promote triticale straw as a future bioethanol feedstock. Additionally, the search for the highest yielding triticale cultivars could also take into consideration the biological

cultivar stability across sites. This is a useful trace of genotype adaptability to different environments in order to better predict performance [26].

In view of this background, this study sought to assess the effects of cultivar selection and site on sugars yield from triticale. This was achieved by combining data obtained from field trials of triticale cultivars, performed by the Department of Genetics (Stellenbosch University), with experimental measurements of straw composition and responses to pretreatment hydrolysis. The combination of these datasets enabled the selection of preferred cultivars to achieve maximal ethanol production through superior traits. The findings presented here provide a foundation for future breeding programs of triticale cultivars in the search for desirable end-quality attributes of straw for bioethanol production.

4.2 Materials and methods

4.2.1 Raw material and sample preparation

4.2.1.1 *Raw material*

Triticale (× *Triticosecale* sp. Wittmack) straw grown on three locations in South Africa in 2009 was used for the study. The field trials and collection of the straw material were performed by the Department of Genetics at Stellenbosch University. Straw from twenty sprint cultivars that generated sixty straw samples (20 cultivars × 3 locations) was used for the study. For convenience of referencing, the identification of the cultivars was rearranged to numbers 1-20. The technical cultivar entries, respective identifiers, specific geographical locations, soil type and growing season precipitation are given in Table 4-1. The plants were rain fed and no fertilizer was applied. The cultivars were selected to represent the most commonly used cultivars at the time of the trials in the year of plantation. All trials were designed as randomized block designs with four replicates.

Table 4-1: Cultivar entries, geographic locations and agroclimatological information.

Cultivar entry	Identification used in this work	Cultivar entry	Identification used in this work
US2010	1	27ITYN32	11
US2009	2	27ITYN36	12
US2008/AgBeacon	3	27ITYN39	13
US2007	4	98T376	14
IBIS	5	00T196	15
REX	6	US2007-5	16
BACCHUS	7	36ITYN27	17
TOBIE	8	36ITSN139	18
01T43	9	00T207	19
37ITSN43	10	04T74	20

Location				
Name	Latitude	Longitude	Elevation	**Soil type**
Mariendahl	33.7166° N	18.6333° E	42	Sandy/loamy sand
Overberg	34.3333° N	19.6666° E	221	Sandy/ windblown
Swartland	33.2166° N	18.8166° E	94	Shale/gravel/granite

4.2.1.2 *Harvesting of grain and collection of straw*

The grain of all field trials was harvested at time of reaching ripeness and after harvesting the grain the straw was chopped manually. The straw material from each trial was baled after the harvesting process, labelled and taken to the research facility.

4.2.1.3 *Sample preparation of straw*

The moisture content of the straw material as received (AR) differed mainly due to location of origin. The average AR of straw from Mariendahl, Overberg and Swartland was 6.7, 8.6 and 12.3%, respectively. The material was stored in a temperature and moisture controlled room set at 20°C and relative humidity of 65% for no longer than 12 months until needed for pretreatment. Before preparing the material for analysis, the extraneous (foreign) matter present in each bale was manually separated from the straw and the material was homogenised in defined lots. Preparation of the material was carried out by coarsely grinding the straw in a Condux-Werk type mill (Wolfgong bei Honou, Germany) and sieved to obtain particle sizes between 3.8-10 mm. The grinded material was suitable for further milling in a laboratory ultra-centrifugal mill model ZM200 basic (Resch GmbH, Germany) to obtain particle size between 425 and 825 µm used for the pretreatment study. In addition, a particle size retained on the 80-mesh sieve (-20/+80 mesh fraction), was used for compositional analysis.

4.2.2 Pretreatment

Pretreatment was carried out at 0.6% (w/w) of acid concentration at 180°C and 10 min. The pretreatment condition was chosen to give a pretreatment severity of 3.36, as a combination of moderate temperature and time, ensuring relatively mild conditions that enable the detection of differences in pretreatment response between straws. This combination of pretreatment temperature and residence time has been reported to result in high yields of overall fermentable sugars from the combined process of pretreatment followed by enzymatic hydrolysis [27;28].

The process was started by soaking 1.5 g of prepared straw dry raw material (DRM) in acid solution using 50-ml Corning tubes and holding it overnight at room temperature. Prior to pretreatment, the pre-soaked material was vacuum-filtered and then for pretreatment loaded in Hastelloy tubular reactors (18 cm long and 1.27 cm of internal diameter) at 30% solid loading. Two fluidized sand baths were used to heat the tubular reactors up to the target temperature and perform pretreatment, minimizing the effect of thermal transients in the reactors, as described elsewhere [29]. The heat up time in the baths varied between 1 and 2 min, which was not included in the stated residence time for pretreatment. After pretreatment, the reactors were rapidly quenched by submersion into a cold water bath. The cooling time (drop in the reactor temperature from hot to a temperature of about 30°C) varied between 1–2 min and it was not included in the stated residence time either. The slurry was removed from the reactors by pushing out the treated solids with a piston-like metal rod; the reactor washed out with 100 ml of distilled water, the washing liquid collected and mixed with the solid fraction and finally vacuum-filtered (Whatman GF/F-pore size 0.7 µm, Piscataway, NJ) to separate the solids from the liquid. Since the released sugars after pretreatment in the liquid fraction were considered as part of the yield of combined sugars, the diluted filtrate was collected, volumetrically measured and stored at 4°C for further analysis of sugars. The treated solids were washed with distilled water using a weight of volume equal to 10 times the biomass weight used for pretreatment. These treated solids are subsequently referred to as water insoluble solids (WIS). All experiments were performed in duplicate, with average data reported.

DA in tubular reactors was the reactor configuration used in the present dissertation due to its advantages over other configurations. Tubular reactors are more effective to ensure uniform temperature across the tube diameter, which is met by the use of tube reactors with diameter of less than 0.5 inches [30]. Pretreatment is generally carried out over a temperature range of approximately 140–180°C when dilute sulfuric acid is used, or from approximately 170 to 220°C when water only is employed [30]. In this study, batch tubular reactors of total volume of about 14.3 ml made from 0.5

in. OD × 0.035 in. wall thickness Hastelloy C276 tubing (developed by Charles E. Wyman's research group at the Center for Environmental Research and Technology (CE-CERT) of the Bourns College of Engineering, University of California, USA) were used (See Figure 4-1). The biomass (1.5 gram of dried prepared material) was firstly soaked overnight in a 30 ml volume of the targeted dilute acid concentration or water (in case of zero acid concentration). The excess of water was squeezed out by filtration to a final solid loading of 30% (w/v) prior to being loaded into the reactors. A two-heated fluidized sand bath system that provides high heat transfer rates, for rapid heating of the batch reactors, was used (See Figure 4-1, Insert B). An ice-bath to cool down the reactor contents by submerging them in an ice-bath was also included. Batch tube pretreatments were run at least in duplicate so that the solids and liquid from one of the tubes could be used for analysis of sugar recovery and compositional changes of the solid substrate during pretreatment. In addition, this also provided treated material to study the effect of pretreatment on cellulose digestibility by enzymatic hydrolysis.

Figure 4-1: (A) Bench-scale tubular reactors used for dilute-acid pretreatment of triticale straw. (B) Dual sand-bath heating system to perform bench-scale pretreatment.

The presence of acid during pretreatment causes the release of monomeric and oligomeric sugars, by improving the reactivity of the biomass while changing the pH of the aqueous environment where pretreatment takes place. Severity of DA is then calculated based on the acidity of the aqueous phase (generally after pretreatment) by the combined severity expression (Subsection 2.2.2.3). Depending on the combined severity of the pretreatment, the sugars can be converted to aldehydes such as furfural and HMF.

4.2.3 Enzymatic hydrolysis

The effect of pretreatment on the enzyme accessibility of the pretreated material was assessed by enzymatic hydrolysis of WIS for all the straw samples. The enzymatic assays were carried out using 24-ml screw-on cap glass tubes loaded with 200 mg (dry basis) of WIS, 10 ml of 0.05 M citrate buffer (pH 4.8), sodium azide (antimicrobial, final concentration of 0.2 $g.L^{-1}$) and two commercial enzyme preparations [31]. The enzymes used were Spezyme CP (Genencor-Danisco, Denmark) with protein concentration of 140 mg/ml (cellulase activity of 65 FPU/ml) and Novozym 188 (Novozymes A/S, Denmark) with protein concentration of 95 mg/ml (β-glucosidase activity of 700 IU/ml). Protein concentration and enzymatic activities of the commercial enzymes (undiluted) were assayed by following the methodology described by Ghose (1987) [32]. Spezyme CP was added at an enzyme loading of 32.31 mg protein/g WIS equivalent to 15 FPU/g WIS, and Novozym 188 was loaded at 2.02 mg protein/g WIS (corresponding to 15 IU/g WIS). The experiments were carried out under agitation at 90 revolutions per minute in a waterbath shaker held at 50°C for 72 h. The samples withdrawn after 72 h were analyzed for sugars concentration by High Performance Liquid Chromatography (HPLC) as described below. All enzymatic assays were run in duplicate and reported as averaged values.

4.2.4 Chemical composition determination and analysis

The starch content of the grain was determined by the Department of Genetics using the Total Starch Assay Kit (Megazyme International Ireland Ltd.). The compositional analysis of the straw was done on air-dried samples following two-step acid hydrolysis of the carbohydrates according to the National Renewable Energy Laboratory (LAPs-NREL) procedures for determination of structural carbohydrates and lignin in biomass [33]. Ash content determination in the raw material was carried out by calcination of 1.5 g extractive-free sample at 575°C in a furnace for 4 h following the NREL procedure for determination of ash in biomass [34]. Released sugars from acid hydrolysis, pretreatment and enzymatic hydrolysis were quantified for monomeric sugars (glucose, xylose and arabinose) by HPLC as described below. The glucan, xylan and arabinan contents in raw material were determined by applying the following conversion factors: (0.95 × cellobiose + 0.9 × glucose), 0.88 × xylose and 0.88 × arabinose, respectively.

The concentration of sugars in the liquid fractions was quantified on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). The conditions for running the column, mobile phase characteristics, type of detector used and flow are described elsewhere [29].

4.2.5 Calculations

The yields of xylose, glucose and arabinose in the liquid streams (pretreatment liquor and enzymatic hydrolysis) were calculated according to expression (4-1).

$$Sugar\ yield_{(monosaccharides)} = \frac{C_{sugar} \times V}{RM} \times 100 \quad (4\text{-}1)$$

Where *sugar yield* denotes either the yield of xylose, glucose or arabinose in monomeric form expressed in grams per 100 g DRM, C_{sugar} is the concentration as either monomers of xylose, glucose, or arabinose (in $g.L^{-1}$), *V* is the total volume (in litres) of the washing liquid collected and measured after pretreatment, and *RM* is the weight of raw material (in grams) used for pretreatment (on dry basis).

The yield of total sugars (combined sugars) was calculated as the sum of the measured monomeric sugars in the pretreatment liquor (arabinose, xylose and glucose) and the same sugars after enzymatic hydrolysis of the WIS at the solid loading of 2% (w/v).

Ethanol yield from starch potential in litres per mega-gram (L· Mg $^{-1}$) = starch content (%) in dry matter × grain dry yield (kg· ha^{-1}) × 0.567 (conversion factor of ethanol from starch, g of ethanol per g starch) × 10/0.789 (specific gravity of ethanol, kg· L^{-1}). (4-2)

Ethanol yield potential from cellulose and hemicellulose **in the straw** ***(L·Mg^{-1})*** = (Glucose content (%) × 0.44 (conversion factor of ethanol from glucose)) + ((Xylose) content (%) × 0.44) (conversion factor of ethanol from C5-sugars)) × 1000/0.789 (specific gravity of ethanol, kg·L^{-1}) × 1000/0.789 (specific gravity of ethanol, g·ml^{-1}). (4-3)

Straw to grain ratio: The ratio of straw to grain for triticale cultivars was calculated as straw yield ($kg.ha^{-1}$)/ grain yield ($kg.ha^{-1}$). (4-4)

Theoretical ethanol yield from released sugars from combined pretreatment and enzymatic hydrolysis (L·Mg^{-1}) = (Glucose yield (g.100 g^{-1}DRM) × straw yield (kg.ha^{-1}) × 0.44 (conversion factor of ethanol from glucose (g of ethanol.g^{-1} glucose)) × 100/0.789 (specific gravity of ethanol, kg·L^{-1}) + ((Xylose) yield (g.100 g^{-1}DRM) × straw yield (kg.ha^{-1}) × 0.44 (conversion factor of ethanol from C5-sugars (g of ethanol.g^{-1} of C5-sugars)) × 100/0.789 (specific gravity of ethanol, kg·L^{-1})/straw yield (Mg.ha^{-1})). (4-5)

Cultivar and site variability in yields of grain and straw were estimated from the average agronomic data supplied by Department of Genetics, Stellenbosch University.

4.2.6 Statistical analysis

Pairwise comparison analysis and one-way-analysis of variance (ANOVA) at a significance level of 95% were performed on the experimental agronomic data of grain and straw yields and chemical composition of the straw, as well as the sugars yield after pretreatment and enzymatic hydrolysis to determine whether the observed differences between cultivars and locations of origin were statistically significant. The statistical analysis was carried out using Design Expert software version 8.0.3 (State Ease Inc., Minneapolis, United States).

4.3 Results

Variability in agronomic properties was assessed. These included overall biomass yields (grain and straw yields), sugars content in biomass materials (starch from grain, and cellulose and hemicellulose from straw), and the accessibility of such sugars in straw for release by the combined pretreatment and enzymatic hydrolysis process. Cultivars with significantly higher sugar yields were identified, and cultivar and site-related variations with statistical significance were determined. The impact of the agronomic properties on improved ethanol yield per hectare was also studied.

4.3.1 Grain and straw yields

Grain yield, as the primary feedstock for ethanol production from triticale as cereal, was considered for selection of the cultivar which provides the highest areal ethanol yields. Cultivar selection was thus aimed at maximising ethanol yield per hectare from straw, without compromising on the best observed performance in ethanol yield per hectare from grain. Figure 4-2 shows the average yields of grain and straw for each of the cultivars across sites of study. Considerable variations

in grain and straw yields among cultivars and sites were found. Grain yield was in the range 2.8 - 4.3 $Mg.ha^{-1}$ among cultivars. The cultivars with the highest yields of grain were 9, 10, 11, 12, 14, 15 and 18, situated in the Overberg site with outputs above 3.9 $Mg.ha^{-1}$. Specifically cultivars 9 and 18 showed the maximum values of 4.3 and 4.2 $Mg.ha^{-1}$, respectively (Figure 4-2). Consistent superior performance in grain yield of cultivars 9, 10 and 11 on Mariendahl and Overberg sites was observed, although these cultivars demonstrated nearly 10, 8 and 5% lower yields respectively on Swartland (Figure 4-2). Average grain yield per location was higher on Overberg and Mariendahl sites with 3.7 $Mg.ha^{-1}$ and 3.5 $Mg.ha^{-1}$, respectively, whilst Swartland site gave the lowest average yield of 3.3 $Mg.ha^{-1}$. In general, only 20% of the samples originated in Swartland resulted in yields higher than 3.4 $Mg.ha^{-1}$ and the remaining 80% with superior yield was evenly provided by Mariendahl and Overberg sites.

Average straw yields were 0.99 (±0.35), 2.09 (±0.22) and 2.76 (±0.47) $Mg.ha^{-1}$ for Mariendahl, Overberg and Swartland, respectively. Variations in the straw yields between cultivars and sites (maximum 3.33 Mg ha^{-1}) were higher than for grain yields (maximum 1.5 Mg ha^{-1}), with straw yields varying from as low as 0.43 $Mg.ha^{-1}$ (cultivar 7, Mariendahl) to 3.76 $Mg.ha^{-1}$ (cultivar 2, Swartland). Straw yields exhibited a clear dependency on site of origin, which was more significant than the impact of cultivar on straw yield. For example, cultivar 7 resulted in the lowest straw yield (0.4 $Mg.ha^{-1}$) in Mariendahl, while it resulted in a relatively high yield of 3.42 $Mg.ha^{-1}$ in Swartland. Specifically, the highest yielding straw cultivars were associated with Swartland with cultivars 2, 7 and 14 providing straw yields of between 3.21 and 3.76 $Mg.ha^{-1}$. Similarly, cultivars 4, 5, 10, 15, 16, 17, 18 and 19, with outputs of between 2.89 and 3.13 $Mg.ha^{-1}$, were also cultivated in the Swartland site.

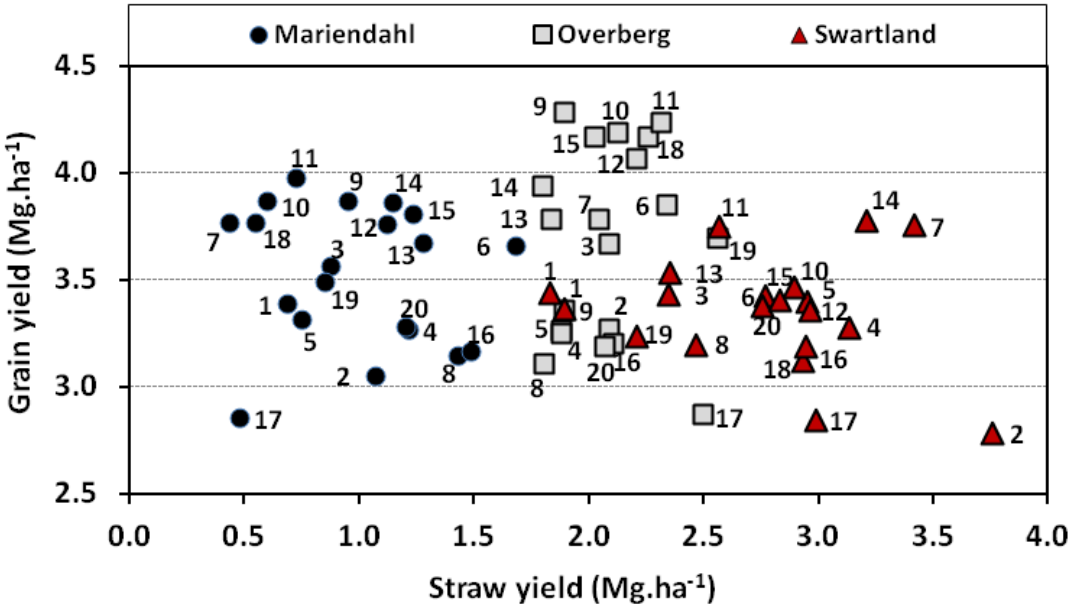

Figure 4-2: Mean values of grain and straw yields expressed in Mg.ha-1 for all of the triticale samples in the trials per cultivar and site of origin. Numbers 1-20 next to the marker symbol on each site correspond to the code label for the particular cultivar.

The top 5 cultivars in terms of straw yield in the Mariendahl region were 6, 8, 13, 15 and 16 (1.23-1.68 Mg.ha^{-1}). Straw yields in the Overberg site ranged from 1.8 to 2.56 Mg.ha^{-1} and the best performers in straw output in this location were cultivars 6, 11, 17, 18 and 19 as shown in Figure 4-2 Swartland, as the site with the highest straw yields, had the major number of cultivars (70% of the cultivars) with yields above 2.57 Mg.ha^{-1} (Figure 4-2).

Straw yield of cultivars within a particular site (maximum variation of 0.52 Mg.ha^{-1}) showed less variability than differences in grain yields between cultivars (maximum variation of 1.13 Mg.ha^{-1}). High grain yields across multiple locations were observed for cultivars 7, 10, 11 and 14. Some cultivars such as 9 and 15 showed greater variability in grain yields between different locations, but with high yields of 3.8 and 4.2 Mg.ha^{-1} at Mariendahl and Overberg respectively. These specific cultivars yielded less than 3.4 Mg.ha^{-1} of grain when cultivated in Swartland. Low grain yields across sites were observed for cultivars 1, 3, 4, 5, 8, 13, 16 and 17 (Figure 4-2). On the other hand, little cultivar variability in straw yield (relatively steady yield) for straw samples 1, 9 and 11 was observed within the Overberg and Swartland sites (Figure 4-2).

High grain yield is always a desirable trait in the selection of cereal cultivars for ethanol production. However, preference is given to cultivars that can combine high grain yields with high straw yields, to

benefit 2G ethanol production and provide additional income for farmers. Thus, cultivars with high grain yields in multiple locations in combination with higher straw-to-grain ratios are of interest. Variations in yields between cultivars and sites led to considerable variations in the ratio of mass yields between straw and grain. Straw: grain ratios between 0.12 and 1.35 were observed between cultivars and between 0.28 and 0.83 as site-specific averages for variations (Figure 4-2). Cultivars 2, 4, 7, 15, 16, 17 and 18 gave straw: grain ratios above 0.9. Particularly cultivar 2 cultivated in Swartland, yielded the highest straw-to-grain ratio among all of the cultivars with a value of 1.35. Cultivars 16 and 17 both showed consistently high ratios across sites, but consistently very low grain yields (Figure 4-2). Considerably low straw output on average in the Mariendahl site led to the lowest straw to grain ratios per site with 0.28 on average for this location (Figure 4-2). Cultivars grown in the Swartland site provided the highest straw: grain ratios, with an average of 0.83 per site, nearly 30% higher than the average ratio obtained for Overberg. Cultivars 7 and 14 originated in Swartland presented high straw: grain ratios of 0.91 and 0.85 respectively at similarly good grain yields (> 3.75 $Mg.ha^{-1}$) (Figure 4-2). The agronomic data of grain and straw yield was used to establish any correlation among cultivars and locations. However, neither grain yield among cultivars nor sites were found correlated to straw yield (R^2 of 0.03 for analysis between cultivars and R^2 of 0.02, 0.005 and 0.04 for analysis of cultivars at specific sites Mariendahl, Overberg and Swartland, respectively).

4.3.2 Chemical composition of straw samples from triticale cultivars

Chemical composition. The chemical composition of the sixty straw samples on a dry weight basis is shown in Figure 4.3, as average values of three replicate measurements per sample. Straw composition was calculated as grams of the measured component per 100 grams DRM, which is equivalent to percentage (w/w). Table 4-2 summarizes the statistics of the chemical composition analysis performed on the straw from the cultivars and sites under study. Glucan was the major carbohydrate in straw composition which ranged between 33.5 and 38.7 $g.100\ g^{-1}$ DRM, followed by arabinoxylan which ranged from 20.6 to 25.8 $g.100\ g^{-1}$ DRM. Xylan was the major C5 sugar (up to 89%) of the entire arabinoxylan measured. Lignin (insoluble) and total extractives (water + solvent extractives) varied from 11.2 to 17.7 $g.100\ g^{-1}$ DRM and from 6.3 to 19.3 $g.100\ g^{-1}$ DRM, respectively, whilst ash content ranged from 1 to 6.3 $g.100\ g^{-1}$ DRM among cultivars.

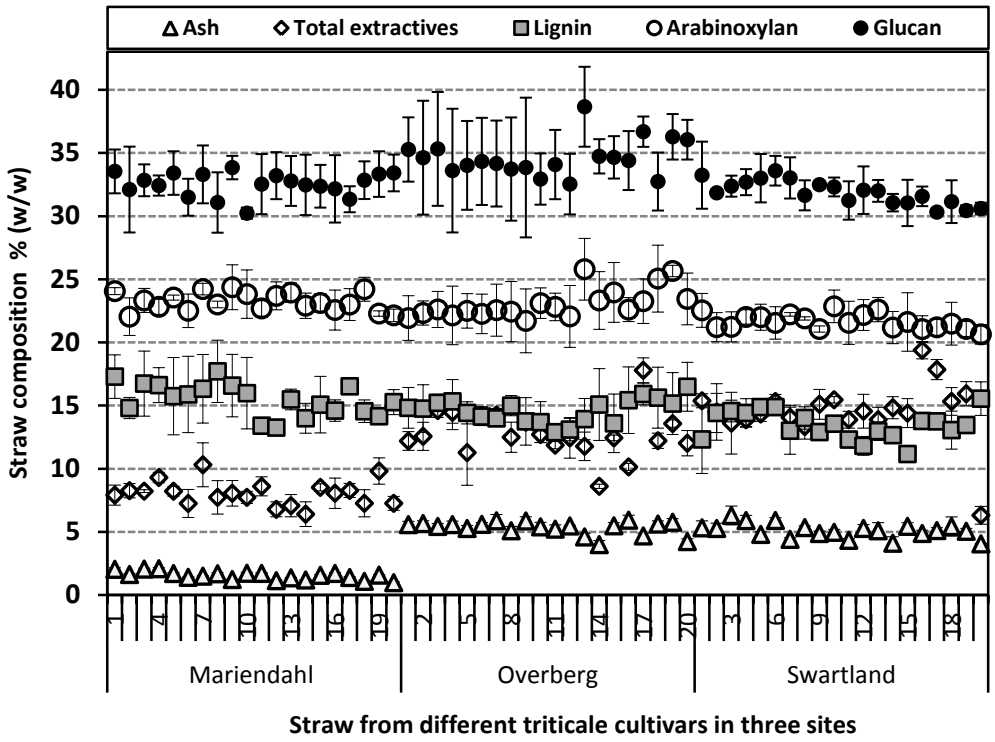

Figure 4-3: Mean of the chemical composition of straw from 20 triticale cultivars in the trials per cultivar and site of origin. Error bars correspond to the variation of three replicates.

ANOVA analysis performed on triticale straw composition to establish significant differences between the means of compositional analysis among cultivars and sites (See Table 4-3), showed similarities on average glucan content between cultivars ($P > 0.05$, Table 4-3). Even though little site-related variations in glucan composition were observed between straw samples, significantly lower glucan content, below 30.6 g.100 g^{-1}DRM (Figure 4-3), was statistically found for straws 17 ($p < 0.001$), 19 and 20 ($p < 0.01$) when cultivated in Swartland (Table 4-3). Cultivar- and site-related variabilities in arabinoxylan were also minor and mostly due to variations in arabinan content (Table 4-3). The total content of structural carbohydrates in the straws, as measured in monomeric form by the analysis method, was estimated at 64.5 g.100 g^{-1} DRM on average for all the cultivars and sites, and neither cultivar- nor site-specific differences in carbohydrate content were found to be statistically significant.

Table 4-2: Statistics summary of chemical composition of straw from 20 triticale cultivars grown at three sites.

Statistics	Site																							
	Mariendahl								Overberg								Swartland							
	Ash	**EtW**	**EtS**	**Lig**	**Xyl**	**Ara**	**Glu**	**TS**	**Ash**	**EtW**	**EtS**	**Lig**	**Xyl**	**Ara**	**Glu**	**TS**	**Ash**	**EtW**	**EtS**	**Lig**	**Xyl**	**Ara**	**Glu**	**TS**
Mean[a]	1.5	6.0	2.1	15.5	21.2	2.0	32.5	62.5	5.3	10.9	1.9	14.6	20.8	2.2	34.6	64.6	5.1	13.4	1.5	13.5	19.9	1.8	31.9	60.0
Min[a,b]	0.9	4.2	1.2	12.9	18.9	1.4	28.6	55.6	3.7	6.4	1.0	11.1	17.1	1.6	28.5	53.7	3.5	10.2	0.5	8.9	18.0	1.2	29.4	55.0
Max[a,c]	2.2	10.4	3.4	20.6	24.0	2.7	35.4	67.9	6.3	17.1	2.5	18.7	29.0	3.0	45.0	86.0	7.1	18.9	2.5	17.1	22.0	3.5	35.9	68.4
SE[d]	0.02	0.14	0.04	0.15	0.07	0.02	0.12	0.19	0.04	0.16	0.03	0.13	0.14	0.03	0.22	0.39	0.05	0.13	0.03	0.11	0.07	0.03	0.10	0.19
SD[e]	0.32	0.08	0.50	2.08	0.99	0.27	1.67	2.60	0.58	2.13	0.37	1.80	1.90	0.34	2.95	5.18	0.66	1.76	0.46	1.47	0.98	0.38	1.34	2.57
Skew[f]	0.10	1.18	0.67	1.00	0.20	0.20	-0.26	-0.33	-0.86	0.13	-0.54	0.63	1.28	0.40	0.57	0.81	0.34	0.76	-0.19	-0.39	0.18	2.06	0.66	0.63
n[g]	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

[a] Expressed in grams per 100 grams of dry raw material. [b] Minimum. [c] Maximum. [d] Standard error. [e] Standard deviation. [f] Skewness. [g] n= 20 cultivars (per location). EtW: water-extractives. EtS: solvent-extractives. Lig: lignin. Xyl: xylan. Ara: arabinan. Glu: glucan. TS: total sugars available in raw material in form of glucan + arabinoxylan.

Table 4-3: Mean square and level of significance for chemical composition of straw from variance analysis on cultivar and site effects.

Source of variation		DF	Mean square						
			Ash	**Et-W**	**Et-S**	**Lignin**	**Xylan**	**Arabinan**	**Glucan**
Mariendahl	Cultivars	19	0.30***	2.55***	0.24ns	4.75ns	1.68ns	0.14*	3.21ns
	Error	40	0.004	0.54	0.25	4.10	1.07	0.14	3.56
Overberg	Cultivars	19	0.88***	11.88***	0.35***	2.77ns	4.31ns	0.18ns	7.83ns
	Error	40	0.083	1.01	0.02	3.45	4.82	0.13	12.13
Swartland	Cultivars	19	1.02***	6.99***	0.41***	3.80**	0.98ns	0.11ns	3.40*
	Error	40	0.146	1.27	0.11	1.37	1.34	0.21	1.65
			Mean square						
Site		2	**Ash**	**Et-W**	**Et-S**	**Lignin**	**Xylan**	**Arabinan**	**Glucan**
Cultivar	1		11.67***	47.07***	1.61**	18.52ns	4.32ns	0.031ns	0.76ns
	2		14.84***	37.83**	0.39ns	0.13ns	1.21ns	0.025ns	8.73ns
	3		15.08***	42.36**	0.57ns	3.72ns	3.31ns	0.097ns	9.30ns
	4		13.40***	30.10***	0.35*	3.66ns	0.43ns	0.130ns	1.42ns
	5		11.25***	40.37**	1.20*	1.35ns	1.80ns	0.05ns	0.96ns
	6		18.97***	68.38***	0.59ns	2.31ns	0.17ns	0.32ns	8.01ns
	7		14.91***	18.84**	0.53*	8.73ns	2.75ns	0.54*	1.30ns
	8		12.50***	29.48***	0.37ns	10.98ns	0.85ns	0.15ns	7.19ns
	9		17.86***	44.67***	0.07ns	11.12ns	9.49ns	0.31ns	2.27ns
	10		12.09***	52.82***	0.28*	5.55ns	1.02ns	0.24ns	7.41ns
	11		9.95***	24.59***	0.19ns	0.90**	0.47ns	0.55*	7.51ns
	12		18.05***	59.81***	0.78*	1.88ns	3.93ns	0.09ns	1.23ns
	13		12.29***	35.31***	0.25*	4.81*	8.14ns	0.16ns	49.05ns
	14		8.14***	58.24***	0.16ns	4.42ns	3.36ns	0.43ns	12.69ns
	15		15.18***	32.22***	1.08***	11.67ns	3.17ns	0.40ns	12.35ns
	16		14.44***	132.9***	1.24*	2.02ns	1.46ns	0.56ns	8.26ns
	17		12.39***	91.14***	0.13ns	6.38**	2.76ns	0.39ns	43.31***
	18		19.72***	51.75***	0.12ns	4.98ns	8.78ns	0.70*	3.34ns
	19		15.03***	44.06***	1.71**	2.18ns	13.33***	1.07***	31.53**
	20		10.01***	30.99**	0.33ns	1.22ns	4.80ns	0.64**	27.53**

Et-W and Et-S stand for water- and solvent extractives, respectively. Significant values are expressed as *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and ns (not significant at $p > 0.05$. DF refers to degree of freedom, for site variation is 2 for all of the cultivars.

Cultivar- and site-related variabilities in lignin content of straw samples were generally not statistically significant. Significantly lower lignin contents were observed for cultivars 11 ($p < 0.01$), 13 ($p < 0.05$) and 17 ($p < 0.01$), while the lignin content of straw samples were lower on average in Swartland ($p < 0.01$) (Table 4-3). No significant variations in lignin contents of straw samples from the other cultivars and sites were observed. Therefore, with only a few exceptions, a lot of similarities were found in the carbohydrate and lignin contents between cultivars and locations. This resulted in the ratio of structural carbohydrate to lignin also not varying considerably between cultivars (3.8-5.3) or locations (3.8-4.8, 4.3-5.2 and 5.1-5.3 for Mariendahl, Swartland and Overberg, respectively). In addition, no correlations between carbohydrate and lignin composition in straw were found, either between cultivars or sites.

Highly significant variations in ash and water-extractives content ($P < 0.001$) between cultivars and sites were found by Pairwise analysis (Table 4-3). Statistical analysis on site-related variations in ash

content revealed Mariendahl to be the lowest ash content site ($p < 0.001$) on average among locations at 1.5 g.100 g^{-1} DRM. Overberg and Swartland also showed significantly different ($p < 0.001$) ash content on average between sites with values of 5.3 and 5.1 g.100 g^{-1} DRM, respectively (Table 4-3). Cultivar 20 showed significantly lower ash content ($P < 0.001$, Table 4-3) compared to other cultivars, with 1.0 g.100 g^{-1} DRM as the lowest value when cultivated at Mariendahl and consistently low content between sites ($P < 0.01$, Table 4-3). Consistently low ash content was also observed for cultivar 14 with comparable ash content to cultivar 20 on average at each site (Table 4-3). Straw from cultivars 6, 9, 12, 13, 17 and 18 cultivated in the Mariendahl site presented ash content below 1.4 g.100 g^{-1} DRM and significantly ($P < 0.05$) higher content than cultivars 14 and 20. The only exception was cultivar 9 which did not differ significantly from cultivars 14 and 20 in ash content (P values of 0.0185 and 0.124 respectively of cultivar 9 against cultivars 14 and 20) as shown in Table 4-3.

Cultivars 12, 14, 6, 13 and 20 were the lowest ash content cultivars, and also exhibited the lowest water extractives content (4.7 - 5.4 g.100 g^{-1} DRM). Site-related variation in water-extractives was also determined by Pairwise analysis. Similarly to ash content, Mariendahl site resulted in the lowest values among sites with 6 g.100 g^{-1} DRM, 1.8- and 2.2-fold significantly lower content ($P < 0.001$) than Overberg and Swartland sites, respectively (Table 4-3).

Solvent extractives were much lower than water extractives and ranged 0.5 – 3.4 g.100 g^{-1} DRM among cultivars. Significant differences ($P < 0.05$), primarily cultivar-related over site, were determined (Table 4-3). Data on extractives content (water and solvent) in straw did not show any correlation with grain and straw yields.

4.3.3 Processibility of the straw samples from different cultivars and sites, measured as combined sugar yields from pretreatment-hydrolysis steps.

Figure 4.4 shows the combined sugar yields from the processing steps of pretreatment and subsequent enzymatic hydrolysis, per 100 grams of raw material on dry basis, for all of the cultivars and sites. Table 4-4 provides the statistical summary of each response.

Table 4-4: Statistics summary of yields of sugars (xylose, glucose and total sugars) and theoretical ethanol (from glucose and total sugars) of straw from pretreatment followed by enzymatic hydrolysis of twenty triticale cultivars grown at three sites.

Yield															
Statistics	Mariendahl					Overberg					Swartland				
	Xylose[d]	Glucose[e]	Total Sugars[f]	Areal ethanol potential		Xylose[d]	Glucose[e]	Total Sugars[f]	Areal ethanol potential		Xylose[d]	Glucose[e]	Total Sugars[f]	Areal ethanol potential	
				from glucose[g]	from xylose[h]				from glucose[g]	from xylose[h]				from glucose[g]	from xylose[h]
Mean[a]	14.8	22.1	40.8	130.4	84.0	13.0	18.0	34.8	223.0	164.7	10.3	21.6	34.4	348.1	163.0
Minimum[a]	12.2	13.4	31.9	52.8	34.4	14.1	13.5	28.9	164.0	137.2	6.5	12.6	25.1	206.1	115.2
Maximum[a]	17.7	30.5	47.8	197.6	149.8	14.9	26.7	45.3	325.9	205.7	15.4	28.3	42.6	518.9	223.6
SD[b]	1.4	4.3	4.2	43.4	32.2	1.07	3.11	3.78	47.5	21.6	2.27	3.96	4.54	90.4	28.5
SE[c]	0.22	0.67	0.67	9.7	7.2	0.17	0.49	0.60	10.6	4.8	0.36	0.63	0.72	20.2	6.4
Skewness	0.29	-0.05	-0.23	-0.13	0.27	-0.01	0.77	0.65	0.99	0.71	0.58	-0.47	-0.36	0.04	0.36

[a] Expressed in grams per 100 grams of dry raw material. [b] Standard deviation; n= 20 cultivars (per location). [c] Standard error. [d] released from pretreatment. [e] released after enzymatic hydrolysis. [f] Total sugars calculated as the sum of monomeric yields of arabinose, xylose and glucose in pretreatment liquor and xylose and glucose after enzymatic hydrolysis. [g] Ethanol yield from glucose from pretreatment and EH in $L.ha^{-1}$. [h] Ethanol yield from total sugars glucose, xylose and arabinose from pretreatment and glucose and xylose from EH expressed in $L.ha^{-1}$.

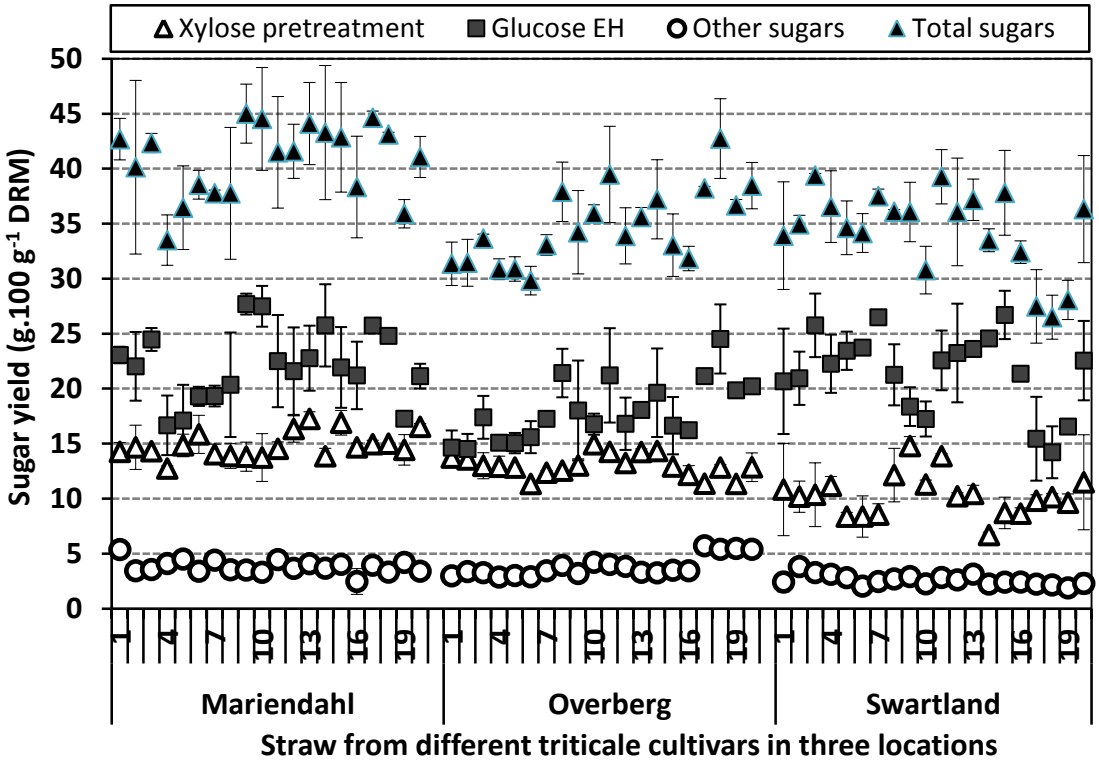

Figure 4-4: Yields of monomeric sugars after dilute-acid pretreatment (180°C, 10 min, 0.6 (% w/w) H_2SO4) followed by enzymatic hydrolysis at 15 FPU/g WIS for straw from 20 triticale cultivars in the trials per cultivar and site of origin. Other sugars refer to yields of glucose from pretreatment and xylose from enzymatic hydrolysis.

Xylose yield. Total xylose recovery, that is the total xylose recovered from pretreatment and enzymatic hydrolysis, ranged between 33.4 and 71.3% of the content in untreated straw, of which more than 90% was obtained from pretreatment (Figure 4-4). Xylose yield from pretreatment varied between 6.7 and 17.3 g.100 g^{-1} DRM for different cultivars (xylose recovery of 30.2 – 70.6%), and varied on average between 10.3 and 14.8 g.100 g^{-1} DRM for different sites (Table 4-4). Significant differences in xylose yields among cultivars and locations ($P < 0.05$) were observed (Table 4-3). The highest xylose yields from pretreatment were observed for the Mariendahl site, with an average value of 14.8 g.100 g^{-1} DRM (Table 4-4), corresponding to a xylose recovery of approx. 62%, which was nearly 10 and 25% higher that xylose recoveries obtained at Overberg and Swartland sites, respectively. Cultivars 13 and 15, as the cultivars with the highest pretreatment yields of xylose, gave values above 16.9 g.100 g^{-1} DRM (Tabl4 4-4). Significantly ($P < 0.05$) lower yields were obtained with straw samples from cultivars 1, 3, 9, 10, 11, 14, 17 and 18, with an average yield of 14.5 g.100g^{-1} DRM and no significant differences between them ($P > 0.05$). The release of xylose after pretreatment was significantly influenced by location ($P < 0.012$). Straw originating from Mariendahl released higher

amounts of xylose on average compared to Overberg and Swartland sites, which showed statistically comparable ($P > 0.05$) xylose yields.

An inverse correlation (polynomial of grade 2) between the yield of xylose from enzymatic hydrolysis (g.100 g^{-1} of straw) and straw yield (Mg.ha^{-1}) for all of the straw samples across the sites was observed in this study ($R^2 = 0.647$). Therefore, more EH xylose yield was released from the studied cultivars at the sites with lower yield of straw (Mariendahl and Overberg) as shown in Figure 4-5.

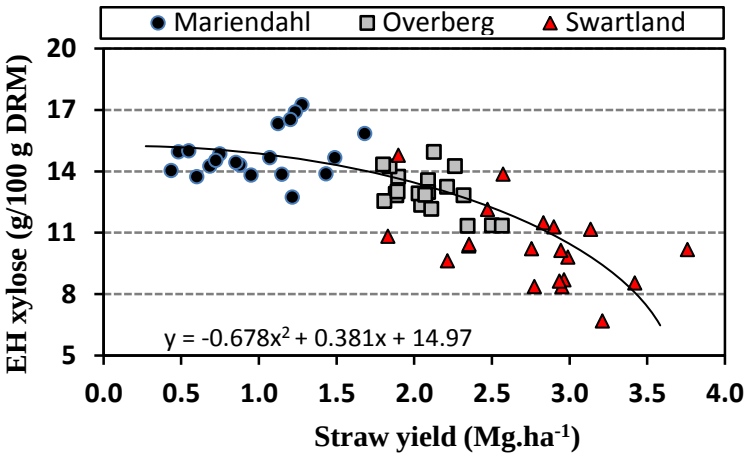

Figure 4-5: Relationship between straw yield (expressed in ton per hectare) and yield of xylose (expressed in gram of sugar per 100 grams of dry raw material) from dilute-acid pretreatment (180°C, 10 min, 0.6 (% w/w) H2SO4) for straw from 20 triticale cultivars in the trials per cultivar and site of origin.

***Glucose yield*.** Glucose yield from enzymatic hydrolysis (EH glucose) was the major sugar fraction yielded from the combined process of pretreatment and enzymatic hydrolysis. The release of glucose from pretreatment represented up to 8.9% of the total glucose yield obtained from the combined process with yields between 0.56 and 2.7 g.100 g^{-1} DRM between cultivars. Glucose yields from pretreatment varied between 0.92 and 1.9 g.100 g^{-1} DRM within locations. On the other hand, EH glucose varied between 14.2 and 27.7 g.100 g^{-1} DRM among cultivars. Site-related variations in EH glucose yields were lower and ranged between 18 and 22.1 g.100 g^{-1} DRM on average (Table 4-4). The cultivars with the highest EH glucose yields were 3, 9, 10, 13, 14, 17 and 18 with statistically comparable ($P > 0.05$) average yields of 25.5 g.100 g^{-1} DRM (yields recorded with straw samples from Mariendahl).

Variations in EH glucose yields between sites were observed, and especially for the highest yielding glucose cultivars 6, 10, 17, and 18. Specifically, cultivar 10 gave significantly higher (P 0.0095) EH

glucose yield in Mariendahl compared to Overberg and Swartland. Cultivars 17 and 18 yielded comparable average glucose yield in Mariendahl and Overberg, but significantly higher than in Swartland (*P* 0.0421 and 0.0306, respectively). Conversely, a significantly higher EH glucose yield (*P* 0.009) was obtained for cultivar 6 in Swartland. The EH glucose yields represented recoveries of 40.7 – 98.1% between the various cultivars, based on the glucose content of straw samples. Mariendahl and Swartland sites showed comparable average recoveries of glucose from EH at nearly 73%, which was nearly 28% higher on average than from Overberg (Table 4-4). Despite the fact that Mariendahl site resulted in straw with high EH glucose yield (good processibility) compared to straw from Overberg, the significantly lower straw yield in this specific site impacted negatively on the available glucose yield that can potentially be released per hectare of triticale. Consequently, Mariendahl site displays a high compromise between better processability and straw resource per hectare.

Total sugars. The yield of total (combined) sugars, calculated as the sum of glucose and xylose yields from both pretreatment and subsequent enzymatic hydrolysis, is shown in Figure 4-4. The combined sugars yield varied between 26.5 and 45 g.100 g^{-1} DRM for all of the cultivars and sites. Straw from Mariendahl yielded consistently more sugars on average at 40.8 g.100 g^{-1} DRM compared to that of Overberg and Swartland (~34.6 g.100 g^{-1} DRM on average, Table 4-4). Thus, straw originating from Mariendahl also provided the highest recoveries between sites with 65.5% of total sugars recovered, whilst Overberg and Swartland sites resulted in recoveries of 53.8 and 57.3% on average, respectively.

Pairwise Analysis performed on the yield of total sugars for all the straw samples revealed little cultivar variability. The analysis per site showed that total sugars yield differed statistically within cultivars at the specific site Overberg (*P* 0.0006) but no cultivar variability was found between cultivars within Mariendahl (*P* 0.1846) or Swartland (*P* 0.0676) sites. Straw from cultivars cultivated in Mariendahl gave the highest yields of total sugars on average (40.7 g.100g^{-1} DRM). Cultivars 9, 10, 13, 17 and 18 from this specific site was a cluster of straws with comparable total sugars yield ($P < 0.05$) which was also the highest for the site, even though these cultivars were significantly lower ($P < 0.05$) in prevalence compared to the other cultivars in the site. These referred cultivars with the highest yields of total sugars also gave the highest recoveries of total sugars of above 66% between straw samples.

The search for the cultivars with the highest yields of ethanol led to the selection of cultivars 1, 3, 9, 10, 11, 13, 14, 15, 17 and 18 with superior performance in total sugars output on average per 100 g DRM that resulted in yields above 41.6 g.100 g^{-1} DRM (See Table 4-5).

Estimated 1G and 2G ethanol yields potential for triticale cultivars

The total potential ethanol yields per hectare were calculated by combining the potential ethanol yields from grain starch (estimated from starch content; 1G ethanol yield) with the potential ethanol yield from release of sugars from straw by the combined pretreatment-hydrolysis process (both glucose and xylose measured experimentally; theoretical yield of 2G ethanol), as shown inFigure 4-6. The potential yield of 1G ethanol per Mg of grain harvested was estimated by combining the maximum theoretical ethanol yield (0.51 g.g^{-1} glucose [35]) with the measured release of glucose from grain starch. Grain starch composition showed little variation among cultivars and sites of origin. Average starch content in grain varied from 59.1 to 65 g.100 g^{-1}DRM between cultivars, with corresponding estimates in the maximum potential ethanol yield of between 424.5 and 467.1 L.Mg^{-1}. All the sites gave average starch content, for grains obtained from that particular site, of 62 g.100 g^{-1} DRM. Therefore, the absence of variation observed in starch content led to little variation in ethanol yield per Mg of grain across sites (443.2 L.Mg^{-1} on average for all of the cultivars and sites).

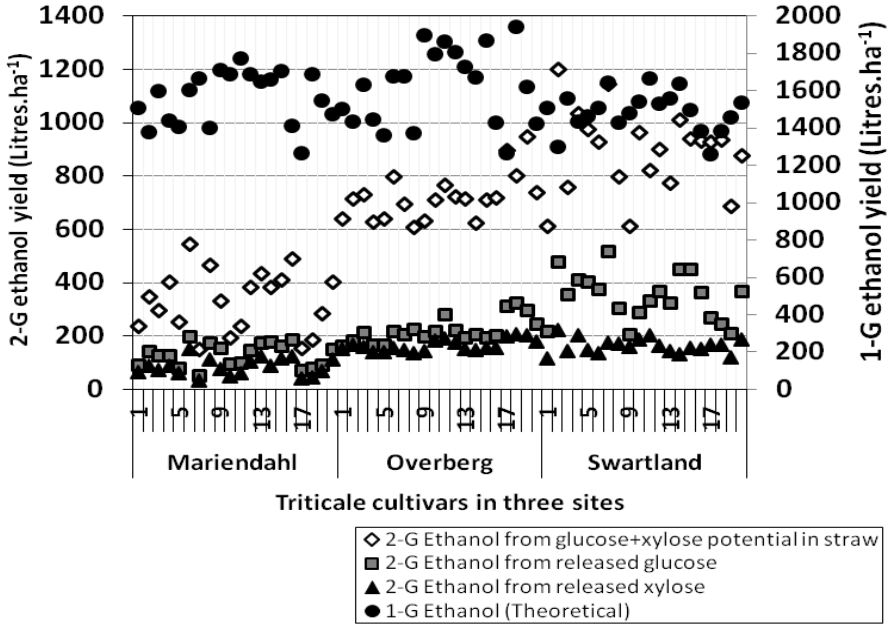

Figure 4-6: Mean values of estimated ethanol yield from starch grain (1G theoretical) and potential glucose and xylose in straw (2G theoretical). Estimated ethanol yields from glucose and xylose potential after pretreatment-enzymatic hydrolysis of straw (2G pot potential) is also given for the triticale cultivars. Cultivars appear in numerical order 1-20 per site.

Table 4-5: Averaged yields of released sugars (pretreatment - enzymatic hydrolysis) for the cultivars with the highest yields of sugars and averaged areal ethanol outputs for the cultivars with the highest yields of areal ethanol.

Cultivars with the highest yields of sugars per Mg^{-1} straw (Mariendahl-originated)										
Parameter	**Cultivars**									
	1	3	9	10	11	13	14	15	17	18
Xylose-pretreatment	14.3±0.85[a]	14.3±0.02[a]	14.8±0.89[a]	14.9±0.00[a]	14.5±0.62[a]	17.3±0.64[b]	14.3±0.00[a]	16.9±1.11[b]	15.0±0.36[a]	15.0±0.00[a]
EH-glucose	23.1±0.75[a]	25.8±2.89[a]	27.7±0.95[a]	27.5±1.86[a]	22.6±2.71[a]	23.6±0.67[a]	25.8±6.74[a]	26.7±2.19[a]	25.7±0.14[a]	24.8±0.59[a]
Total sugars	42.7±1.89[ns]	42.3±0.87[ns]	45.0±2.68[ns]	44.5±4.67[ns]	39.5±2.47[ns]	44.1±3.73[ns]	43.3±6.1[ns]	42.9±4.98[ns]	44.6±0.61[ns]	43.1±0.20[ns]
Ethanol ($L.Mg^{-1}$)	260±11.4[ns]	259.6±5.83[ns]	277.4±16.04[ns]	274.40±4.68[ns]	254.0±32.2[ns]	268.3±23.5[ns]	266.4±40.1[ns]	260.6±33.5[ns]	273.6±3.45[ns]	264.3±1.70[ns]

Cultivars with the highest yields of 2G ethanol per hectare (Swartland-originated)										
Parameter	**Cultivars**									
	2	4	5	7	11	12	14	15	18[1]	20
Ethanol ($L.ha^{-1}$)	704.5±25.3[a]	617±63.8[a,b,d,e,f,i]	551.5±45.3[b,d,e,f,g,i]	695.1±11.1[a,c,]	539±41.6[d,e,f,g,h,i]	534.5±86.3[e,f,g,i]	582.4±20.6[f,g,i]	605.8±69.7[a,g,i]	532±53.5[h]	554.4±149.0[i]
P site-related var.	0.0016	0.0026	0.0012	< 0.0001	0.0048	0.0215	0.0051	0.0106	0.0026	0.0722
Grain compromise	Very high	High	High	No	No	Low	Low	Low	No	Low
Ethanol yield ($L.ha^{-1}$) across other sites for the cultivars with the highest areal yields of ethanol										
Mariendahl	230.6	215.8	145.0	87.2	160.3	250.5	267.2	282.7	127.4	265.0
Overberg	347.5	309.7	309.5	357.6	473.7	396.3	357.2	353.8	414.8[2]	425.3
Improved ethanol yield[2]	50~67	50~65	44~73	48~87	12~70	26~53	38~54	41~53	22~76	23~52

Sugars (Xylose-pretreatment, EH-glucose and total sugars) are given as yield in gram of sugar per 100 gram dry raw material. Average ± standard deviation. The values in each row having similar superscript letters do not differ between each other at a significance level of 0.05. *P* site-related var. refers to the *P* value for significance of site-related variation at 95% of confidence.

[1] Cultivar 18 Overberg-originated. [2] Averaged value for Swartland site.

[2] Refers to the improved ethanol yield of the cultivars with the highest yields in Swartland, except for cultivar 18 which is Overberg-originated, with respect to the ethanol outputs in other sites, expressed in percentage.

The potential 1G ethanol yields per hectare were also estimated and ranged from 1264.8 to 1942 $L.ha^{-1}$ between cultivars (Figure 4-6) and 1489 – 1621.6 $L.ha^{-1}$ on average between sites. These observed variations in potential ethanol yields were primarily due to differences in grain yields (Figure 4-2), as the starch content in grains remained fairly constant. Overberg site demonstrated a higher potential 1G ethanol yield on average with 1622 $L.ha^{-1}$; nearly 4 and 8% more than yields on average in the Mariendahl and Swartland regions, respectively. In particular, grain yield for cultivars 9, 12 and 18 seemed to be negatively influenced by Swartland specific conditions, resulting in lower grain yields and consequently 1G ethanol yield at this specific site, as shown in Figure 4-2 and Figure 4-6, respectively.

Maximum 2G ethanol yield per Mg of straw was estimated. This corresponds to the theoretical 2G ethanol yield that could be obtained per Mg of straw, based on its glucose and xylose composition (0.44 g ethanol/g sugar consumed). Maximum 2G ethanol yield ranged between 310 and 388.5 $L.Mg^{-1}$ between cultivars. However, no statistically significant differences in maximum 2G ethanol yield were found by Pairwise analysis ($P > 0.05$); neither between cultivars nor across sites. Xylose composition alone made up between 35.4 and 41% of the maximum 2G ethanol yield. Thus, the potential xylose content in straw was demonstrated to contribute considerably to improved sugars yield, if it is accounted for.

Total 2G ethanol yield per Mg of straw, estimated from glucose and xylose released by the combination of pretreatment and subsequent enzymatic hydrolysis, varied in the range 140.9 – 242.8 $L.Mg^{-1}$ between cultivars. Variations of 177.8 - 242.8, 159.7 - 229.5, and 140.9 - 212.3 $L.Mg^{-1}$ were observed for the sites Mariendahl, Overberg and Swartland, respectively. Despite these variations, Pairwise analysis revealed no statistically significant differences in total 2G ethanol yield per Mg of straw either across cultivars nor across sites ($P > 0.05$), possibly due to the high standard deviation in the supplied experimental data.

Total 2G ethanol yield per hectare was also estimated based on glucose and xylose released from the combined process pretreatment-hydrolysis (0.44 g ethanol/g sugar consumed) and straw resourced per hectare. The statistics summary of the areal cellulosic ethanol potentially resourced for all of the cultivars across the sites of study is given in Table 4-4. Generally high cultivar- and site-related variations in straw yield (Figure 4-2), greatly impacted on cellulosic ethanol potentially resourced per hectare, as determined by the analysis among cultivars and across sites. Estimated minimum and maximum total ethanol yields for all of the cultivars were 87.2 and 742.5 $L.ha^{-1}$ (total of

minimum and maximum yields of areal ethanol yield from glucose and xylose in Mariendahl and Swartland sites, respectively, Table 4-4). A noticeable influence of site-related variation in straw yield on areal ethanol yield was observed.

Estimated areal 2G ethanol yield potential from glucose and xylose in straw composition was also calculated (Figure 4-6). This was calculated in order to quantify the proportional recovery of 2G ethanol potential after pretreatment – enzymatic hydrolysis of the maximum potentially (theoretically) achievable. Figure 4-5 shows that a tendency was observed of Mariendahl sourced straw being more amenable after the combined process with a resultant potentially higher 2G ethanol production. On average 65.1% of the total potential (theoretical) ethanol available in the straws on Mariendahl site may be recovered. On the other hand, the lowest recovery on average was estimated for Overberg (53.5%), whilst Swartland site averaged 57.2%.

Variability related to site was observed for the estimated 2G ethanol yield per hectare ($P < 0.005$) for all of the straw samples except for cultivars 1 and 20 (P 0.0907 and 0.0722, respectively). The statistical analysis indicated that estimated 2G ethanol yield from cultivar 1 remained steady across sites whilst 2G ethanol yield from cultivar 20 was significantly lower in Swartland compared to other sites ($P < 0.0325$). Swartland- originated straw gave the highest areal 2G ethanol yield of 511 $L.ha^{-1}$ among locations, representing improved areal productivity of 25 and 59% compared to estimations for Overberg and Mariendahl sites, respectively. The cultivars 2, 4, 5, 7, 11, 12, 14, 15, 18 and 20 were the highest areal 2G ethanol yielding cultivars (prominent trait of favourable 2G ethanol yield). Swartland was the preferred location of origin for these cultivars, with the exception of cultivar 18, which showed superior performance in the Overberg site. Table 4-5 shows the average outputs of areal ethanol for the highest areal 2G ethanol yielding cultivars and the compromise with grain production expected in these cultivars. The compromise between sugar yields from straw and grain productivity was rated for the selected cultivars. This was done according to the expected reduction in grain yield for the cultivar at the site of selection, in comparison to the output determined on the other sites.

The preferred cultivars would give significantly higher ($P < 0.05$) 2G ethanol yields per hectare, above 530 $L.ha^{-1}$, compared to non-preferred cultivars. The highest estimated 2G ethanol yield of 704.5 ± 25.3 $L.ha^{-1}$ was obtained for cultivar 2 from straw cultivated in the Swartland site. However, even though cultivar 2 showed very good performance in 2G ethanol yield, very low grain yield of 2.8 $Mg.ha^{-1}$ was also associated with this cultivar at the specific Swartland site (Figure 4-2). Thus, selection

of straw sample 2 as one of the preferred cultivars resulted in a large compromise between the yields of areal 2G ethanol and grain. Similarly, preferred cultivars 4 and 5 resulted in large compromise between 2G ethanol yield per hectare and grain output, as fairly stable but yet low grain yield was observed at the specific sites for both cultivars (Figure 4-2). On the other hand, selection of straw samples 7, 11 and 12, as preferred cultivars with high 2G ethanol yield, demonstrated little or even no compromise between yields of areal 2G ethanol and grain. This is due to the relatively stable but yet high grain yield of these referred cultivars at the three studied sites, as shown in Figures 4-2 and 4-4. In the case of the selection of cultivars 14, 15 and 20, the highest 2G ethanol yield per hectare was only associated to Swartland. In this case, little compromise between areal 2G ethanol yield and grain yield represented little compromise at this particular site while other sites gave lower 2G ethanol yields and higher grain yields, as shown inFigure 4-2. Cultivar 18 also was selected as a sample with high 2G ethanol yield per hectare. However, its superior performance in 2G ethanol yield representing low compromise with grain yield, was associated only with the Overberg site with better grain yield (4.2 $Mg.ha^{-1}$) for this specific cultivar (Figure 4-2).

The total ethanol yield (1G + 2G) per hectare ranged between 1379 and 2474 $L.ha^{-1}$ on average for all of the cultivars. Cultivar variability in the potential total ethanol yield (1G + 2G) per hectare was high although site-related variability was even higher. Site-related variation was more accentuated on Mariendahl with the lowest average yield of 1778 $L.ha^{-1}$, whilst ethanol productivity in Overberg and Swartland sites did not show much divergence between them with average values of 2010 and 2000 $L.ha^{-1}$, respectively. As a result of the observed trends in areal ethanol yield potential from straw, the highest total ethanol yielding cultivars were found to be 2, 4, 7, 10 and 14 originated in the Swartland site, with outputs of above 2609 $L.ha-^{1}$. These cultivars were also found to be the highest 2G ethanol yielding cultivars, with the exception of cultivar 10.

4.4 Selection of straws for further study under pretreatment optimization (second selection)

A second selection was performed on the screened straws to choose the final top 5 samples, with selected samples covering all the mega-regions of study. The selected straws underwent pretreatment optimisation at bench-scale (Chapter 5), with the aim to determine the specific pretreatment requirements and these cultivars' responses to pretreatment in terms of total fermentable sugars yield as well as the effect of site on these parameters. In this way straws with superior processability in terms of sugar productivity and pretreatment requirements would be revealed for additional differentiation. The criteria for selection were based on pretreatment performance in terms of sugars

recovery and additionally on sensible criteria for the selection that covered farmers' preference for the cultivars and commercial status. These criteria were applied under straw availability constraint due to larger requirements of straw material needed for the planned second stage of pretreatment optimisation at pilot-plant scale. Table 4-6 summarises the desirable traits of the top 10 selected straws according to their higher estimated total ethanol output (1G + 2G), better processability in terms of sugars yield after pretreatment under constraint to straw availability for further study.

Table 4-6: Straw from top 10 cultivars selected for further pretreatment optimisation stage

Straw	Ethanol yield ($L.ha^{-1}$)-rank	Pretreatment sugars[(1)] -rank	Availability	Sample for further study
O-18[2]	2473.6 (1)	42.7 (5)	**Limited**	Not selected
S-7	2339.4 (2)	37.5 (8)	**Plenty**	**Selected**
O-11	2336.1 (3)	39.5 (6)	**Limited**	Not selected
O-10	2199.1 (4)	35.9 (9)	**Limited**	Not selected
S-15	2103.3 (5)	37.8 (7)	**Limited**	Not selected
S-6	2024.4 (6)	34.2 (10)	**Limited**	Not selected
M-15	1985.9 (7)	42.9 (4)	**Limited**	Not selected
M-13	1952.5 (8)	44.1 (2)	**Plenty**	**Selected**
M-9	1941.6 (9)	45.0 (1)	**Plenty**	**Selected**
M-14	1925.6 (10)	43.3 (3)	**Plenty**	**Selected**

(1) Expressed in g/100g of raw material (dry basis).
(2) No straw from Overberg could be selected into the top 10 selection due to the little availability of the material; instead the sample O19 with no major differences in pretreatment was selected.

Straw from cultivars with good pretreatment response, ethanol yield and in sufficient amount to undergo further pretreatment optimizations were selected from each particular site from the top 10 cultivars given in Table 4-6. This selection aimed at evaluating the best performer straws from each site to observe differences in total sugars yield between straws covering all the mega-regions as well as as identify preferred sites for maximum sugars and ethanol from straw. Three straws from Mariendahl with technical entries *01T43*, *27ITYN39*, and *98T376* (M9, M13 and M14) and one straw sample from each of the locations Overberg (straw sample with entry *00T207* corresponding to O19) and Swartland (entry *BACCHUS* corresponding to straw S7) were selected. Pretreatment optimisation with dilute-acid at bench-scale of these straws was performed on maximising total fermentable sugars from the combined process pretreatment-enzymatic hydrolysis. A clear differentiation in straw processability regarding sugars yield and pretreatment response between straws from different sites was observed; in general straws originated in Mariendahl gave higher total fermentable sugars compared to straw from the other two sites. The methodology and results of the pretreatment

optimisation performed at bench-scale for the straws M9, O19 and S7 are given in Chapter 5 to illustrate those referred differences in processability between straws from different sites. The results of the bench-scale optimisations for straw samples M13 and M14 are omitted to facilitate comparisons in straw performance between sites; even though these results are explained and compared to those obtained at pilot-plant optimization in chapter 7.

4.5 Discussion

Triticale straw yield was found to be strongly influenced by location and to a lesser extent by cultivar. Straw yield was also more influenced by location in comparison to grain yield. Site specific influences of soil-climatic growth conditions may possibly help to explain the greater grain yield variability observed between sites. Average grain yield in the Swartland region was lower than average yields in Mariendahl and Swartland where soils of the shallow sandy type are found. Shale-based soils, as in Swartland, are characterised by permanent draining, which is facilitated by the layers of shale and results in there being less water available for plant intake. Grain yield in wheat has been found to be negatively influenced by low water use and especially soil water loss and water use efficiency due to excessive rain early or late in the crop growth period [36]. Nitrogen uptake has also been found to be an influencing factor [37]. Grain yield has been found to be site-dependent in some triticale cultivars and biological instability of cultivars in specific locations has been associated with poor adaptation to low organic matter soils and semi-arid conditions [7;9].

Mariendahl gave the lowest average straw yield, at a relative high variability between cultivars, compared to other sites. However, the highest variability in straw yield between cultivars was observed in the Swartland site (Figure 4-2). Ability of the cultivars (robustness) to keep straw yield stable across sites was not observed whereas robustness in grain yield across sites was only observed for cultivar 10. Lesser variability in straw yield within locations was found in the Overberg site which was 1.6 and 2.5-fold less variable than Mariendahl- or Swartland- originated straws, respectively. This stability observed in straws from Overberg could be derived from favourable environmental conditions, such as better quality of soil and plenty precipitation during the season on this specific location, which resulted in a generally better and steady cultivar output. Environmental factors such as location of origin and, to a large extent, weather conditions have been found to have a large influence on straw yield in cereals such as wheat, barley and oats [38]. The observed general variability in straw yield between cultivars across sites may infer a possible cultivar × location interaction or the presence of genotypic features that are influenced by environmental conditions to a large amount [9]. In the light of inferring cultivar robustness as an indication of the adaptability by the

cultivar, the influence of environmental conditions in Swartland and Mariendahl on those assumed cultivar × location interactions/genotypic features was greater which result in less adapted cultivars than in Overberg (Figure 4-2). These influences were also observed in the cultivars with the highest straw yields (Table 4-5).

In the present study, no general association between grain and straw yields neither within cultivars nor at specific sites was observed. For example, cultivar 7 gave high grain yield (3.76 $Mg.ha^{-1}$) and low straw yield (0.43 $Mg.ha^{-1}$) compared to others cultivars in the Mariendahl site (Figure 4-2) while cultivars 16 and 20 showed low grain yields (below 3.4 $Mg.ha^{-1}$) and high yields of straw (above 1.2 $Mg.ha^{-1}$) in the same site (Figure 4-2). Although cultivar 7 showed fairly stable grain yield across sites (~3.4 $Mg.ha^{-1}$), very high straw yield was observed at Swartland site (3.4 $Mg.ha^{-1}$) in contrast to the poor straw yield observed in Mariendahl for this cultivar (Figure 4-2). Thus, negative associations between yields of grain and straw reported for triticale cultivars [26] or even positive correlation as found for wheat cultivars [17] were not observed for the cultivars under study. Straw-to-grain ratio varied significantly as a result of high yield variations of grain and straw. Mariendahl gave the lowest average ratio of 0.3 and Swartland the highest value of 0.8.

Straw-to-grain ratios of around 2.47 have been reported on average for some triticale cultivars at Mediterranean climate conditions and in limestone/chalky clay soil types [39]. Average straw-to-grain ratio within cultivars across the sites in this dissertation corresponded to 0.56. This ratio was in close agreement with the average value of 0.52 reported for triticale grown in sandy, clayey soil and in a combination of Atlantic and continentally influenced climate in Denmark with temperatures below 24°C over the whole year [17]. This shows the important effects of specific climate and geographical conditions on biomass yield in triticale.

Triticale was found to be very steady in carbohydrate composition: starch, cellulose and to a lesser extent hemicellulose. In general, straw samples from various triticale cultivars exhibited comparable sugars composition regarding major sugars (glucan + arabinoxylan), with few exceptions. Cultivars with traits of potentially higher carbohydrate content could therefore not be identified for the field trials and growing/harvest season evaluated in this study. Cultivar-related variation in cellulose and hemicelluloses composition and site-related variation in hemicellulose composition have been reported for wheat cultivars grown on sandy, clayey soils in a specific growing season [19]. Site-related variability in hemicellulose would also be expected in triticale cultivars evaluated, as found for wheat straw, since the local growing conditions differed considerably between sites [19].

Despite the fact that little cultivar variation in lignin content of straw was observed for triticale, straw originated in the Swartland site showed the lowest average lignin composition (Table 4-3). Thus, little variation in the carbohydrate to lignin ratio was also observed for the triticale cultivars. In contrast, ash and water extractives content differed among cultivars. Thus, differences in straw composition were mainly related to differences in ash and extractives composition and to a minor extent to lignin content. Variation in ash content of straw was found to be highly site-related for the triticale cultivars under study. The expression of 73-80% less ash content in straw was possible when cultivars were grown in the Mariendahl site. Thus, the Mariendahl site, with sandy type of soil, seemed to result in the lowest ash content of triticale biomass (Table 4-3). Similar effects of type of soil on ash composition have been observed for switchgrass when grown on sandy soils with a reduction of ash content of 51-73% [40]. Ash content is associated with mineral uptake by the plant and consequently local environmental conditions, such as type of soil and climate of the growing season, would have a major impact on cultivars [41]. However, the cultivar-specific variability observed for ash composition could possibly be associated with variation in the physiological function of mineral uptake by the cultivars [41] which is reflected in cell wall structure.

A clear association between ash content in straw and the yield of total sugars per site was observed. Straw originated in Mariendahl was more amenable to pretreatment – enzymatic hydrolysis and up to 62% of the xylose and 73% of the glucose recovery potential in straw were achieved by pretreatment and enzymatic hydrolysis, respectively Table 4-4). Thus significantly more combined sugars were recovered from Mariendahl straws. Therefore, Mariendahl was the preferred site for cultivars due to the highest yields of combined sugars obtained there (Table 4-5). The better overall sugar release (better processability) observed for cultivars with reduced ash content in straw, may have been the result of the lower neutralising capacity, which has been found to improve the effectiveness of sugar release by dilute acid pretreatment of wheat straw [19], corn stover, poplar and switchgrass [42]. The selection of cultivars with lower ash/lignin contents as attributes for improved straw quality would also result in higher sugars yield at milder pretreatment conditions and lower enzyme dosages as combined economic benefits [43].

Sugars release from the combined process, pretreatment – hydrolysis, was studied on triticale as basis for the selection of cultivars with traits for improved ethanol output. Variations in total sugars release were mostly attributed to site (Mariendahl for cultivars with lower ash content in straw resulting in improved response to pretreatment) as a result of environmental influences on cultivar-related variations. Estimated 2G ethanol yield from total potential sugars yield resourced per hectare

was conclusive in the final selection of top performer cultivars for improved areal bioethanol outputs. Selection of cultivars with the highest areal ethanol yields resulted from the systematic combination of agronomic measurements with the response to pretreatment - hydrolysis based on total sugars yield.

It was clearly observed that environmental conditions (site-related influences) were predominant over triticale cultivar variability for the expression of lower ash content in straw, as a desirable feature for maximum sugar release per Mg^{-1} of straw. Thus, selection of a preferred site demonstrating both the highest potential ethanol yield per Mg of straw and overall biomass yield (grain + straw) could not be achieved. It is consequently expected that cultivars with the highest sugars yield per Mg of straw (preferred cultivars with the highest sugar yields, (Table 4-5) would provide considerably lower sugars resourced per hectare and vice-versa as a result of the high influence of variations found in straw yield across sites.

Cultivars that met high 2G ethanol output potential with high grain yield per hectare, or accordingly high total ethanol potential as grain starch, were selected as preferred cultivars (Table 4-5). The selected top 2G ethanol cultivars would potentially be able to yield an estimated 530 to 700 litres of 2G ethanol per hectare, embodying up to 87% improvement in ethanol yield as an outcome of the specific cultivar-site selection (Table 4-5).

Selection of top cultivars with improved 2G ethanol yield under low compromise between yields of 2G ethanol and grain was possible. On the other hand, larger variation in straw yield, particularly observed in Swartland compared to other sites, had a large influence on the data and led to the high compromise between ethanol yield and grain yield observed for the top cultivars 2, 4 and 5 (Table 4-5). Yields of sugars from straw and areal ethanol of cultivars 11, 14, 15 and 18 were highly influenced by site, which also resulted in high compromise between yields of grain and areal ethanol. However future work on maximising total ethanol yield per hectare from these referred cultivars may be advantageous as a result of better performance showed in Mariendahl or Swartland sites. Conversely, cultivar 2 presented superior areal 2G ethanol output but limited grain yield. Thus, selection of cultivar 2 as preferred cultivar was made for future work on grain yield improvement. Specific attributes related to locations were also identified. This included the fact that more yields of grain (up to 4.3 $Mg.ha^{-1}$), straw (up to 3.8 $Mg.ha^{-1}$) and total sugars from the combined process pretreatment-hydrolysis (up to 0.47 Mg total sugars.Mg^{-1} straw) could be expected from Overberg, Swartland and Mariendahl, respectively.

Finally, the study on cultivar and site variability carried out in the present study may represent an especially narrow variation where no large geographical, climatic or annual variations were assessed. Therefore, field trials under more robust influences (e.g. larger number of cultivars over extended growing seasons) would be required to corroborate a more stable grain starch and straw composition in triticale cultivars evaluated in this study compared to that of wheat. This could possibly enable the determination of significant variability in the estimated ethanol yield per Mg^{-1} of straw, with emphasis on the ten cultivars with the highest sugars yield per Mg^{-1} of straw (Table 4-5).

4.6 Conclusions

Cultivar and site variabilities in agronomic yields, straw composition and response to pretreatment regarding sugars yields was demonstrated for 20 triticale cultivars grown under South African environmental conditions. Agroclimatological conditions specific to sites had a large influence on the reduction of ash content in triticale straw. The Mariendahl site in South Africa was found to have the environmental conditions necessary to be the preferred region for the expression of low ash content in straw, which was found to be a determinant for improved processability with regards to pretreatment-hydrolysis. Triticale cultivar selection based on agronomic yields of grain and straw coupled with better straw processability can positively impact 2G ethanol yield under no compromise with grain yield.

Acknowledgements

The authors gratefully acknowledge laboratory technicians Solly Solomon at Department of Forestry Science for carrying out the extensive work of sample preparation of all the straw material. Manda Rossouw and Levine Simmers at Department of Process Engineering, University of Stellenbosch are greatly acknowledged for laborious HPLC analysis during the experimentation. The Technology Innovation Agency (TIA), an entity of the Department of Science and Technology (DST) of the Republic of South Africa is also thanked for funding support that made possible this study.

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Chapter 5

5 Optimisation of dilute acid pretreatment for differentiation in maximal combined sugar yield from preferred triticale straw

Large fraction of the information supplied in Chapter 5 is part of the following manuscript submitted to Applied Biochemistry and Biotechnology.

Title: "Optimization of fermentable sugars yield by dilute-acid pretreatment and enzymatic hydrolysis of straw from different triticale (× *Triticosecale* sp. Wittmack) cultivars"

Authors: Roberto A. Agudelo, Maria del Prado Garcia-Aparicio, and Johann Görgens

Objective of dissertation and summary of findings in present chapter

Chapter 5 addresses **Objective 2** after preceding the selection of top cultivars successfully accomplished in **Objective 1**. Results of the present chapter will be applied in **Chapter 6** to refine the selection of top cultivars to straws with better-quality response to pretreatment – enzymatic hydrolysis that guarantee higher sugar output through better bio-convertibility at larger scale.

Abstract

This chapter's goal was to perform pretreatment optimisation of the top five straws from preferred cultivars on the combined sugars yields that enabled a comprehensive cultivar differentiation in response to pretreatment between straw samples for final selection of the top three straws for further evaluation at pilot-plant scale. The preferred five straws from the prior cultivar selection (Chapter 4) were subjected to dilute-acid pretreatment optimization at bench-scale using tubular reactors; this aiming at estimating maximum potential total sugars yield and consequently estimated 2G ethanol yield from preferred cultivars. This pretreatment configuration allows for good temperature control, high solids concentrations similar to those commercially operated, accurate mass closure and the screening of large number of samples. Additionally, dilute-acid pretreatment is an efficient technology to improve enzymatic digestibility and the recover of a high fraction of hemicellulose-derived sugars as takes place during steam explosion pretreatment.

The material corresponded to straw from cultivars originated in three different sites, three straws Mariendahl-originated (*01T43*, *27ITYN39*, and *98T376*), and one straw from each of the locations

Overberg and Swartland (*00T207* and *BACCHUS*, respectively). In this chapter, pretreatment optimisation at bench-scale is shown for the straw samples with entries *01T43* (M9), *00T207* (O19) and *BACCHUS* (S7). The results of the bench-scale optimisations for straw samples M13 and M14 are omitted to facilitate comparisons in straw performance between sites; even though these results are explained and compared to those obtained at pilot-plant optimization in chapter 7. Pretreatment was performed in the range of temperatures 170 - 190°C, acid concentration 0 - 0.6% (w/w) and residence time 6 - 18 min at bench-scale. The monomeric yields of glucose, xylose and arabinose from pretreatment and glucose from enzymatic hydrolysis were totaled, referred to as total fermentable sugars (TFS) and considered as pretreatment response for optimization. In this particular chapter, the release of sugars from pretreatment – enzymatic hydrolysis in the only form of fermentable monomers was studied; other chapters into this dissertation work were based on the measurement of the combined sugars yield, in which not only fermentable monomer sugars but also oligomeric sugars were totaled and referred to as combined sugars yield (CSY). A Central Centred Face Experimental Design (CCFD) under Response Surface methodology (RSM) was applied to develop predictive models for the maximization of the total sugars yield. Straw composition of the preferred samples was in general similar in potential carbohydrate although significant differences in ash content were common for straws from different sites. Straw originated in Mariendahl displayed statistically comparable ash content between cultivars in this specific site ($P \geq 0.05$) although significant lower content compared to cultivars with straw originated in Overberg and Swartland sites ($P < 0.0001$). Significant higher yields of TFS were experimentally observed as well as predicted after optimization for cultivars originated in Mariendahl at lower pretreatment requirements against straws originated in Swartland and Overberg. In order to facilitate comparisons in terms of TFS yields and sites, the straw *01T43* was selected as representative sample from Mariendahl site and its performance was weighted against straws *00T207* and *BACCHUS* from Overberg and Swartland, respectively. The predicted optimum TFS yields from the pretreatment optimization were 50.5, 46.7 and 44.9 g/100 g DRM for the straw *01T43*, *00T207* and *BACCHUS*. These sugar yields represented estimate recoveries of 80.4, 77.8 and 68% of the original TFS content (accounted as xylose + glucose) in the feedstocks, respectively. Straw sample *01T43* that gave the highest TFS yield also showed lower predicted requirements of pretreatment to maximise TFS (182°C, 0.39% w/w and 15.4 min). Predicted pretreatment conditions to maximise TFS yield from straws *00T207* and *BACCHUS* demonstrated to differ with each other predominantly on acid concentration and residence time as optimal conditions were found to be 190°C - 0.53% - 13 min and 189°C - 0.60% - 18.0 min for *00T207* and *BACCHUS*, respectively. These results suggest that improved sugar yield at reduced pretreatment requirements is possible by selection of triticale cultivars grown on Mariendahl site

featuring low ash content in straw and through pretreatment optimization to pick up these attributes in feedstock quality.

Candidate declaration

With regard to chapter 5 page numbers 108-140 of this dissertation, the nature and scope of my contribution were as follows

Name of contribution	**Extent of contribution (%)**
Planning of experiments	60
Executing laboratory experiments	100
Interpretation of results	60
Writing the chapter	100

The following co-authors have contributed to chapter 5 page numbers 108-140 of this dissertation.

Name	**e-mail address**	**Nature of contribution**	**Extent of contribution**
1. Maria del Prado Garcia-Aparicio	Garcia@sun.ac.za	- Planning of experiments - Providing inputs - Interpretation of results - Reviewing the chapter	20 60 10 60
2. Johann Görgens	jgorgens@sun.ac.za	- Planning of experiments - Interpretation of results - Providing inputs - Reviewing the chapter	20 20 60 40
3. Willem Botes	WCB@SUN.AC.ZA	- Experimental field trials -Agronomic measurements -Interpretation of results	100 100 10

Signature of candidate……

Date……

Declaration by co-authors

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 5 page numbers 108-140 in the dissertation,
2. No other authors contributed to chapter 5 page numbers 108-140 in the dissertation besides those specified above, and
3. Potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in to chapter 5 page numbers 108-140 of this dissertation.

Abstract

BACKGROUND: Triticale, a man-made small grain cereal developed by crossing wheat with rye and displaying high straw yield, has gained interest for second-generation fuel ethanol. This study aimed at assessing variability in straw properties from triticale cultivars grown in South Africa and the impact on straw procesability for optimal yield of total fermentable sugars (TFS) from dilute-acid pretreatment followed by enzymatic hydrolysis.

RESULTS: The optimum pretreatment conditions 182°C-0.39% (w/w)-15.4 min maximised total sugar yield with 50.5 g/100 g for straw from cultivar *01T4*3, representing total sugars recovery of 80.4% of original TFS contents (xylose + glucose) in the straws. Conditions of 189°C-0.60%-18 min and 190°C-0.53%-13 min maximised total sugar yield for cultivars *00T207* and *BACCHUS* at 46.7 and 44.9 g/100 g which represented total sugars recoveries of 80.4, 77.8 and 68% of theoretical TFS (xylose + glucose) in the straws, respectively.

CONCLUSIONS: Pretreatment conditions impacted accordingly to ash content as most important feedstock quality feature between straws. A negative association between ash content in straw and glucose yield after enzymatic hydrolysis was observed. The results show that low ash content in straw is highly recommended for improved sugars release at reduced pretreatment requirements by dilute-acid pretreatment-hydrolysis of triticale straw.

Keywords: Cultivar variability; Dilute-acid pretreatment; Feedstock quality; Optimization; Sugar maximization; Triticale straw.

5.1 Introduction

Triticale (× *Triticosecale* sp. Wittmack) is a non-food based cereal crop with high tolerance to drought and pests, good adaptability to environments and low inputs requirements such as fertilizers [1]. More importantly, triticale displays high grain yield and straw potential [2], highly desirable traits for bioethanol production [3]. Thus, triticale straw constitutes a promising feedstock for cellulosic ethanol production, possibly in combination with ethanol from grain. Production and demand for triticale are increasing worldwide with a global production nearly 14.6 million metric tons in 2013 [4]. Several triticale cultivars with specific agronomic characteristics are commercially well-established [5].

Industrial production of cellulosic ethanol requires reduction of production cost, to be competitive with gasoline and first generation bio-ethanol. Different aspects could be targeted to maximise ethanol production per feedstock including cultivar selection, utilization of whole plant, and process optimization. The potential use of triticale straw as bioethanol feedstock would face similar challenges of cultivar variability and agronomic features such as biomass properties, commonly referred to as biomass quality, as found in other cereal species such as wheat [6;7]. Variation in quality of biomass relates to changes in structure and tissue composition of compounds such as carbohydrates, lignin and ash that finally have impact on recalcitrance and limit the availability of the structural carbohydrates for recovery and at last lead to reduced processability of the biomass. Processability encloses the efficacy in deconstructing plant cell walls, and transforming their polysaccharides to fermentable sugars [8]. Variations in biomass quality have resulted in differentiations in process requirements (e.g. pretreatment severities) as found in varieties of sugarcane [9] and similarly is expected in triticale. Generic varieties and agronomics are responsible of possible differences in the response of triticale straws to pretreatment and hydrolysis [6], having also potential for feedstock engineering to minimize processing requirements and costs for ethanol production, while maximizing yields.

Studies on variability in feedstock quality of different herbaceous such as canarygrass and switchgrass [10], maize biomass [11], sugarcane bagasse [9] and wheat straw [7] have found with significant effects on their response to pretreatment; however cultivar variability in straw quality and its influence on the output of sugars from pretreatment-hydrolysis has not been yet assessed for triticale. The variability in responses to pretreatment-hydrolysis by straw from different triticale cultivars provide opportunity for variety-specific optimization of pretreatment conditions, and to select varieties with reduced pretreatment process requirements and costs, while maximizing yields.

Pretreatment is an essential step to make the cellulose in the biomass more accessible and susceptible to enzymatic hydrolysis and provide high yield of monosaccharides for further fermentation. With cellulosic biomass typically containing up to 35% hemicellulose as a sugar heteropolymer the recovery of hemicellulose-derived sugars from pretreatment can contribute to achieve high sugar yields as demanded at industrial levels. Current pretreatment technologies such as alkaline, steam explosion, ionic liquids and dilute-acid differ considerably with respect to pH, temperature, residence time and additives used, and consequently in pretreatment severities and the effects on sugar yields [12].

Pretreatment of cellulosic materials by dilute-sulfuric acid at high temperatures (>160°C) and acid concentrations usually below 4% (w/w) has shown to effectively hydrolyze hemicellulose to xylose and enhance the enzymatic digestibility [13]. Different reactor configurations are currently available to perform dilute-acid pretreatment of biomass [14]. Bench-scale tubular reactors exhibit excellent uniformity in the temperature profile across the reactor diameter, rapid heat-up and are suitable for the use of high solids concentrations comparable to those required at commercial operations [15]. Other advantages include good temperature control and flexibility to perform large number of experiments at low cost as preferred for process optimization over a wide range of pretreatment conditions.

The conversion of total reducing sugars from pretreatment and enhancement of enzymatic digestibility, aiming at identifying pretreatment conditions that maximize sugars from pretreatment or glucose yield after subsequent EH of the treated solids, has been studied for dilute-acid pretreatment of herbaceous materials such as corn stover [16] and wheat straw [17]. On the other hand, improved sugar output can be achieved by targeting the maximization of the "combined" yield of sugars from pretreatment and enzymatic hydrolysis as a compromise of conditions from both stages. This latter approach has been adopted for improved sugar yield from corn stover [18], switchgrass [19] and sugarcane bagasse [20]. Even though extensive work has been done on dilute–acid pretreatment of a variety of feedstocks, to the authors' knowledge no work has been reported in literature for the maximization of the total fermentable sugars yield from dilute-acid pretreatment of triticale straw.

Positioning triticale as second generation feedstock for ethanol production depends greatly upon performance by its straw under leading pretreatment technologies such as dilute-acid. This study aimed at assessing variability in straw composition from triticale cultivars grown in South Africa and the impact on straw procesability for optimal yields of total fermentable sugars (TFS) from dilute-acid pretreatment followed by enzymatic hydrolysis.

Straw from three triticale cultivars originated in three distinctive locations with high potential for bioenergy crops production in South Africa was selected for the study. The straw samples under evaluation showed advantage in attributes per site in a previous stage where a large number of cultivars were screened regarding agronomics, straw quality, response to pretreatment and farmers' preference for commercialization (Chapter 4). Response Surface Methodology (RSM) was applied on TFS maximization from dilute acid pretreatment – enzymatic hydrolysis. Predictive models for

maximal TFS yields were developed that enabled to compare predicted maximal yields and optimal pretreatment conditions between straws to disclose triticale straw with superior response to pretreatment at reduce pretreatment requirements and site differentiation.

5.2 Materials and methods

5.2.1 Raw material and sample preparation

Straw from three triticale (X *Triticosecale* ssp.) cultivars was collected in 2009 and used as raw material. The cultivars under study were grown in the distinctive regions Mariendahl (latitude: 33.7166° N; longitude: 18.6333° E; elevation: 42 masl) with sandy/loamy sand-type of soil, Overberg (latitude: 34.3333° N; longitude: 19.6666° E; elevation: 221 masl) presenting a windblown-type of soil, and Swartland (latitude: 33.2166° N; longitude: 18.8166° E; elevation: 94 masl) with a varied shale/gravel/granite soil type in South Africa. Field trials were crop managed similarly and triticale grain was harvested at the same ripeness before collecting the straw. The straw material from Mariendahl, Overberg and Swartland were coded with the entries *01T43*, *00T207* and *Bacchus*, respectively. The locations of origin were selected as the most representative potential mega-regions for triticale cultivation as second generation bioenergy crops in South Africa. All the straw material was supplied by Department of Genetics, Plant Breeding Laboratory (PBL), Stellenbosch University, South Africa.

After collection the straw was baled, labeled and transported to the research facility for sample preparation. The average moisture content of the straws as received varied from 5.8 to 8.6%. Before preparing the material for pretreatment and analysis, the extraneous (foreign) matter and other anatomical parts different than straw present in each bale were manually separated from the straw and the material was homogenized in defined lots. All the straw material was firstly coarsely grounded with a Condux-Werk type mill (Wolfgong bei Honou, Germany), and sieved to obtain particle size between 3.8-10 mm. This fraction was grinded in a laboratory ultra-centrifugal mill model ZM200 basic (Resch GmbH, Germany) to a final particle size retained between 425 and 825 µm and homogenized according to sampling procedures for further pretreatment. The material for pretreatment was packed in zipped plastic bags and stored in a temperature and moisture controlled room set at 20°C and relative humidity of 65% until needed. The total storage time of the samples did not exceed 6 months.

20-g samples from these lots were convection-oven dried at 40±2°C for 48 h and milled in the same ultra-centrifugal mill using 1 mm screen. The milled material was sieved in a vibratory shaker for 15 min and the fraction retained on the 80-mesh sieve (-20/+80 mesh fraction) was selected for compositional analysis.

5.2.2 Dilute sulphuric acid pretreatment

Dilute sulphuric acid pretreatment was carried out on duplicate in the range of conditions 170 - 190°C, 0 – 0.6 %w/w H_2SO_4 and 6 – 18 min by increments of 10°C, 0.3% (w/w) and 6 min for pretreatment temperature, acid concentration and residence time, respectively. This range of conditions was based on preliminary experimentation at 180°C, 0.6% (w/w) acid concentration and 10 min which facilitated differentiation in TFS yields from pretreatment – enzymatic hydrolysis of a large number of triticale cultivars (data not shown). Hastelloy small tubular reactors (18 cm long and 1.27 cm of internal diameter) were used to perform pretreatment, according to Yang and Wyman [15] and pair of tube reactors was treated similarly. 1.5 gram of dried prepared material was used as experimental unit for pretreatment. The samples for pretreatment were soaked in 30 ml of acid solution at the targeted dilute acid concentration and left overnight. Two sulphuric acid solutions at concentration 0.3 and 0.6 % (w/w) were made from a stock 72 ± 0.1% H_2SO_4 solution and distilled water was used for soaking the material to be tested at 0% (w/w) acid concentration. Soaked samples were filtered to a final solid loading of 30% (w/v) prior to being loaded into the reactors. Pretreatment was carried out by using a sand bath heating system described elsewhere [9]. After the target residence time for each pretreatment condition was passed, the reactors were immediately transferred to an ice water bath to stop the reaction.

The pretreated material was removed from the reactors and washed with 100 ml of distilled water and vacuum-filtered using a Buchner funnel and qualitative filter paper grade 1 (Whatman) to separate the soluble solids from the solid fraction. The liquid fraction containing the soluble solids, referred to as pretreatment liquor, was volumetrically measured and analyzed for monomeric sugars (glucose, xylose, and arabinose) by HPLC as described below. The solid residue was washed with 300 ml of distilled water to remove residual soluble matter. The washed solid fraction is further referred to as water insoluble solids (WIS).

The yield of monomeric sugars from pretreatment was calculated as the amount of glucose, xylose or arabinose released as a percentage of the maximum theoretical release possible of each

sugar; based on the composition of the respective monomeric sugars in the material in dry weight and expressed in gram of glucose, xylose or arabinose per 100 grams of dry raw material (DRM).

5.2.3 Enzymatic hydrolysis

The WIS was used as substrate for enzymatic hydrolysis (EH) to be able to determine the effect of pretreatment on the cellulose accessibility to the enzymes. EH was performed by duplicate using screw-on cap glass tubes of 24 ml of total volume. 200 mg-WIS sample (dry basis) was used with 10 ml of enzyme solution in 0.05 M citrate buffer (pH 4.8) and incubated at 50°C under agitation at 90 rpm in a water-bath shaker for 72 h. The enzyme loading was 15 FPU/g dry WIS of Spezyme CP (protein concentration of 140 mg/ml and cellulase activity of 65 FPU/ml) and Novozym 188 (protein concentration of 95 mg/ml and β-glucosidase activity of 700 IU/ml), obtained from Genencor-Danisco and Novozymes A/S, (Denmark), respectively. After completion, the supernatant was centrifuged, and the supernatant was treated sequentially with PCA 35% (w/w) and 7N KOH for protein precipitation, filtered with nylon filters 0.22 µm and analyzed for cellobiose, glucose, xylose and arabinose by HPLC as described below.

The yields of monomeric glucose, xylose and arabinose after EH were calculated as the amount of the respective monomeric sugars released from hydrolysis, and expressed as percentage of the original glucose, xylose or arabinose content in the straw considering the insoluble solids recovery after pretreatment in the calculations. The original (theoretical) monomeric sugars in the raw material were determined by compositional analysis and were expressed in dry basis.

5.2.4 Chemical composition of the raw material and hydrolysate

The extractives content of untreated biomass was carried out using 5 g (dry basis) of milled straw subjected to successive water and solvent (95% (v/v) ethanol solution) extractions, according to the National Renewable Energy Laboratory (NREL) standard laboratory analytical procedures (LAP) [21]. The carbohydrate and lignin compositional analyses were performed according to NREL procedure for determination of structural carbohydrates and lignin in biomass on 300 mg extractive-free sample subjected to double acid hydrolysis [22]. The resulting hydrolysate was analyzed for glucose, xylose and arabinose by HPLC as described below. The solid residue was oven-dried at 105°C for 24 h and used for acid-insoluble lignin (AIL) analysis by calcination in a furnace at 575°C for 4 h. AIL was determined as the difference between the weight of the sample before and after incineration and

was expressed in percentage [23]. Compositional analysis was performed by four replicates and expressed as average.

5.2.5 HPLC analysis

The hydrolysates from untreated material, pretreatment liquor, and supernatant from EH were analyzed for sugars (cellobiose, glucose, xylose and arabinose) on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) with column temperature set to 65°C, a mobile phase of 5 *mM* H_2SO_4 and a flow rate of 0.6 ml/min, as described elsewhere [9]. The sugar concentrations were measured with a RI detector (Shodex, RI-101) operated at 45°C and determined by comparison against a set of sugar standards with known concentrations.

5.2.6 Statistical analysis

***Chemical composition of straws*.** One way analysis of variance (ANOVA) at 95% confidence interval was performed on the chemical composition data. Carbohydrates, extractives, insoluble lignin and ash contents, as well as the xylose to arabinose ratio were analysed to find statistically significant differences in the means of the composition and degree of hemicellulose branching among straw samples which could derive in compositional variability of the raw material.

Experimental design for pretreatment. A central composite face-centered design (CCCF) into Response Surface Methodology (RSM) was applied for the dilute acid pretreatment to find conditions that maximise the yield of total fermentable sugars (TFS) under optimization. TFS yield was calculated as the sum of the yields of monomeric glucose, xylose and arabinose in pretreatment liquor and the yields of the same monomeric sugars after EH of the washed pretreated solids. The yields of xylose in pretreatment liquor (as major hemicellulose-derived sugar released from dilute-acid pretreatment), glucose after EH (main sugar released by enzymatic treatment) of the treated solid, and TFS yield were considered as responses into the experimental design. These responses were assumed to be influenced by the independent variables (referred to as factors) temperature (T), acid concentration (c) and residence time (t). A polynomial expression of second order in the form of the following equation was assumed to represent the responses.

$$Y = b_0 + b_1T + b_2c + b_3t + b_{12}Tc + b_{13}Tt + b_{23}ct + b_{11}T^2 + b_{22}c^2 + b_{33}t^2$$

Where *Y* denotes the response variables (xylose yield from pretreatment liquor, EH glucose yield or yield of TFS) in gram of sugar/100 g DRM, b_0 is a constant gave it by the model, b_1, b_2 and b_3 are the regression coefficients for linear effects, b_{11}, b_{22} and b_{33} are the quadratic coefficients and b_{12}, b_{13} and b_{23} are the interaction coefficients. *T*, *c*, and *t* are the independent factors pretreatment temperature, acid concentration and residence time, respectively.

Three factors, two levels (2^3) full factorial design with six axial points, and three replicates at the centrepoint gave a total number of 17 experiments. The same experimental design was applied for pretreatment – enzymatic hydrolysis of all the straw samples and the experiments were run in random order to minimize the effects of unexpected variability. Centrepoint and intervals of variation of temparture, acid concentration and residence time were selected based on preliminary experimentation with a larger number of triticale straw samples. The coded values for axial and factorial points corresponded to -1 and +1 at the lowest and the highest points respectively, with respect to the centrepoint which had coded value of zero (0) for all the points. Table 5-1 shows the coded and uncoded independent factors, levels and experimental design of the CCFD applied on pretreatment – enzymatic hydrolysis. All the experiments were performed in duplicate except for the centrepoint condition which was performed in triplicate. The statistical significance of the simple factors, and the interactions between factors were examined by ANOVA at a significant level of 95% and lack-of-fit analysis. Contours representing the monomeric yields of xylose, EH glucose and TFS as response variables were plotted as function of two independent variables whilst holding the third one at a constant value (stationary point). The experimental data was analyzed by Design Expert software version 8.0.3. (Stat ease, Inc. Minneapolis, USA).

Table 5-1: Factors and their levels for central composite design.

Variable	Symbol	Coded factor levels		
		-1	0	1
Pretreatment temperature (°C)	T	170	180	190
Sulfuric acid concentration (% w/w)	*c*	0	0.3	0.6
Residence time (min)	*t*	6	12	18

Finally, numerical optimization of the TFS yield was performed based on the developed model equations that depicted the response for the feedstocks. For the maximization, upper and lower boundaries were set in the range of outcome for the yields of xylose, glucose and TFS. These limits were able to create the desirability function for the simultaneous optimization. The desirability

function was then used to combine the responses xylose and glucose yields into one single response of interest by choice of value from 0 (unacceptable TFS yield) to 1 (TFS is acceptable and on target).

5.3 Results and discussion

5.3.1 Raw material composition

The chemical compositions of straw materials provide essential information on the maximum potential for release of fermentable sugars by pretreatment-hydrolysis. The chemical compositions of the straws used in this study, together with the results of the ANOVA analysis on the compositional data, are listed in Table 5-2.

Table 5-2: A. Chemical composition of triticale straws (g/100 g DRM). B. ANOVA analysis to test the statistical difference of the main components in chemical composition between straw samples (p 0.05).

A. Composition (in % of dry matter)			
Component	Straw sample		
	01T43	***00T207***	***BACCHUS***
Glucan (%)	33.9 (0.84)	36.3 (1.62)	33.0 (1.47)
Xylan (%)	22.2 (1.56)	22.9 (0.40)	20.6 (0.12)
Arabinan (%)	2.42 (0.24)	3.1 (0.28)	1.9 (0.45)
Extractives[1](%)	8.1 (1.01)	13.5 (0.29)	14.0 (0.96)
Extractives W	6.0 (0.27)	12.2(0.38)	13.0(0.9)
Extractives S	2.1 (0.86)	1.3 (0.11)	1.0 (0.18)
Lignin[2] (%)	16.5 (2.86)	15.3 (2.73)	15.0 (1.96)
Ash (%)	1.2 (0.03)	5.8 (0.3)	4.4 (0.13)
Xylan/Arabinan ratio	10.5 (0.55)	8.4 (0.72)	12.9 (2.81)
Mass closure	84.34	96.9	88.9
B. ANOVA *t-test*	*p*-values		
Component	***01T43*** vs ***00T207***	***01T43*** vs ***BACCHUS***	***00T207*** vs ***BACCHUS***
Glucan	0.0816	0.4474	0.0613
Xylan	0.5047	0.1384	**0.0006**
Arabinan	**0.0320**	0.1398	**0.0158**
Extractives W	**2.01E-05**	**2.02E-04**	0.2344
Extractives S	0.2134	0.1103	0.0664
Lignin	0.6170	0.1423	0.2816
Ash	**1.02E-05**	**2.07E-06**	**0.0016**
Xylan/Arabinan ratio	**0.0174**	0.2178	0.0559

[1]Total Extractives (water plus EtoH).
[2]Insoluble lignin.
Standard deviations are given in parenthesis; The *p*-values in bold (less than 0.05) are considered to be significantly different between the straws at 95% confidence interval.

The carbohydrate fraction in the feedstocks represented 58.6, 61.9 and 55.3% of the composition in *01T43*, *00T207* and *BACCHUS*, respectively. The main measured carbohydrates (glucan + xylan) in

00T207, *01T43* and *Bacchus* straws totalled 59.2, 56.1 and 53.6% (Table 5.2. Insert A), which represent promising potential as monomeric sugars of 66.4, 62.9 and 60.1% for the straw samples, respectively. Comparable glucan content between straws but significant lower xylan content compared to *00T207* and *01T43* and lower arabinan content than *00T207* was found in *BACCHUS* straws (Table 5-2. Insert B). Minor contents of arabinan carbohydrates were found, ranging from 1.9 to 3.1 g/100 g DRM. The carbohydrate fractions of the straws in this study were comparable to other compositions reported for triticale straw [24].

Total extractives (water + solvent extractions) varied from 8.1 to 14 g/100 g DRM and insoluble lignin content only between 15 and 16.5 g/100 g DRM, whilst ash content varied in a broader range from 1.2 to 5.8 g/100 g DRM (Table 5-2). Differences in mass balance closure of the chemical composition determinations were observed between straws (84.3 – 96.9), which could be attributed to minor components that were not quantified (such as galactan, mannan and soluble lignin), but commonly found in triticale straw. The ratios of xylan to arabinan found in all the straws varied from 8.4 to 12.9 (Table 5-2) and were into the range of those reported for triticale straw [24]. Statistically significant differences in water-extractives and ash content were observed between straw samples (Table 5-2, values in bold). Particularly, ash content in *01T43* straw was 4.8 and 3.7-fold lower than *00T207* and *BACCHUS* straws, respectively. The ANOVA also showed significant lower xylan to arabinan ratio (8.4) in the straw *00T207*, compared to *01T43* and *BACCHUS*.

Mariendahl-originated straw (*01T43*) consistently resulted between 73 and 80% reduced ash content compared to Swartland- (*BACCHUS*) and Overberg-originated (*00T207*) straws. Ash content in plants is associated to the physiological function of mineral-uptake and is highly influenced by environmental conditions during growing season [25]. The observed significant variability in ash content between straws may be specially related to the type of soil in the studied sites. Sandy soils, as the characteristic in Mariendahl region (South Africa), are low in soluble silica and have been found to reduce ash deposition in switchgrass at levels between 51-73% [26].

5.3.2 Dilute-acid pretreatment

Dilute-acid pretreatment of triticale straw was carried out according to the experimental design shown in Table 5-1. Two fractions were obtained from pretreatment, i.e. the pretreatment liquor containing the hemicellulose derived sugars (mostly xylose) and the pretreated solid enriched in glucan that was further hydrolyzed enzymatically to yield glucose as main fermentable sugar. The yields of the responses xylose from pretreatment liquor (as major sugar in pretreatment liquor), EH

glucose (as major glucose from enzymatic hydrolysis) and TFS were measured for the different straw samples across the pretreatment conditions. Table 5-3 shows the full design matrix of experiments with variables in coded and actual values, including the replicates at the centrepoint (runs 15-17) for the response TFS yield.

5.3.2.1 *Xylose recovery in pretreatment liquor*

Dilute acid pretreatment can effectively hydrolyze hemicellulose resulting in the solubilisation of hemicellulose-derived sugars, mostly in monomeric form, in the pretreatment liquor [27]. The measured yields of xylose (g/100 g DRM) for the straw samples are summarized in Table 5-3. The maximal experimental yields of xylose were 12.3 (run 4), 11.7 (run 14) and 11.1 g/100g DRM (run 7) for the straws *BACCHUS*, *01T43* and *00T207*, respectively.

Although comparable maximum experimental xylose yield of about 11 g/100 g DRM was found for *00T207* and *BACCHUS* at the same pretreatment condition (run 7; Table 5.3), the straw samples differed in pretreatment requirements to reach that maximum yield in the pretreatment liquor (Table 5-3). Differences in pretreatment requirements to realize maximum xylose yield were generalized between straws with *00T207* as the straw with lower requirements (170°C). Acid concentration dependence by the straws was consistent for *00T207* and *BACCHUS* (0.6% w/w) whilst concentration of 0.3% (w/w) resulted in maximum xylose yield in *01T43* (11.7 g/100 g DRM) near 5% lower and 6% higher than maximal experimental yields reached by *BACCHUS* and *00T207*, respectively (Table 5.3).

Pretreatment conditions including no acid (runs 1, 2, 5, 6 and 1; Table 5-3) resulted in the lowest xylose yields from all of the straw samples with the exception of *01T43* straw on run 6 (190°C, 0% (w/w) acid and 18 min) that showed superior processability with no acid included resulting in comparable yield (8.0 g/100 g DRM) at 180°C, 0.6% (w/w) acid and 12 min (run 12; Table 5-3). It was found that the mildest severity corresponding to the pretreatment condition 170°C- 0% acid - 6 min (run 1; Table 5-3) gave the lowest output of xylose for all the straw samples. On the other hand, the presence of acid was required to maximize yield of xylose whilst pretreatment temperature did not show a clear effect on the xylose yield maximization for the straw samples.

Differences in pretreatment requirements for the release of xylose from pretreatment may be explained by differences in straw composition and mostly regarding distinction in ash content between cultivars. Straw *01T43* with the significant ($p < 0.001$) lowest ash content displayed better

processability at no acid conditions but also quite alike maximum xylose yield at half of the acid concentration required by *00T207* and *BACCHUS*. Ash content has been directly associated with neutralising capacity in biomass (ability of the material to hold the pH of the environment almost invariable to changes in the acidity for instance during pretreatment) and the effectiveness in sugar release from dilute acid pretreatment of wheat straw [7], corn stover, poplar and switchgrass [28]. Thus reduced-ash biomass may be require less acid or make use of the acidic environment more effectively for the release of sugars during pretreatment.

The experimental design applied for the dilute-acid pretreatment in this study resulted in monomeric xylose recoveries not higher than 53% of potential xylose in the raw material, with the maximum value found for *BACCHUS* straw at 190°C, 0.6% (w/w) and 6 min (run 4; Table 5-3). Recoveries of monomeric xylose near to 62% of theoretical from dilute acid pretreatment have been reported for corn stover at much higher pretreatment severity (200°C, 32 min and 0.49% w/w) by a different reactor configuration and lower solid loading [29] than the referred conditions in the present study were used. Higher xylose recoveries near 91% of theoretical content has been reported for dilute-acid pretreatment of wheat straw [30] although comparisons are difficult owing differences in applied pretreatment conditions that highly influence dilute acid such as reactor configurations [31] and solid loading [32]. The effects of the temperature, acid concentration and residence time and the statistical significance on the response xylose yield are discussed in Section *5.3.5 Statistical analysis of the pretreatment responses* below.

5.3.3 Glucose yield from enzymatic hydrolysis

The yields of glucose after enzymatic hydrolysis (EH glucose yield) of pretreated straw samples are listed in Table 5-3. Pretreatment optimization resulted in improvement of EH glucose in nearly 25 g/100 g DRM and also noticeable differences in glucose yield between straws. Measured EH glucose yields were in the ranges 14.9-39.3, 6.2-31.2 and 8.6-32.7 g/100 g DRM for straws *01T43*, *00T207* and *BACCHUS*, respectively (Table 5.3). The maximum EH glucose yield from pretreated straw *01T43* corresponding to theoretical glucose recovery (100% recovery of glucose in the straw) was significantly higher than maximal EH-glucose from *BACCHUS* and *00T207* which represented 89.3% and 77.4% of recovery of glucose in the respective straws.

Table 5-3: Measured monomeric yields of xylose, EH glucose and total fermentable sugars (TFS).

run	Temp [*T*] (°C)	Acid conc. [*c*] (% w/w)	Time [*t*] (min)	Xylose yield[a] (g/100 g DRM) Straw			EH Glucose yield[a] (g/100 g DRM) Straw			TFS[a,b](g/100 g DRM) Straw		
				01T43	***00T207***	***BACCHUS***	***01T43***	***00T207***	***BACCHUS***	***01T43***	***00T207***	***BACCHUS***
1	170	0.0	6	0.1 (0.2)	0.2 (0.7)	0.4 (1.6)	14.9 (39.6)	6.2 (15.2)	8.6 (23.5)	19.5 (31.1)	9.60 (14.4)	13.2 (22.0)
2	190	0.0	6	0.4 (1.7)	0.3 (1.3)	0.4 (1.7)	13.2 (35.1)	12.8 (31.7)	18.5 (50.5)	17.8 (28.3)	21.0 (31.7)	26.7 (44.4)
3	170	0.6	6	7.5 (29.8)	4.8 (18.3)	5.0 (21.2)	21.9 (58.0)	20.3 (50.2)	21.1 (57.6)	33.6 (53.5)	30.6 (46.1)	31.4 (52.3)
4	190	0.6	6	6.1 (24.3)	8.6 (33.0)	**12.3 (52.7)**	20.6 (54.6)	28.7 (71.2)	29.1 (79.4)	28.4 (45.2)	40.4 (60.9)	**46.5 (77.4)**
5	170	0.0	18	0.1 (0.5)	0.2 (0.9)	4.7 (20.1)	25.2 (67.0)	11.1 (27.6)	12.9 (35.1)	26.9 (42.7)	18.3 (27.6)	25.2 (41.9)
6	190	0.0	18	8.0 (31.6)	1.2 (4.7)	2.8 (12.0)	21.3 (56.5)	23.0 (56.9)	31.3 (85.4)	32.9 (52.4)	30.6 (46.2)	37.9 (63.1)
7	170	0.6	18	9.1 (36.0)	**11.1 (42.5)**	11.0 (47.2)	27.5 (73.0)	22.4 (55.5)	27.1 (73.9)	39.3 (62.5)	39.9 (60.1)	42.9 (71.3)
8	190	0.6	18	5.9 (23.2)	6.4 (24.5)	9.7 (41.2)	27.8 (73.8)	25.8 (63.9)	**32.7 (89.2)**	35.7 (56.8)	35.1 (52.9)	45.1 (75.1)
9	170	0.3	12	4.99 (19.8)	1.7 (6.5)	1.6 (6.7)	25.9 (68.7)	17.2 (42.5)	16.5 (44.9)	36.6 (58.2)	30.0 (45.2)	23.6 (39.3)
10	190	0.3	12	7.7 (30.5)	1.1 (4.3)	8.6 (36.6)	**39.3 (104.3)**	**31.2 (77.3)**	23.1 (62.9)	**51.9 (82.5)**	**49.4 (74.4)**	36.8 (61.2)
11	180	0.0	12	0.9 (3.6)	0.1 (0.3)	0.6 (2.4)	21.2 (56.3)	6.9 (17.0)	16.9 (46.1)	31.7 (50.4)	10.8 (16.3)	24.1 (40.1)
12	180	0.6	12	8.0 (31.8)	0.7 (2.8)	10.4 (44.6)	36.4 (96.7)	29.9 (74.2)	24.9 (67.9)	49.6 (78.9)	47.2 (71.1)	36.6 (60.9)
13	180	0.3	6	3.1 (12.2)	2.2 (8.6)	3.0 (13.0)	25.8 (68.5)	19.9 (49.3)	26.1 (71.3)	35.2 (56.0)	27.9 (42.1)	33.6 (55.9)
14	180	0.3	18	**11.7 (46.5)**	4.0 (15.3)	8.5 (36.1)	33.7 (89.5)	24.0 (59.6)	30.3 (82.7)	50.8 (80.8)	37.2 (56.1)	43.2 (72.0)
15[c]	180	0.3	12	8.6 (34.0)	4.0 (15.4)	7.3 (31.2)	36.3 (96.5)	21.5 (53.3)	25.5 (69.5)	49.3 (78.4)	31.2 (47.0)	37.0 (61.6)
16[c]	180	0.3	12	7.0 (27.6)	5.8 (22.4)	5.5 (23.4)	34.1 (90.4)	22.8 (56.6)	25.4 (69.3)	45.6 (72.5)	35.0 (52.7)	36.5 (60.8)
17[c]	180	0.3	12	7.2 (28.7)	2.2 (8.5)	6.4 (27.3)	38.7 (102.7)	20.2 (50.0)	27.0 (73.6)	49.6 (78.8)	27.4 (41.3)	38.2 (63.6)

T: temperature *c*: acid concentration *t*: temperature.

[a] average of duplicates runs excepts for design points 15–17.

[b] calculated as the sum of monomeric sugar yields (glucose, xylose and arabinose) from pretreatment liquor and same sugars after enzymatic hydrolysis.

[c] centre point conditions.

Values in brackets represent the percentage of recovery of corresponding sugar(s) of theoretical sugar content in the raw material. Maximum actual sugar yield for each straw material is highlighted in bold.

Pretreatment condition of 190°C, 0.3% (w/w) acid and 12 min (run 10; Table 5-3) maximized the glucose yield after EH from both *01T43* and *00T207* straws, whilst a higher pretreatment severity (run 8: 190°C, 0.6% (w/w) acid and 18 min) was required to maximize the EH-glucose yield with *BACCHUS* straw (Table 5-3). The high glucose recovery determined for *01T43* straw may infer some experimental error in the determination. On the other hand, the lowest yield of glucose was found at the mildest severity evaluated (170°C, 0% (w/w) acid and 6 min; run 1) in all of the straw samples. The similarities in pretreatment temperature required for maximum output of glucose for all of the straws is apparently related to the rate of cellulose conversion to glucose, which is favored by pretreatments at high temperature [33].

The effect of ash content was more evident on the measured maxima EH glucose yield. Straw samples with lower ash content reached highest glucose yields. Ash content of the straws (Table 5-2) followed the trend *01T43* < *BACCHUS* < *00T207* while the highest measured EH glucose yield the straws samples developed *01T43* > *BACCHUS* > *00T207* (Table 5-3). Similar adverse effects of high ash content on the yield of fermentable sugars have been reported for straw from different wheat cultivars [7], as well as bagasse from sugarcane varieties [20]. High ash content in lignocellulosic biomass such as wheat straw has been found negatively correlated to glucose, xylose and overall sugar release from pretreatment-enzymatic hydrolysis [7]. Even though possible explanations for these relationships of sugars released with ash content are not given in the referred study, ash content in herbaceous has been found affect neutralising capacity of the materials under aqueous pretreatment in presence of acid as catalyst and consequently negative effects on sugars release are expected (See subsection 2.1.4 Ash).

The maximum measured glucose recovery achieved between straws samples corresponded to complete recovery for straw *01T43* (Table 5-3), in close agreement with EH glucose recoveries higher than 96% reported for diluted-acid pretreatment of wheat straw [34] where lower solid loading (10% w/w) and higher enzyme dosage (46 FPU) were employed. Dilute-acid pretreatment of bagasse from sugarcane varieties was study under similar reactor configuration, solid loading and enzyme dosage [20] as used in the present study, which resulted of utility for comparisons between bagasse and straw performance after pretreatment. When the highest experimental outputs of EH glucose of *01T43* straw (39.3 g/100 g DRM; run 10. Table 5-3) and the bagasse with the highest glucose yield were compared, 3.3-fold higher acid concentration was required by sugarcane bagasse at lower temperature (180°C) and reasonably similar residence time (10 min) resulting in comparable values. The observed differences in amenability to enzymes of the treated solids that favored straw over

bagasse could not be attributed to higher contents of ash or lignin as the reported inorganic and lignin compositions in bagasse were significant lower (0.8 ± 0.1 and 12.2 ±0.2 g/100 g DRM, respectively) compared to *01T43* straw (Table 5-2). However, higher xylan to arabinan ratio (equal to 12) in sugarcane bagasse than in *01T43* straw (equal to 10.5) could have had negative impact on amenability of bagasse to require more acid in pretreatment. The degree of xylan substitution has major influence on the properties of carbohydrates and subsequently in the properties of biomass, and has been associated with the recalcitrance of the biomass and affect the enzyme reactivity [35]. In our study, the effect of the degree of xylan substitution on the glucose yield between straws was not clear, and the improved amenability to pretreatment - enzymatic hydrolysis found on *01T43* straw was therefore attributed to feedstock properties.

Regarding *00T207* and *BACCHUS* straws that yielded lower EH glucose compared to *01T43* straw, the experimental design applied in this study led to yields yet well in excess of the glucose yield of 23 g/100 g DRM reported for wheat straw pretreated at 121°C, 0.75% (w/v) of acid concentration, and residence time of 1 h [17]. The incidence of the pretreatment conditions on glucose yield and the predictive equations developed by the extended ANOVA analysis the experimental data are given in Section *5.3.5*.

5.3.4 Effect of pretreatment on TFS yield

Although the primary fermentable sugar from lignocellulose is glucose (Table 5-2), the conversion of xylose to ethanol could make the process more economically feasible when a suitably engineered microorganism is available. Therefore it is important to determine the optimum pretreatment conditions for maximum recovery of both sugar streams as a total. The sugar recovery of the whole process (pretreatment liquor and EH), referred to as total fermentable sugars (TFS), is shown in Table 5-3. While the EH glucose yield from pretreated *01T43* straw was significantly higher than the glucose yields obtained from *BACCHUS* and *00T207*, less considerable differences between straws were observed in the TFS yield. The maximum TFS yields were 51.9 (run 10), 49.4 (run 10) and 46.5 g/100 g DRM (run 4), corresponding to 82.5, 74.5 and 77.4% of the total xylose and glucose in the straw samples *01T43*, *00T207* and *BACCHUS*, respectively (Table 5-3). The maximum measured TFS yields were achieved for straws *01T43* and *00T207* by pretreatment at 190°C - 0.3% (w/w) acid concentration and 12 min (run 10; Table 5-3), whilst higher acid concentration (0.6% w/w) and shorter residence time (6 min) (run 4. Table 5-3) resulted in maximum TFS yield for *BACCHUS* straw.

Pretreatment conditions that resulted in the highest yields of xylose in pretreatment liquor, maximum EH glucose of pretreated straws, and TFS for the combined sugar yields, did not match between straws. These pretreatment conditions for maximal yields were determined individually for each of the straws, and showed clear divergence in pretreatment requirements to be achieved, as found with other feedstocks [18]. Thus maximization of the combined yields of TFS required of a compromise between pretreatment temperature and residence time for the straw *01T43*; temperature, acid concentration and time for *00T207* and only in residence time for *BACCHUS*, when compared to conditions required for maximization of either xylose or glucose yields only.

The influence of EH glucose yield as major sugar fraction in the TFS yield was reflected in the conditions that maximized the TFS. However, when the conditions with the highest EH glucose yields for each feedstock and the respective xylose yields achieved at these conditions (Table 5-3) were compared, a clear contribution of xylose from pretreatment to build up the yield of total sugars was observed for straws *01T43* and *00T207* (Table 5-3).

The inclusion of an acid catalyst improved significantly TFS yields from the combined pretreatment – enzymatic hydrolysis process. Pretreatment conditions at the lowest and highest temperatures, and the shortest and longest residence times, but with no acid added (runs 1, 2, 5, 6 and 11; Table 5-3), provided the lowest TFS outputs, with the exception of run 6 (190°C - 0% (w/w) - 18 min), where the TFS yield reached values over 30 g/100 g DRM for all of the studies straws. On the other hand, comparable experimental TFS yields for the straws *01T43* and *00T207* were reached at the more severe conditions with the highest acid concentration (runs 7, 8 and 12; Table 5-3). The influence of the pretreatment conditions as single factors and their interaction on TFS yield, as well their statistical significance found by the extended ANOVA analysis is discussed below.

Maximum total monomeric sugars yield from wheat straw near 56 g/100g DRM was achieved by dilute sulphuric acid pretreatment at 121°C - 0.75% (v/v) acid concentration and 1 h of residence time followed by enzymatic hydrolysis [17]. In this study, yield 8% lower was achieved at 190°C with 2.5-fold lesser acid and 5-fold shorter residence time, which represents substantial reductions in pretreatment requirements. The highest TFS yield found with triticale straw in this study (obtained at 190°C-0.3% (w/w)- 12 min) was nearly 22% lower than the highest output of combined sugar yield (CSY) from dilute acid pretreatment – enzymatic hydrolysis of sugarcane bagasse (at 180°C-0.65% (w/w)- 10 min) [20]. However, all the sugars (glucose, xylose, and arabinose) in monomeric and

oligomeric form were included in CSY calculations of the bagasse [20], while only monomeric xylose and glucose yields were included in the TFS calculation in the present study.

5.3.5 Statistical analysis of the pretreatment responses

Predictive models for the monomeric yields of xylose, EH glucose and TFS as response variables for dilute-acid pretreatment-hydrolysis of triticale straw were developed based on the values of the input variables. The measured yields of xylose, EH glucose and TFS (Table 5-3) were fitted into quadratic model expressions to assess the effect of the independent process variables (factors) on the responses. The statistical significance of models and the effect of each factor on the responses were determined by an extended ANOVA analysis at 95% of confidence interval. Likewise, the fit of the models to describe suitably the experimental data was assessed by the lack-of-fit analysis. The ANOVA showed statistical significance ($P < 0.05$) for all the predictive models of the responses and second order polynomial expressions were fitted to describe the experimental data according to the lack-of-fit analysis, except for the yield of xylose for *00T20* straw which could only be described by a linear model. Table 5-4 shows the results of the extended ANOVA and predictive models of the responses monomeric xylose, EH glucose and TFS yields The model expressions were simplified to the model terms with statistical significant for which "Prob > F" was lesser than 0.05 according to the *P*-values at significance level 95% of confidence (ANOVA). Predictive models in Table 5-4 depict the interaction effects of the independent parameters *Temperature*, *acid concentration* and *residence time* on the yields of xylose, glucose from EH and total fermentable.

Table 5-4: RSM based predictive models for the yields of monomeric xylose (X_Y), EH glucose (G_Y) and TFSy. Temperature (T), acid concentration (c) and residence time (t) are given in coded form.

	Model equations		Model equations	
Straw	Xylose	R^2	Glucose and TFSy	R^2
01T43	$X_Y = 7.23+2.71c+1.75t$	0.84	$G_Y = 35.66+3.83c+3.91t$	0.86
			$TFS_y = 48.68+5.78c+5.10t$	0.91
00T207 [a]	$X_Y = 2.39+2.95c$	0.55	$G_Y = 22.12+4.43T+6.72c$	0.89
			$TFS_y = 33.59+10.27c$	0.84
BACCHUS	$X_Y = 5.88+3.96c+1.55t$	0.88	$G_Y = 24.68+4.85T+4.67c+3.09t$	0.95
			$TFS_y = 35.18+5.67T+7.53c+4.29t$	0.96

[a] Linear model.
R^2 represents the determination coefficient.

The lack of fit for the entire modeled responses was not significant, which imply that the experimental data could reasonably be described by the models. Additionally, the coefficients of determination (R^2) of the models for EH glucose and TFS were satisfactory (0.84–0.96) to attribute the variability in the results to the variables of the process for all the responses except for the model describing xylose yield for *00T207* straw (R^2 0.55. Table 5-4). In this case other factors such as sugar degradation products or oligomeric yields of sugars from pretreatment liquor could have had influence on the experimental data of monomeric xylose to be not consistent by the model depiction. The statistical significance of temperature, acid concentration and time, as simple factors, and their interaction on the responses were examined by the lack-of-fit analysis. Acid concentration was the factor that exerted the main influence for the responses of all the straw samples.

5.3.5.1 *Xylose yield in pretreatment liquor*

The summary of the ANOVA for monomeric xylose is given in Table-5.5. The model equations that predicted xylose yields (Table 5-4) based on the experimental data were plotted in three-dimensional surface responses (See Figure 5-1) as function of two independent variables whilst holding the third variable at a constant value (stationary point). Acid concentration showed to enhance xylose yield for all straws, particularly for *BACCHUS* (Figure 5-1 C). Residence time impacted the xylose yield in lesser extent but only on *01T43* and *BACCHUS* (Figure 5-1. Inserts A and C). The maximization of monomeric xylose in pretreatment liquor seemed to differ between straws regarding the pretreatment requirements. The maximal of xylose yield predicted by the models was comparable (11.7 – 12.3 g/100 g DRM) for *01T43* and *BACCHUS*, although *BACCHUS* straw seemed to demand higher acid concentration (>>0.6% (w/w)) than *01T43* if pretreatment temperature is held at 180°C. *BACCHUS* straw appeared to require double the acid (0.6% (w/w)) and longer residence times (18 min) to reach the highest predictive yield of xylose (nearly 12 g/100 g DRM) at a pretreatment temperature of 180°C (Figure 5-1. C) while *01T43* and *00T207* straws showed predictive yields lesser than 8 g/100 g DRM (Figure 5-1. Inserts A and B).

5.3.5.2 *EH Glucose yield*

The predictive model equations developed for EH glucose yield are summarized in Table 5-4. The respective results of the ANOVA for EH glucose is given in Table 5-6. The pretreatment parameters influencing the yield of glucose depended on the type of straw (Table 5-6). For example the glucose yield on *BACCHUS* was positively influenced mainly by temperature, but also by acid and residence time (Table 5-6). Higher values of glucose yield were therefore obtained by increasing these parameters up to certain values of temperature, after which point is a reduction in the glucose yields (quadratic negative effect). On the other hand, acid concentration was a major influence on the

response for *00T207*, whilst time did not have statistical significance effect (*p* 0.1556) for this straw (Table 5-6). Time and acid concentration significantly affected the glucose yield for *01T43* (Table 5-6).

Table 5-5: Summary of the estimate of model term significance (P-values; P>F) at significance level 95% of confidence for monomeric xylose yield after pretreatment

Factor	Xylose yield		
	01T43	***00T207***	***BACCHUS***
Model	0.0414	0.0129	0.0152
Temperature	0.3826	0.9731	0.1300
Acid	0.0054	0.0018	0.0005
Time	0.0373	0.3852	0.0472
Temperature × Acid	0.0735	-	0.2150
Temperature × Time	0.3881	-	0.1072
Acid × Time	0.3332	-	0.5799
Temperature^2	0.6559	-	0.7352
Acid^2	0.1004	-	0.9997
Time^2	0.7414	-	0.8484
Lack of Fit	0.1112	0.3986	0.1373
	F-values		
Model	3.97	5.33	5.79
Lack of Fit	8.29	1.88	6.57
P-values lesser than 0.05 indicate that model terms are statistically significant.			

Table 5-6: Summary of the estimate of model term significance (P-values; P>F) at significance level 95% of confidence for the yields of glucose from enzymatic hydrolysis (EH glucose) and total fermentable sugars (TFS).

Factor	**EH Glucose**			**TFS**		
	01T43	*00T207*	*BACCHUS*	*01T43*	*00T207*	*BACCHUS*
Model	0.0247	0.0112	0.0010	*0.0062*	0.0387	0.0005
Temperature	0.6513	0.0066	0.0003	*0.5050*	0.0586	0.0004
Acid	0.0316	0.0007	0.0004	*0.0073*	0.0019	< 0.0001
Time	0.0291	0.1556	0.0039	*0.0132*	0.1827	0.0019
Temperature × Acid	0.7261	0.5445	0.0578	*0.3730*	0.3580	0.3005
Temperature × Time	0.9597	0.9944	0.3740	*0.5208*	0.4944	0.1299
Acid × Time	0.6789	0.1675	0.2865	*0.5153*	0.4739	0.1389
Temperature^2	0.3852	0.5035	0.0264	*0.1509*	0.3320	0.0833
Acid^2	0.056	0.1040	0.0870	*0.0260*	0.1637	0.0956
Time^2	0.0931	0.7874	0.0152	*0.0815*	0.5147	0.0266
Lack of Fit	0.1773	0.0908	0.1042	0.1401	0.2102	0.0682
	F-values					
Model	4.84	6.45	14.31	7.90	4.07	17.42
Lack of Fit	4.93	10.31	8.89	6.42	4.04	13.97
P-values lesser than 0.05 indicate that model terms are statistically significant.						

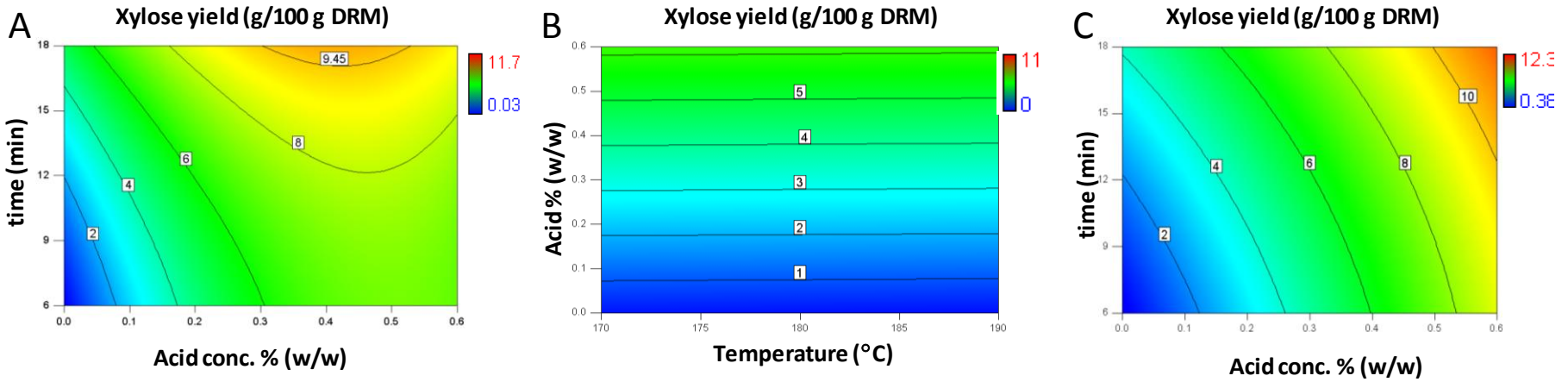

Figure 5-1: Contour plots for the yield of monomeric xylose from pretreatment liquor for the straw samples (A) 01T43, (B) 00T207, and (C) BACCHUS. Yields are plotted as a function of acid concentration and residence time (holding pretreatment temperature at 180°C) in (A) and (C), and pretreatment temperature and acid concentration (holding residence time at 12 min) in (B). Yields are expressed in gram per 100 grams of dry raw material (DRM).

The maximum predictive values for EH glucose yields and the pretreatment conditions that enable these yields can be examined from the contours plotted for the response (See Figure 5-2, inserts A, B and C). A complete recovery of EH glucose from *01T43* straw is predicted by the analysis (highest predictive yield near 39 g/100 g DRM; Figure 5-2. A). The predicted conditions leading to optimize EH glucose yield are temperature of 180°C, acid concentration near 0.4% (w/w) and residence time of 12 min (Figure 5-2. A). Predictive maximal of EH glucose yields from *00T207* and *BACCHUS* straws are reasonably comparable between them (31.1 – 32.7 g/100 g DRM), corresponding to maximal recoveries of 72 and 79% of theoretical, respectively. Pretreatment conditions that result in the highest predictive yields from *00T207* and *BACCHUS* are temperatures between 185 and 190°C, acid concentration between 0.4 and 0.5% (w/w) and residence time between 12 and 15 min (Figure 5-2).

Total fermentable sugars yield.

The model equations for the prediction of TFS yield are given in Table 5.4. The acid concentration (linear effect) exerted the main influence ($P < 0.008$) on the TFS yield of all the straw samples (Table 5-6). The TFS response for *BACCHUS* was mainly affected by acid concentration, followed by temperature and time in linear fashion, as well as quadratic effects of time (Table 5-6). The effect of time was also statistically significant together with the quadratic term of acid concentration TFS response of *01T43* straw (Table 5-6). TFS yield for *00T207* was only influenced by acid concentration in a linear fashion significant at $p < 0.05$ and pretreatment temperature $p < 0.1$ (Table 5-6).

The predictive models generated could represent reasonably the measured TFS yields for all the straw samples as indicated for the not significance of the lack-of-fit (Table 5-6). However, the coefficients of determination (R^2) that measured the variability in the outcome of the prediction around the mean (Table 5-4) indicated that 4, 9 and 16% of the variation of the models for *BACCHUS*, *01T43* and *00T207* could not be attributed to the process variables.

The unexpected variability of the prediction by the models could have possibly be generated by the presence of other components as oligomeric sugars and sugar degradation products that were present in the pretreatment liquor as well as xylose production after enzymatic hydrolysis but not quantified in the study. Levels of EH xylose yields up to 11.3 g/100 g DRM have been found using the same enzyme combinations at similar enzyme and solid loading with steam exploded triticale straw [36].

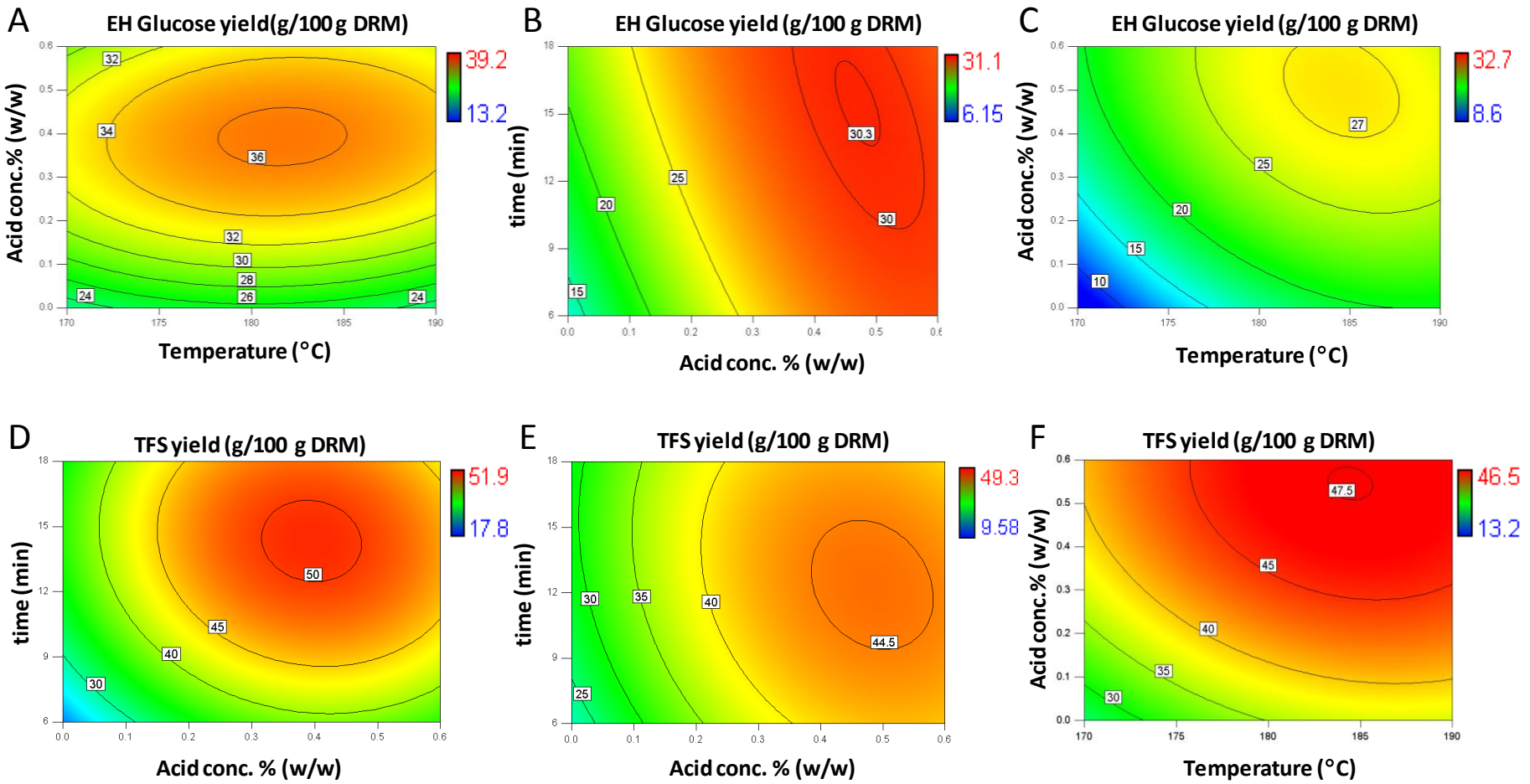

Figure 5-2: Contour plots for yields of EH glucose and total fermentable sugars (TFS) for the feedstocks (A and D) 01T43, (B and E) 00T207, and (C and F) BACCHUS. EH glucose yield as a function of temperature and acid concentration (holding residence time at 12 mi) (A) and (C), and acid concentration and residence time (holding pretreatment temperature at 190°C) in (B). TFS yield as a function of acid concentration and residence time (holding pretreatment temperature at 180°C) in (D) and (holding temperature at 190°C) in (E), and pretreatment temperature and acid concentration (holding residence time at 18 min) in (F). Yields expressed in gram per 100 grams of dry raw material (DRM).

In general, the maximization of TFS yield from *01T43* reached higher values and required less severe pretreatment conditions, followed by *00T207* and *BACCHUS*. Pretreatment conditions that predictably maximize the TFS yield for each straw sample, as the main goal in the study, could be obtained by inspection of the contour plots for the TFS (Figure 5-2. Inserts D, E and F). Acid concentration of 0.4% (w/w), temperature of 180°C and residence times between 13 and 15 min will maximize TFS yield from *01T43* straw (Figure 5-2. D). Acid concentrations near 0.5% (w/w), temperature and residence time of 190°C and 12 min respectively will result in maximum yield for *00T207* straw (Figure 5-2. E). In the case of *BACCHUS* straw, acid concentration near 0.55% (w/w), temperature between 185 and 190°C Pretreatment temperatures between 185 and 190°C, 0.6% (w/w) acid concentration and residence and of 18 min will maximize the response TFS (Figure 5-2. C).

Numerical optimization was performed on the second order model equations obtained for TFS yield to find the optimum dilute-acid pretreatment conditions that enable the release of the maximum yield of total fermentable sugars from pretreatment-hydrolysis of each straw under study. The differentiation in pretreatment requirements to maximize each of the sugar yields (xylose, EH glucose and TFS) observed by the contour plots of the predicted model between straws was expected to be accentuated by optimization.

5.3.6 Numerical optimization of TFS yield

The study aimed at assessing variability in feedstock quality and the possible impact on the maximization of total fermentable sugars from pretreatment-hydrolysis. Numerical optimization was then performed to identify optimal pretreatment conditions that suit with the specific feedstock quality of the straw samples as well as maximize the output of total fermentable sugars. After optimization, the requirements in pretreatment severity between straw samples were also considered as important factors impacting the economic feasibility of the straws as bioethanol feedstock.

The process for TFS optimisation of all the straws was done by allocating higher weight of importance (≥ 4 in a scale from 1 to 5) to EH glucose yield than the one assigned to xylose yield, due to the better adjustment in the prediction (higher determination coefficients of the models (Table 5-4). The design response TFS resulted from optimization displaying desirability function values closer to 1 (more desired output of the response) were preferred. Table 5-7 shows the predicted pretreatment conditions that maximize the TFS yield, as well as the predicted yields of xylose and EH glucose that would be expected when TFS is optimized at these predictive conditions.

Table 5-7: Predictive pretreatment conditions for maximum TFS yield (TFS_Y) from pretreatment and enzymatic hydrolysis. Desiderability function given in values between 0 and 1.

Straw	Optimum condition[a]			Response (g/100 DRM)			Desirability of the model
	Temperature (°C)	Acid conc. (% w/w)	Time (min)	X_Y[b]	G_Y	TFS_Y[c]	
01T43	182	0.39	15.4	(9.0)	36.7	50.5	0.88
00T207	190	0.53	13.0	(6.0)	30.2	44.9	0.87
BACCHUS	189	0.60	18.0	(11.5)	33.4	46.7	0.98

[a]Predictive pretreatment conditions for maximization of TFS_Y.
[b]Pretreatment variable not optimized. Values in brackets represent the yields of monomeric xylose at the optimum pretreatment conditions that maximized TFS yield.
[c]Optimum yield.

The predicted optimum TFS yields were 50.5, 46.7 and 44.9 g/100 g DRM for the straw *01T43*, *00T207* and *BACCHUS*, respectively, representing predictive recoveries of 80.4, 77.8 and 68% of the theoretical TFS (xylose + glucose) in the straws. Variability in response to pretreatment regarding output of TFS yield was again consistent after optimization. Interestingly, differences in pretreatment requirements by the optimized response TFS was even more obvious after optimization. The highest expected yield between straws was found for *01T43* straw (better performer straw) is predicted to be achieved by the lowest requirements in acid concentration, pretreatment temperature and residence time when the predicted pretreatment conditions are compared within straws (Table 5-7). Apparently, the observed inconsistencies for the prediction of monomeric xylose from *00T207* straw and possible EH glucose lost negatively affected the prediction of the sugar yields for the referred straw which was the lowest outputs after the optimization process. This fact could be consequently reflected in the ability of the model to optimize the responses leading to a low, but still acceptable, desirability value but in all cases the desirability was ≥ 0.80, which can derive in good fit of the models.

5.4 Conclusions

Differentiation in straw composition between triticale cultivars was markedly associated to ash content. Differences in composition of xylan, arabinan and water-extractives were found less generalized within straws. Dilute-acid is an efficient pretreatment for releasing monomeric sugars from pretreatment-hydrolysis of triticale straw. The studied straws required different dilute-acid pretreatment conditions to maximize the yields of xylose, EH glucose and TFS. Likewise, pretreatment conditions seemed to impact different accordingly to ash content as most important feedstock quality feature between straws. A clear negative association between ash content in straw and glucose yield after enzymatic hydrolysis was observed. The results and previous observations on larger number of straws from triticale cultivars show that low ash content in straw is highly

recommended for improved processibility on sugars release at reduced pretreatment requirements by dilute-acid pretreatment-hydrolysis of triticale straw.

Acknowledgments

The authors would like to thank Dr. W. Botes from Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa for performing the field trials and providing the triticale straw material used in this study. We would like to extend our gratitude to the Technology Innovation Agency (TIA), an entity of the Department of Science and Technology (DST) of the Republic of South Africa, for funding support of this study. The authors extend the acknowledgement to Genencor and Novozymes for kindly supplying the enzymes.

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Chapter 6

6 Screening of steam explosion pretreatment conditions for realizing areas of maximal sugars release and improved digestibility from triticale straw

The complete content of Chapter 6 was published in *New Biotechnology* 33 (2016) 153 - 163. The content presented in Chapter 6 represents the reviewed version of the manuscript accepted for publication. The schematic diagram of the steam explosion unit originaly presented in the manuscript was replaced with colour photographs of the unit for better illustration.

Title:

"Steam explosion pretreatment of triticale (× *Triticosecale* Wittmack) straw for sugar production"

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The work done in the present chapter was motivated by the lack of scientific information pertinent to steam explosion (SE) of triticale straw and more specifically aimed at the release of sugars from the combined pretreatment-hydrolysis process and the extent of sugar degradation that would be potentially expected from autohydrolysis steam explosion.

Objective of dissertation

Chapter 6 assists in addressing the **Objective 3** by establishing optimal steam explosion (SE) pretreatment ranges for maximal release of hemicellulose-derived sugars, enhanced cellulose digestibility and maximum combined sugars yield (CSY) from the combined process pretreatment - enzymatic hydrolysis. Additionally, production of inhibitory compounds after pretreatment was measured to realise the extent of sugar degradation and observe the levels of inhibitor concentrations that can be obtained at the range of conditions evaluated. The assessment of these specific goals was required as a foundation for further pretreatment optimization of the CSY under constraints of inhibitors production performed in **chapter 7**.

Summary of findings in present chapter

The efficient use of the entire sugar content in biomass has been pointed as crucial to realize economic feasibility of lignocellulosic ethanol. However, one of the major limitations on reaching maximum sugar yields relies on the mismatch between pretreatment conditions that maximize hemicellulose sugars release and those for maximum digestibility and thus yield of combined sugars. SE pretreatment is effective in solubilising significant portion of hemicellulose-derived sugars while increasing cellulose digestibility of the pretreated solid although pretreatment conditions can impact negatively on hemicelluloses leading to sugar degradation. Thus sugar productivity may substantially be affected and toxicity of sugar streams turns relevant if not sufficient knowledge in pretreatment requirements by the feedstocks is guaranteed.

In this chapter, a range of uncatalyzed SE conditions between severities 3.05 to 4.12 was compared in order to distinguish conditions that result in the highest recovery of hemicellulose-derived sugars, cellulose digestibility or the combined sugars yield (CSY) from the pretreatment-enzymatic hydrolysis. Maximum hemicellulose-sugars recovery (52% of the theoretical content in the straw) was realized at a severity 3.64 that matched with pretreatment severity for maximum CSY (nearly 77% of theoretical content). The harshest severities above 3.94 resulted in the highest cellulose digestibility (>92%) at similar high extent of sugar loss after pretreatment. However, the concentration of sugar degradation products (HMF, furfural and formic acid), as well as acetic acid observed across the studied pretreatment severities were at levels below tolerance limits of the downstream biological conversions. These findings suggest that the maximization of combined sugars yield by uncatalyzed SE of triticale straw entails compromise of pretreatment conditions with those for maximum digestibility and conditions for reduced hemicellulose sugar loss (or low inhibitors production).

Candidate declaration

With regard to chapter 6 page numbers 141-172 of this dissertation, the nature and scope of my contribution were as follows

Name of contribution	**Extent of contribution (%)**
Planning of experiments	100
Executing experiments	100
Interpretation of results	70
Writing the chapter	100

The following co-authors have contributed to chapter 6 page numbers 141-172 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Maria del Prado Garcia-Aparicio	Garcia@sun.ac.za	- Providing inputs - Interpretation of results - Reviewing the chapter	70 20 70
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Signature of candidate……

Date……

Declaration by co-authors

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 6 page numbers 141-172 in the dissertation,
2. No other authors contributed to chapter 6 page numbers 141-172 in the dissertation besides those specified above, and
3. Potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in to chapter 6 page numbers 141-172 of this dissertation.

Abstract

Triticale, a non-food based, low-cost crop that is well-adapted for cultivation on marginal lands has been considered as a potential feedstock for 1G and 2G bio-ethanol production. In this work, triticale straw was evaluated as a source of fermentable sugars by combination of uncatalyzed steam explosion and enzymatic hydrolysis. Pretreatment conditions with severities from 3.05 to 4.12 were compared in order to identify conditions that favour the recovery of hemicellulose-derived sugars, cellulose digestibility or the combined sugars yield (CSY) from the pretreatment-enzymatic hydrolysis.

Xylose oligosaccharide was the major sugar in hydrolysates from all pretreatment conditions. Maximum hemicellulose-sugars recovery (52% of the feedstock content) was obtained at 200°C and 5 min. The highest cellulose digestibility (95%) was found at 200°C – 15 min, although glucose recovery from hydrolysis was maximised at 200°C – 10 min (digestibility $>$ 92%) due to higher mass yield of pretreated solids. The maximum CSY (nearly 77% of sugar content of straw) was obtained at 200°C - 5 min. Sugar loss after pretreatment was observed to higher extent at harsher severities. However, the concentrations of sugar degradation products and acetic acid were at levels below tolerance limits of the downstream biological conversions.

Steam explosion pretreatment without acid impregnation is a good technology for production of fermentable sugars from triticale straw. This work provides foundation for future autohydrolysis steam explosion optimization studies to enhanced sugars recovery and digestibility of triticale straw.

***Keywords**:* Triticale straw; steam explosion; autohydrolysis; combined sugar yield; cellulose digestibility; inhibitors

6.1 Introduction

The well-established technology for production of the so-called first generation biofuels (1G) through sugar and starch fermentations is based on different feedstocks, depending on the availability in specific regions worldwide. At present, maize and sugar cane are the major feedstocks for 1G ethanol production; the former a starch-rich crop of which 365.8 million metric tons were produced in the United States in 2014 [1], and the second a sucrose-rich crop of which 619 million metric tons were produced in Brazil during the same year [2]. Alternative crops with significant 1G ethanol production are wheat [3], grain sorghum [4], rye and triticale [5]. The concerns about 1G ethanol production in terms of land use and competition with food agriculture, in addition to

societal and environmental implications such as greenhouse-gas emissions and carbon balance [6] have triggered the transition from 1G to more advanced and beneficial biofuels. Second generation (2G) biofuels are intended to be produced primarily from lignocellulosic biomass, plant materials that have gained importance over the last two decades. Lignocellulose represents the most abundantly and renewable raw material available on earth and its composition is rich in carbohydrates (cellulose and hemicellulose), making it ideal to fill the gaps of 1G feedstocks [7]. The recalcitrance of cellulosic biomass together with land-use concerns have been identified as primary potential barriers to realizing cellulosic ethanol on a large scale [8].

Triticale, a genetic cross between wheat and rye, has shown better grain yields than wheat when cultivated in marginal lands not well suited for wheat production, thus realising potential as an energy crop from combined 1G (grain) and 2G (straw) ethanol production. Triticale features higher biomass and straw production than other cereals like wheat [9; 10] and barley [11] as well as better energy yields per hectare than the available yields from forest biomass, resulting in an efficiently energetic use of land [12]. Triticale world production is in constant growth; around 14.6 million metric tons of grain were produced from 3.9 million ha around the world in 2013 [13], mostly for use as animal feed, while the straw production is even higher with a straw to grain ratio of 2.47 [14]. Triticale straw is usually not utilized for value-adding, but rather left in the field after harvesting.

Whereas 1G ethanol production from triticale grain is similar to other grain-based processes, the application of triticale straw as lignocellulosic feedstock for ethanol production requires development of a pretreatment step, to make the carbohydrate fraction (sugar monomers of C5 and C6) available to enzymes and microorganisms for conversion by fermentation. It is also expected that such pretreatment should enhance the cellulose digestibility of the lignocellulose, minimise sugar loss and consume low amounts of energy [15]. Various pretreatment technologies have been extensively studied on a variety of lignocellulosic feedstocks prior to enzymatic hydrolysis, with their distinctive effects on cellulose, hemicellulose and lignin reported as either advantageous or disadvantageous [16; 17].

Steam explosion (SE) is one of the most promising technologies for pretreatment of lignocellulose [18], primarily due to suitability for application under industrial conditions. SE is mainly characterized by good fractionation of components predominantly in the way of solubilisation of hemicellulose and e enhancement of the digestibility of the material [19] without major changes in lignin. After pretreatment, the pretreated material, commonly referred to as whole slurry, consists

of water and solids (water-soluble and water-insoluble). Water-soluble solids (WSS) are found in pretreatment liquor, together with mostly hemicellulose-derived sugars and sugar degradation products, among other soluble matter. The water-insoluble solids (WIS) are comprised of the digestible cellulose-rich fibres. SE can be performed uncatalyzed or in presence of catalyst (SO_2, diluted H_2SO_4 or CO_2) to increase the removal of hemicellulose (mostly in the way of monomeric sugars) and improve the digestibility of the material [20]; however, uncatalyzed (water-only) SE exhibits interesting advantages, regarding the inclusion of no chemicals except water and high yields of hemicellulose conversion into hemicellulosic sugars with low by-product generation among other features [21].

Uncatalyzed SE is commonly known as “autohydrolysis” due to the catalytic action of water and acetic acids released from xylans, which catalyze hemicellulose hydrolysis. Acetic acid and other acids produced from acetyl or other functional groups of hemicelluloses contribute to the autohydrolysis by creating more acidic conditions taking place during pretreatment [22]. Uncatalyzed SE has been studied with different lignocelluloses such as sugarcane bagasse [23], wheat straw [24] and sunflower stalks [25], although to the authors’ knowledge it has not been studied on triticale straw and more specifically targeted at the release of sugars from the combined pretreatment-hydrolysis process and the extent of sugar degradation that would be potentially expected from autohydrolysis steam explosion of triticale straw.

In this study, uncatalyzed SE was evaluated on straw from a particular triticale cultivar that showed superior performance regarding response to pretreatment in a previous study. A screening of pretreatment conditions in the range 180°C - 200°C and 5 – 15 min was carried out aiming at identifying conditions that lead to the highest recovery of hemicellulose-derived sugars, or the most digestible cellulose in pretreated material or the highest combined sugar yield (CSY) from the pretreatment and enzymatic hydrolysis (EH) process steps as different approaches for downstream application. The selected range of pretreatment conditions were based on pretreatment severities that cover mild and harsh conditions for other genetically related materials such as wheat straw with plenty information in literature [15; 17], due to the lack of reported studies on triticale straw.

The local maxima in sugar yields in these distinct three regions were balanced against the need to limit the production of inhibitors to acceptable levels. The extent of sugar degradation and fermentation inhibitors (hydroxymethylfurfural -HMF-, furfural, formic acid and acetic acid) production during pretreatment was thus analyzed. The results from the different approaches

considered in the study find usefulness for future works on pretreatment optimization where sugar maximization at reduce inhibitors production (sugar loss) are commonly sought.

6.2 Materials and methods

6.2.1 Raw material

Straw from sprint triticale (x *Triticosecale Wittmack*) crop 2010 was obtained from Welgevallen Experimental Station (Stellenbosch University). The straw material came from a field trial planted in the Western Cape Province in South Africa where 20 triticale cultivars were assessed. The average moisture content (MC) of the harvested straw as received was 9.7%. After collection, the straw was baled and stored in a temperature and moisture controlled room set at 20°C and relative humidity of 65% for further processing. The material was coarsely grounded with a Condux-Werk type mill (Wolfgong bei Honou, Germany), sieved to obtain a homogenous sample containing material with a particle size between 3.8-10 mm for pretreatment.

6.2.2 Pretreatment

6.2.2.1 *Steam Explosion Unit*

Steam pretreatment was carried out in a batch pilot steam explosion unit (IAP GmBH, Graz, Austria) equipped with a 19-L reaction vessel and a blow tank constructed of stainless steel and a boiler capable of producing saturated steam up to 40 bar (Figure 6-1). A control panel comprised of a PC based HMI/SCADA system with PLC's was used to control steam conditions, valves operation, as well as the processing temperature and residence time for each steam explosion run. Pressure monitoring inside the reactor was done by a redundant system comprising two Norgren type electronic pressure switches (33D-0863412) with range of sensing up to 40 bars, (Norgren-GmbH Werk Fellbach. Stuttgart, Germany). The bottom of the vessel tapers down gradually to a 78.5 mm line where a ball-type discharge valve is attached to. This valve is capable of opening within less than 0.5 s and is automatically actuated. The pretreatment temperature in the reactor was controlled by supplying steam through automatic manipulation of two air-actuated needle control valves (Samson AG, Frankfurt, Germany). A condensate tank is connected to the bottom of the reactor vessel to allow condensate purge during preheating of the pipes and reactor before feeding the material. Two gate valves (manhole-type) were installed in the blow tank, one in front of and another at the bottom for easy accessibility to remove the exploded material.

6.2.2.2 *Pretreatment*

Uncatalyzed SE was carried out using 500 g of milled triticale straw (9.7% MC) as experimental unit. Initially, the straw was soaked in buckets of 25 L by adding 10 litres of distilled water, left overnight and then dewatered to approximately 65% of residual MC. Pretreatment was carried out by varying temperature in 10°C increments from 180 to 200°C and 5 min increments from 5 to 15 min. Range of pretreatment conditions was based on uncatalyzed SE conditions reported for wheat straw in literature [15;24].

The weighed sample was fed into the reactor and injected with saturated steam at 30 bars. Upon reaching the pretreatment temperature in less than 90 sec, the pretreatment time commenced. After the target pretreatment time was passed, the discharge valve was automatically opened causing an explosive depressurisation inside the reactor and the biomass discharged into the blow tank.

The severities of the different steam explosion pretreatments were determined by using the severity factor (SF), which combines the pretreatment conditions temperature and residence time in a single factor. The following expression (equation 6-1) was used to define SF, as described [26].

$$SF = \log_{10}\left(t \times \exp\left(\frac{T - T_R}{14.75}\right)\right) \qquad (6\text{-}1)$$

Where, t is the residence time (min), T is the process temperature (°C), T_R is the reference temperature which is assumed to be 100°C and the constant 14.75 corresponds to the activation energy value for processes of first order kinetics following Arrhenius law. The pretreatment conditions in this study were in the range of severities 3.05 – 4.12.

6.2.3 Characterization of raw and pretreated materials

The obtained slurry was collected in buckets; 50-g wet sample was taken in triplicate and pressed by using a press filter with a hydraulic jack to separate the liquid fraction from the solids. The pH and volume of the obtained pretreated liquor was then measured.

The solid residue was then washed with excess of distilled water (500 ml) and filtered by a standard laboratory filtration system using filter paper Whatman No. 1 to remove residual sugars and other soluble matter from the solids. The washed solid residue, referred to as water-insoluble

solids (WIS) was then weighed and used for enzymatic hydrolysis. A 5 gram sample of WIS was oven dried at 40°C for 72 h for further chemical composition analysis.

6.2.3.1 *Compositional analysis of solids*

The samples for compositional analysis of the straw and the WIS were milled in a laboratory ultra centrifugal mill model ZM200 basic (Resch GmbH, Germany) and sieved in a vibratory sieve shaker model AS200 basic (Resch GmbH, Germany) to obtain a particle size between 425 and 825 µm and further dried at 40°C for 48 h in a convection oven (EcoTherm, Germany). The structural carbohydrates glucan, xylan and arabinan as well as lignin in the straw were determined with extractive-free milled samples by double acid hydrolysis following the standard laboratory analytical procedures (LAP) for biomass analysis from the National Renewable Energy Laboratory –NREL- [27]. Sugars (cellobiose, glucose, arabinose and xylose) as well as acetic acid acetyl groups were measured by HPLC, as described below. Acetyl group composition was obtained by applying a factor of 0.983 on the acetic acid composition [27].

Extractives and ash contents were determined using the standard NREL LAP standards [28; 29]. Acid-soluble lignin (ASL) content was measured by UV-spectrophotometer at a wavelength of 240 nm [27]. The samples for AIL determination were passed through 0.22 µm filters to remove suspended solids and undesirable turbidity before the spectrophotometric analysis. Filtered samples were diluted with 4% (v/v) H_2SO_4 solution until reaching absorbance readings in the range of 0.7-1.0. An absorptivity (ε) of 55 L g^{-1} cm^{-1} was used to convert absorbance to mass values.

6.2.3.2 *Pretreatment liquor*

Pretreatment liquor and the liquid from washing the solid residue were analyzed for cellobiose and monosaccharides, glucose, arabinose and xylose, as well as sugar degradation products and inhibitors compounds 5-hydroxy-2-methylfurfural (HMF), furfural and formic acid and acetic acid as described elsewhere [30]. All sugars, HMF, furfural, acetic acid and formic acid were measured by HPLC as described subsequently under HPLC analysis subsection. Monomeric sugars yields were calculated by the following expression (equation 6-2).

$$Sugar\ yield_{(monosaccharides)} = \frac{C_{sugar} \times V}{RM} \times 100 \qquad (6-2)$$

Where *sugar yield* denotes either the yield of xylose, glucose or arabinose in monomeric form expressed in grams per 100 g of dry raw material (DRM), C_{sugar} is the concentration as either monomers of xylose, glucose, or arabinose in ($g.L^{-1}$), *V* is the total volume of pretreatment liquor (in Litres) recovered, and *RM* is the weight of raw material (in grams) used for pretreatment (dry basis). The oligosaccharides content in the pretreatment liquor was determined by mild acid hydrolysis by addition of 72% H_2SO_4 to final acid concentration of 4% (w/w) and autoclaving for one hour at 121°C, according to NREL procedure [30]. The concentration of xylose oligosaccharides (XOS), glucose oligosaccharides (GOS) and arabinose oligosaccharides (AOS) were then calculated by subtracting the initial monomer content from the total hydrolyzed content for each sugar and expressed as their monomer equivalent by using conversion factors of 1.136 for the conversion of XOS and AOS to their monomeric sugars and 1.111 for GOS to glucose.

The total yield of glucose, xylose or arabinose was obtained as addition of the sugar monosaccharides and the respective oligomeric sugar present in the pretreated liquor expressed as its monomer equivalent. Similarly, sugars in raw material (theoretical sugars) were expressed in its monomer equivalent to refer to sugar recovery. Hemicellulose-derived sugars yield was defined according to the equation (equation 6-3). All the yields of sugars were expressed in g of sugar (mono or oligosaccharides) per 100 grams of dry raw material (DRM).

$$Yield_{Hemicellulose} = (Total\ xylose\ yield) + (Total\ arabinose\ yield) \qquad (6-3)$$

6.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out on WIS to determine the digestibility of cellulose in the fibres after pretreatment, thus resulting in glucose yield after enzymatic hydrolysis (EH). Two commercial enzymes were used, Spezyme CP (Genencor-Danisco, Denmark) and Novozym 188 (Novozymes A/S, Denmark) with cellulase and β-glucosidase activities of 65 FPU/ml and 700 IU/ml, respectively. An enzymatic solution made of 0.05 M citrate buffer (pH 4.8), sodium azide at 0.02% (w/v) as antimicrobial growth and the enzymes was used for all the enzymatic experiments. Wet WIS from washing was used as substrate with the solution at enzyme loadings of 15 FPU of cellulase (32.21 mg protein) and 15 IU of β-glucosidase (2.02 mg protein) per gram of dried WIS. Protein concentration and activities of the enzymes used were measured by following the methods described elsewhere [31].

All enzymatic experiments were carried out by duplicate with a working volume of 50 ml at 2% of solid loading based on the dried weight of the material in 250-ml Erlenmeyer flasks by incubation for 72 h at 50°C in a water-bath with agitation at 90 rpm. A control of EH using 1 g (dry weight) of Avicel at the same enzyme loadings was included. After completion the time, liquid samples were taken and then prepared for monomeric sugars (glucose, xylose and arabinose) analysis, as described for WIS analysis. Cellulose digestibility was defined as the percentage of cellulose (glucan) converted to glucose after incubation for 72 h with cellulase and β-glucosidase enzymes, and referred to as conversion ratio of cellulose by the following expression (equation 6-4).

$$Digestibility\ (\%) = \frac{glucose\ from\ EH \times V\ \times 0.9}{glucan_{WIS} \times m} \times 100 \qquad (6-4)$$

Where *glucose from EH* is the concentration of glucose in the supernatant after completion of EH ($g.L^{-1}$), *V* is the volume of EH supernatant (L), $glucan_{WIS}$ is the composition of glucan in the WIS used for EH (g/100 g WIS), and *m* is the oven-dry mass of WIS used for EH (g). A correction factor of 0.9 was used for the conversion of monomeric glucose to glucan. Glucose release after EH of 100 g of WIS was calculated by (equation 6-5), and expressed in g/100 g DRM:

$$Glucose\ yield\ (\%) = Glucose\ \times 5 \times WIS_{recovered} \times 100 \qquad (6-5)$$

Where *glucose* is the concentration of glucose from the HPLC analysis ($g.L^{-1}$) and $WIS_{recovered}$ is the WIS recovered after the pretreatment of 100 g DRM. A factor of 5 was employed considering the solid loading used for EH, the volume of enzymatic solution loaded and the dilution factor used for preparing the samples for HPLC analysis. The yield of xylose (in g/100 g DRM) after EH were also calculated by using the same expression as for glucose yield with the same values for the members of the equation except for (glucose) which was substituted for the respective xylose concentration in $g.L^{-1}$.

The combined sugars yield (CSY) was calculated as the sum of the xylose, glucose and arabinose in monomeric and oligomeric forms in pretreated liquor and in monomeric form obtained from EH, and was expressed in gram of total sugars per 100 g DRM (equation 6-6).

$$CSY = [Y_X + Y_G + Y_A]_{mono} + [Y_X + Y_G + Y_A]_{oligo} + [Y_X + Y_G + Y_A]_{mono\ in\ EH} \qquad (6-6)$$

Where Y_X, Y_G, *and* Y_A are respectively the yields of xylose, glucose and arabinose in the monomeric and oligomeric fractions in the pretreated liquor and monomeric fraction from EH expressed in g/100 g DRM.

6.2.5 HPLC analysis

Monomeric sugars as well as formic and acetic acids were analyzed on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa), with column temperature set to 65°C, a mobile phase of 5 mM H_2SO_4 and a flow rate of 0.6 ml/min. Sugar concentrations were measured with a RI detector (Shodex, RI-101, Munich, Germany) operated at 45°C. Additional HPLC analyses were conducted since xylose and galactose, and mannose-arabinose co-eluted in the described H-column. To perform these analyses the HPLC system was equipped with an Xbridge™ Amide column (Waters Corporation, Massachusetts, United States) (4.6 × 250 mm, 3.5 µm particle size) and a Xbridge™ Amide precolumn (Waters) set at 30°C using 0.05% ammonium hydroxide in water (A) and 0.05% ammonium hydroxide in 90% acetonitrile (B) as mobile phase with a flow rate of 0.7 ml/min. Sugars were detected by a Varian 380-LC evaporative light-scattering detector (Agilent Technologies, California, United States).

HMF and furfural were analysed on a Phenomenex Luna C18(2) reversed phase column equipped with a Phenomenex Luna C18(2) precolumn (Separations, Johannesburg, South Africa) with column temperature set to 25°C and a flow rate of 0.7 ml/min. The mobile phases used for elution were 5 mM trifluoroacetic acid in water (A) and 5mM trifluoroacetic acid in acetonitrile (B). HMF and furfural concentrations were measured with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm.

6.2.6 Statistical Analysis

A Pairwise comparison analysis and one-way-analysis of variance (ANOVA) at a significance level of 95% were performed across all the level of pretreatment on the responses: yields of monomeric and oligomeric sugars and inhibitors released from pretreatment, WIS and monomeric sugars after enzymatic hydrolysis and total sugars from the combined process. Cellulose digestibility and chemical composition of the WIS were also analysed. The Analysis looked for differences statistically significant of each response at the different levels of the pretreatment conditions. The statistical analysis was carried out using Design Expert software version 8.0.3 (State Ease Inc., Minneapolis, United States).

6.3 Results and Discussion

6.3.1 Raw material

The chemical composition of triticale straw raw material was analysed before pretreatment, as is shown in Table 6-1. The main sugars in the straw (glucan, xylan and arabinan) were determined, while minor sugars such as galactan and mannan were present at very low content, and were thus not given further consideration. Acetyl groups and ash (as inorganic matter) were also quantified as these components have shown to affect lignocellulose pretreatment [32]. Acetyl groups play an important role in catalysing the xylan depolymerisation, by serving as precursors for the formation of acetic and other acids during pretreatment. Additionally, extractives content in the straw should be considered when measuring the mass loss in solids during pretreatment.

The overall carbohydrate composition of the straw found in this study (64%) was in agreement with other reported compositions for triticale straw [30; 31]. Total lignin (quantified as the sum of insoluble and soluble lignin contents; 13.5%) and ash content (1.5%) were present in lower levels than lignin (17-2%) and ash (4.4-7.5%) contents reported for triticale straw [30]. Acetyl group content in the straw (3.3%) showed to diverge 1.8-fold with respect to composition of 1.82% for triticale straw reported previously [33].

6.3.2 Steam pretreatment

Three different approaches for maximisation of sugar recovery by combination of SE pretreatment with subsequent enzymatic hydrolysis were considered, each offering an alternative strategy for process optimisation, based on downstream applications. Firstly, the pretreatment conditions leading to maximization of hemicellulose solubilisation (combination of xylose and arabinose) and recovery in pretreatment liquor were identified. Secondly, the conditions that provide the highest digestibility of cellulose in the pretreated fibres were determined and finally, the pretreatment conditions that resulted in the highest yields of sugars for the combined pretreatment-hydrolysis process (combined sugars yield, CSY) were found, while also taking into consideration the formation of inhibitors.

Uncatalyzed SE of triticale straw was carried out by varying pretreatment temperature in the range 180 - 200°C and residence time from 5 - 15 min. The range of pretreatment conditions included severities from 3.58 to 3.88, to favour the recoveries of hemicellulose-derived sugars in the pretreatment liquor, and severities around 3.94, to maximise the glucose yield from EH.

6.3.2.1 *Pretreatment liquor*

Autohydrolysis (water-only) SE pretreatment is characterized by high hemicelluloses solubilisation, which is favoured by acidic conditions during pretreatment caused by the formation of organic acids, mainly acetic acid, from the feedstock [34]. Figure 6-2 shows the main sugars present in the pretreated liquor as either monomers or soluble oligomers. As expected, the sugars recovered in pretreatment liquor from all the (autohydrolysis) process conditions were predominantly oligosaccharides. The recovery of glucose in the pretreatment liquor was the lowest among the quantified sugars and it was mainly released in form of glucose oligosaccharides (GOS). The total yield of glucose from pretreatment remained in the range of 1.5 to 3 g of glucose/100 g DRM for all the conditions. The recovery of XOS was greater than the other monomeric or oligomeric sugars for all the tested conditions, and reached the maximum yield of 10.6 g/100g DRM at 200°C and 5 min, although comparable yield was also found at 180°C and 10 min (Figure 6-2). The lowest XOS yield of 4.4 g/100 g DRM was found at the most severe conditions (severity 4.12) and was comparatively similar to the yield at the mildest conditions (severity 3.05), which represented only 16.6% recovery of the hemicellulose in the feedstock. The output of XOS was poorly related to severity factor whereas AOS, although low in yields across all the pretreatment conditions, showed more consistency in decreases as severity factor increased. On the other hand, the severity factor showed to be robust enough correlating monomeric xylose with a regression coefficient (R^2) 0.90, and less related to monomeric arabinose with R^2 0.44 (data not shown).

Increasing either the temperature and/or residence time of pretreatment had a positive impact on the recovery of sugars from hemicellulose, with the highest hemicellulose recovery achieved at the highest temperature and longest the time, with the exception of 15 min where some hemicellulose loss possibly by sugar degradation was observed. Except for the severity 3.53 and up to a severity of 3.64, the recovery of hemicellulose-derived sugars was positively correlated to the severity factor (Figure 6-2). Pretreatment severities of 3.82 and beyond led to hemicellulose degradation. Thus the severity factor showed poor capability to predict hemicellulose-derived sugars yield in the range of uncatalyzed SE conditions tested in this study. The observed trend in reduction of hemicellulose-sugars yield may most probably be the outcome of pentoses degradation, mainly xylose, which has been shown to be related with increments in pretreatment residence time [35].

The maximum recovery of hemicellulose-derived sugars was 52% of the theoretical maximum, based on the chemical composition of the straw (27.2%, Table 6-1), and found at severity of 3.64 (200°C - 5 min), with nearly 70% of liberated sugars as XOS. Slightly inferior hemicellulose recovery,

around 47.3% of the theoretical, was also found at a severity of 3.35 (180°C – 10 min) with nearly 74% of sugars as XOS.

Although autohydrolysis SE of triticale straw has not been reported, similarities with the preferred pretreatment conditions reported for genetically related cereal straws, such as wheat, were found. Relatively low recoveries of hemicellulose sugars by uncatalyzed SE has been also reported for these feedstocks [22; 24], indicating a limitation in the recoveries of xylose that can be achieved without the addition of an acid catalyst. The maximum recovery of hemicellulose-derived sugars (xylose and arabinose) from uncatalyzed SE of triticale straw (~70%) was comparable to the range of 57-61% for maximum recovery of sugars from hemicellulose reported for wheat straw [24]. Preferred conditions for maximum recovery of hemicellulose-derived sugars from SE pretreatment of wheat straw were reported as 200°C and 10 min [24]. A similar pretreatment temperature of 200˚C, but shorter residence time of 5 min, were found to maximise hemicellulose sugar recovery in the present study (Figure 6-2), possibly due to a higher xylose content in triticale straw than in wheat straw, which may indicate that hemicellulose is more readily accessible during SE. Differences in the technical configurations between steam explosion units (small-scale in [24] versus pilot-plant in the present study) could have also contributed in the observed divergences. Others factors that could have effects on the response to SE pretreatment of the triticale straw, are most presumably the moisture content for pretreatment and the configuration of the SE units used (80% MC in [24] versus 65% in the present study). The presence of more water in contact with the material will facilitate even more the opening of the cell-walls during the depressurisation taking place in steam explosion pretreatment, while different SE units also vary regarding condensate formed during runs, which will impact differently on the outcomes of the experimentation.

6.3.2.2 *Sugar degradation and inhibitors*

Biological conversion of sugars in the pretreatment liquor, to maximise product yields per ton of lignocellulose, is inevitably impacted by the presence of inhibiting compounds in these streams formed during pretreatment. The inhibitors formation of pretreatment should be minimized to an extent where the downstream biological processes such as enzymatic hydrolysis and fermentation are not highly affected, as well as preserving sugars – thus also avoiding the need for detoxification of lignocellulose hydrolysates.

The main inhibiting compounds from sugar degradation, HMF, furfural and formic acid, formed during uncatalyzed SE as a result of degradation of main sugars in straw, in combination with acetic

acid formed by the hydrolysis of acetyl groups in hemicellulose, were quantified. These monitored compounds were measured to realize the extent of sugar loss in first instance and their levels of toxicity expected. Thus other compounds such and lignin-derived phenolics (e.g. vanillin and tannic acid) expected from the softening and partial depolymerizations of lignin during pretreatment were not measured. Table 6-2 shows the yields of inhibitors (g/100 g DRM) across the pretreatment conditions and the equivalent concentrations in gram per litre. Additionally, statistical analysis was performed on the experimental data to establish significant differences between the responses across the different levels of pretreatment conditions.

The variations in the pretreatment severity for the SE conditions evaluated were reflected in the formation of inhibitory compounds. Furfural from xylose was the predominant sugar degradation product, with a maximum yield of 0.83 g/100 g DRM (1.84 $g.L^{-1}$) under the conditions tested (Table 6-2). This value was substantially higher than the maximum yield of HMF from glucose degradation of 0.22 g/100 g DRM (0. 49 $g.L^{-1}$. Table 6-2), which was consistently the sugar degradation product with the lowest yield and consequently present at lowest concentration; apparently due to low levels of glucose release during SE (Figure 6-2)

Increases in both the pretreatment temperature and residence time substantially resulted in increased production of furfural (Table 6-2). Furfural production was linearly correlated with the severity factor with a regression coefficient R^2 0.78 (data not shown). Such sugar degradation could explain the observed reduction in the recovery of hemicellulose-derived sugars when increasing pretreatment residence time up to 15 min.

The production of formic acid, as the result of further degradation of HMF and furfural, also showed linear dependence of the severity factor (R^2 0.90; data not shown). Formic acid was present at higher yields than HMF under all of the tested conditions, and at lower yields than furfural at a residence time of 15 min (at all tested temperatures). This may suggest that larger xylose degradation could take place at the longest residence as a result of higher hydrolysis rate leading to more oligomeric xylose converting into unstable monomers that finally decompose to furfural. Thus it is likely that formic acid was mostly formed from furfural, due to the low values of glucose release and corresponding limits in HMF formation, which could result in an apparent decrease in furfural yields due to conversion to formic acid.

Acetic acid was released at higher yields than the inhibitors from sugar degradation, ranging between 0.27 and 1.38 g/100 g DRM, with yields increasing with pretreatment severity, under all conditions tested (Table 6-2) The production of acetic acid in the pretreatment liquor is thus not only determined by the (high) acetyl group content of triticale straw, as a result of a high xylan content, but also the severity of pretreatment (R^2 0.92 for acetic acid correlation with pretreatment severity), determining the extent of acetyl group hydrolysis under acidic pretreatment conditions. The release of acetic acid may also take part as catalyst in further hydrolytic reactions of glucose or xylose degradation [19].

Although it was found that recovery of hemicellulose-derived sugars in the pretreatment liquor was maximised by increasing severities of SE pretreatment (up to 3.64, Figure 2), these conditions also resulted in higher formation of inhibitory compounds (Table 6-2. The extent of sugar degradation during SE pretreatment of triticale straw under these referred conditions resulted in pretreatment liquor with concentrations of 0.1, 0.34, 0.47 and 1.90 $g.L^{-1}$ HMF , furfural, formic acid and acetic acid, respectively ((Table 6-2). In particular, formic acid at levels about 326 mM has been reported to inhibit 5-20% of the cellulolytic activity in commercial cellulase preparations [36]; yet much lower formic acid concentrations below 12.4 mM ($MW_{\text{formic acid}}$ = 46.02 g. $gmol^{-1}$ applied on maximum concentration of 0.57 $g.L^{-1}$ (Table 6-2) found in the present study. Although these concentrations are lower than reported inhibitory levels for enzymatic hydrolysis and fermentation [37-39], further research is needed to examine the toxicity of the material during EH and/or SSF at high solids loading.

6.3.2.3 *Insoluble solid recovery*

Pretreatment is primarily aimed at providing solids that are rich in highly-digestible cellulose, thus suitable for subsequent enzymatic hydrolysis and fermentation. The highest digestibilities of cellulose in solids from SE pretreatment are commonly obtained at the highest severities, where larger portions of the hemicelluloses have been dissolved in the pretreatment liquor. The associated increase in pretreatment severity also has a negative impact on WIS recovery, and hence on the final sugar yield from pretreatment followed by enzymatic hydrolysis.

The yield of WIS and its chemical composition are shown in Figure 6-3. The WIS was quantified as the percentage of dry insoluble solid residue recovered after pretreatment per 100 g DRM. As expected, the recovery of WIS was dependent on the pretreatment severity, with a substantial

reduction from 90.1 to 60.3% when the severity was increased from 3.05 to 3.65. Higher severities did not result in significant further reduction of the WIS recovery.

These WIS recoveries compare well with the values of 48.6, 45.6 and 45.1% obtained for uncatalyzed SE of wheat straw at temperatures of 180, 190 and 200°C and residence time of 10 min [24]. These differences could be due to higher levels of hemicelluloses solubilisation into the pretreatment liquor during SE of wheat straw and consequently more mass solubilisation during pretreatment. The latter may be a result of the higher moisture content of 80 % for the wheat straw raw material used for SE pretreatment, compared to a moisture contents of 65 % for the triticale straw used in the present study.

Compared to the raw material (Table 6-1), the pretreated solids were enriched in glucan and lignin due to hemicelluloses solubilisation. The hemicelluloses content on the WIS varied from 19.38 to 4.14% which corresponds, respectively, with 64.2 and 8.9% of the hemicelluloses on the raw material (total of 27.2%). Glucan content in WIS corresponded to 85 – 99% of the glucose present in the triticale straw feedstock. The highest glucan content in the WIS (54.63%) was found at the most severe pretreatment conditions (200°C and 15 min). Regarding the lignin content, the values varied from 22 to 30.50% of the dry weight of the WIS, what is in line with reported values from uncatalyzed SE of wheat straw [34]. It has been reported that SE induces structural changes in the lignin, but lignin is not extensively removed by SE at the range of conditions screened in this study [40, 41].

6.3.3 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out on WIS to assess the effect of the pretreatment conditions on the digestibility of cellulose in the pretreated solids, and thus identify pretreatment conditions that maximise cellulose digestibility. The digestibility was defined as the percentage of glucan in WIS converted to glucose after 72 h of EH. A low solid loading of 2% (w/v) was selected to prevent enzyme end-product inhibition that could mask differences in recalcitrance due to severity of pretreatment.

Digestibility of the pretreated solids and the yields of monomeric glucose and xylose after EH are shown in Figure 6-2 and the Summary of the statistical analysis for cellulose digestibility is shown in Supporting Information (Table 6-4). The cellulose digestibility in the WIS was in the range of 43.3 – 95%, for the pretreatment severities evaluated (3.05 to 4.12). Digestibility was highly dependent on

pretreatment temperature and less dependent on residence time, while the most severe pretreatment conditions resulted in the highest digestibility which is in line with uncatalyzed wheat straw [24]. The least severe pretreatment condition (180°C – 5 min) resulted in cellulose digestibilities under 50%, while severities of 3.65 or higher were required to achieve glucan conversions of 70% or more. Pretreatment severities greater than 3.8 were required in order to obtain digestibilities over 80% (Figure 6-2). Digestibility of more than 90% was observed for pretreatment at 200°C – 10 min and 200°C - 15 min (Figure 6-2).

According to observed trends it would seem that completely digestible solids from SE pretreatment of triticale straw (nearly 100%) could be achieved at temperatures above 200°C, similar to values reported for wheat straw [24]. However, the very short residence times required by pretreatment at such high temperatures, to avoid sugar degradation, are impractical to achieve [24]. The removal of xylan during pretreatment had a positive impact on the digestibility and glucose yield from EH, as found for SE of wheat straw [24]. The increase in the removal of xylan during pretreatment increased the glucose yield from EH, despite the higher pentosan degradation observed at high severities such as 3.94 and 4.12 (Table 6-2).

Glucose and xylose yields from enzymatic hydrolysis were also calculated as grams of sugar per 100 g DRM, taking into account the WIS recovery from pretreatment and sugar release during EH of WIS (Figure 6-2). Glucose yields ranged from 17.9 to 35.9 g /100 g DRM for the pretreatment conditions evaluated. The maximum yield of glucose (35.9 g /100 g DRM) was achieved at 200°C and residence times of either 10 or 15 min, although a higher digestibility (95%; severity 4.12) was also found compared to 200°C – 10 min (92%; severity 3.94). However, negative impact on WIS recovery was also observed when increasing pretreatment severity from 3.94 to 4.12. Pretreatment at 200°C – 10 min (severity 3.94) would be preferred as condition to maximise the yield of glucose from enzymatic hydrolysis, due to less severe pretreatment requirements, thus minimising inhibitor formation. SE pretreatment at a temperature of 200°C showed the highest glucose yields (> 34.6 g /100 g DRM) for all the residence times, which were considerably higher than glucose yields at 180 and 190°C.

High cellulose conversion yields were observed in the present study (up to 95% as shown in Figure 2). The use of low solid loadings in EH tests have shown to increase cellulose conversion yield of SE pretreated wheat straw (190°C – 10 min) of 70% at 10% w/v solid loading [24] up to 92.5% with EH tests performed at 5% solid loading (using similar cellulase loading of 15 FPU/g substrate) [42].

Thus possible product inhibition and mixing problems limiting yields at high substrate loadings may be minimized at low solid loading as performed in the present study (2% w/v), which assists us in differentiating variation due to pretreatment.

The release of xylose from WIS during enzymatic hydrolysis was attributed to the xylanase side-activity typically present in cellulose cocktails [31]. The highest xylose yields from EH of WIS were 11.3 and 10 g/100 g DRM at pretreatment severities of 3.36 and 3.35, respectively (Figure 6-2). The lowest yields were observed at the harshest pretreatment conditions with 2.5 g/100 g DRM as the lowest yield at the more severe condition (severity 4.12).

6.3.4 Combined sugar yield and mass balance

The combined sugar yield (CSY) is based on the total yields of sugars (mostly glucose+xylose) obtained from both pretreatment liquor and subsequent EH of the water insoluble solids, calculated on the basis of 100 g of dry raw material. Maximisation of CSY is often considered as a preferred target for optimisation of pretreatment conditions, representing an acceptable compromise between hemicelluloses-derived sugars recovery and digestibility of the cellulose fraction in the WIS. Furthermore, maximisation of CSY would also maximise ethanol yields and concentrations when utilising a xylose-consuming organism in the subsequent hydrolysis-fermentation step(s).

The CSY values for the range of evaluated pretreatment conditions are shown in Figure 6-2 and the corresponding summary of the statistical analysis of this response is provided as supporting information (Table 6-3). The measured CSY ranged from 33.3 to 55.6 g of sugars/100 g DRM for the tested pretreatment conditions. The maximum recovery of CSY of theoretical, based on the chemical composition of triticale straw, was 76.5% found at 200°C - 5 min (severity 3.64).

The relation between CSY and pretreatment severity followed a similar trend as the impact of pretreatment severity on glucose yield from EH, mainly because glucose from EH represented the major mass fraction in the CSY. As observed in Figure 6.2, pretreatment temperatures higher than 180°C required longer residence time of 10 minutes to achieve high CSY (55.31 g/100 g RM), whilst residence times of 5 min were sufficient to reach high CSY (53.51 - 57.4 g/100 g RM) at higher temperatures. The observed tendency of CSY to decrease at the longest residence times may suggest significant negative effects of the pretreatment severity on the sugars integrity, which lead to sugar degradation and consequently reduce the overall sugar yield. It was also important noticing

that comparable CSY of nearly 52 g/100 g DRM was obtained at 180°C-10 min and 190°C - 5 min representing similar severities of 3.36 and 3.35, respectively (Figure 6-2).

Pretreatment conditions that meet the specific goals of providing maximum hemicellulose-derived sugars (200°C – 5 min; severity 3.64), good digestibility of WIS with high mass yield of pretreated solids (200°C – 10 min; severity 3.94) and maximum CSY (200°C – 5 min; severity 3.64) were compared in terms of sugar loss by mass balances and given as supplementary information (Figure 6-4). These calculations indicate the amount of sugars in the raw material that could not be accounted for after the pretreatment-hydrolysis steps, considering the measured yields of sugars in solid and liquid products. The recovery of sugars in the pretreatment liquor was increased with pretreatment at 200°C – 5 min compared to 200°C –10 min. For the pretreatment condition 200°C – 5 min, leading to maximization of both hemicellulose-derived sugars and CSY, the total amount of sugar unaccounted for after pretreatment was 15.2% of the sugars in the raw material, of which nearly 92% was hemicellulose. Pretreatment at 200°C – 10 min, which gave high digestibility of WIS with acceptable levels of inhibitors, resulted in 27.5% of the sugars in the raw material being unaccounted for, of which nearly 76% was hemicellulose. The amount of glucose either converted into degradation products or otherwise unaccounted for, therefore remained rather low, indicating the “preservation” of glucose during pretreatment, while it remained in water-insoluble form. HMF and furfural formation for pretreatment at 200°C-10 min (severity 3.94) were 3.8- and 3.3-fold higher compared to 200°C-5 min (severity 3.64) (Figure 6-4), whereas 18% more formic acid (0.57 $g.L^{-1}$) was produced from pretreatment to realize maximum digestibility (Figure 6-4). The increase in pretreatment severity was thus reflected in the increase in the amounts of sugar degradation products.

The mass amounts of sugar mass degradation products, converted into the amounts of sugar that these quantities represented, could not explain the full amount of unaccounted sugars after pretreatment. This indicated the degradation of sugars to products that were not measured, possibly as volatiles leaving the SE unit through the vent out after the release of pressure. Similar amounts of hemicellulose unaccounted for after SE pretreatment of wheat straw have been reported, suggesting the high volatilization of furfural and recondensation reactions as possible causes [24]. These observations have been also found in other studies where fractions of the hemicellulose degradation and volatile compounds were lost in the outlet steam after SE [43].

6.4 Conclusions

In this study a screening of steam explosion pretreatment conditions for triticale straw was carried in order to identify conditions that favour different targets: maximization of the hemicellulose-derived sugars recovery in the pretreatment liquor, improvement of digestibility of pretreated solids or maximization of the combined sugars from the pretreatment-hydrolysis process.

Maximization of the yields of hemicellulose-derived sugars in the pretreatment liquor and maximisation of the CSY converged into a single pretreatment condition (200°C – 5 min; severity 3.94) within the range of conditions investigated (3.05 - 4.12). All of the preferred pretreatment conditions provided hemicellulose-derived sugars primarily in oligomeric form, which would require extra steps of depolymerisation if fermentation to ethanol is the final target for this stream of sugars. The preferred pretreatment temperature to maximise hemicellulose-derived sugars in the pretreatment liquor for uncatalyzed SE of triticale straw was comparable to reported values for wheat straw. Uncatalyzed SE was effective in improving substantially the digestibility of the material as one of the main targets at the pretreatment of biomass; however, high requirements in severity and sugar loss should be expected.

The main inhibitors derived from sugars degradation monitored in this study were below levels of toxicity reported for enzymatic and fermentation process, although future works on a wider spectrum of inhibitors from SE of triticale straw may account for the presence of other inhibiting compounds of importance such lignin-derived phenolics (e.g. vanillin and tannic acid).

Further studies on pretreatment optimization of triticale straw to maximize any of these goals, i.e. the recovery of hemicellulose sugars, cellulose digestibility or the CSY, may be targeted at a pretreatment temperature of 200°C and residence times between 5 and 10 min, or alternatively pretreatment severities between 3.64 and 3.94 that enclose the optimum area of pretreatment.

Acknowledgements

The authors would like to thank the Technology Innovation Agency (TIA), an entity of the Department of Science and Technology (DST) of the Republic of South Africa, for their financial support of this work, as well as the Claude Leon Foundation. The authors extend the acknowledgement to Genencor and Novozymes for kindly providing the enzymes preparations. Dr. Willem Botes from Department of Genetics, Stellenbosch University, is also acknowledged for supplying the straw material subject of this study.

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Table 6-1: Chemical composition of triticale straw

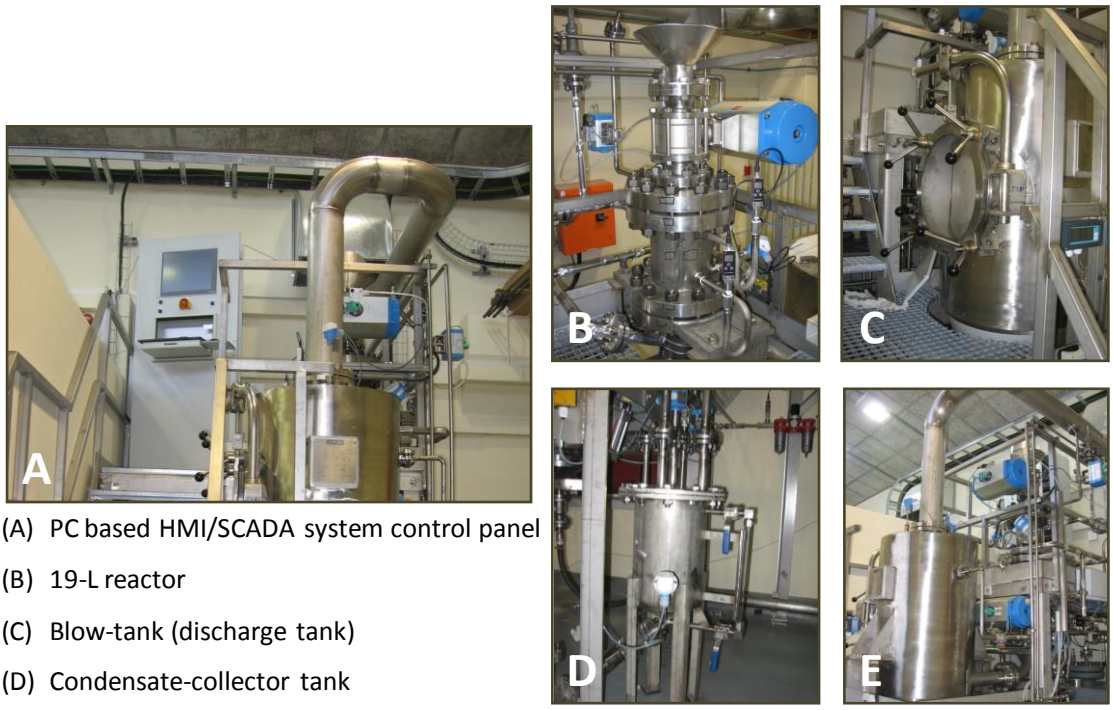
Carbohydrates	Composition (g/100 g dry basis)
Glucan	37.6 (±1.52)
Xylan	25.0 (±0.93)
Arabinan	2.2 (±0.14)
H_2O-extractives	9.0 (±0.28)
EtOH-extractives	2.0 (±0.14)
Insoluble-lignin	13.2 (±0.98)
Soluble-lignin	0.3 (±0.03)
Acetyl groups	3.3 (±0.41)
Ash	1.5 (±0.35)

Composition in dry basis. Standard deviation in brackets.

Table 6-2: Yields of inhibitors in pretreatment liquor from uncatalyzed steam explosion

Temperature (°C)	Time (min)	Severity factor (Log Ro)	**Acetic acid** (g/100 g DRM)	Sugar degradation products expressed in g/100 g DRM (and $g.L^{-1}$)		
				HMF	**Furfural**	**Formic acid**
180	5	3.05	0.27±0.041 (0.72)^{a}	0.01±0.001 (0.02)^{a}	0.02±0.001 (0.05)^{a}	0.06±0.011 (0.17)^{a}
	10	3.36	0.46±0.020 (1.08)^{b}	0.02±0.001 (0.04)^{a,b}	0.06±0.010 (0.15)^{a,b}	0.09±0.010 (0.21)^{a,b}
	15	3.53	0.72±0.181 (2.06)^{c}	0.07±0.060 (0.21)^{c}	0.23±0.150 (0.65)^{c}	0.14±0.021 (0.41)^{a,c}
190	5	3.35	0.35±0.001 (0.97)^{a,b,d}	0.01±0.001 (0.03)^{a,b,d}	0.03±0.001 (0.09)^{b,d}	0.07±0.001 (0.18)^{b,d}
	10	3.65	0.59±0.050 (1.48)^{e}	0.02±0.001 (0.06)^{a,b,e}	0.11±0.010 (0.27)^{a,b,e}	0.14±0.021 (0.35)^{e}
	15	3.83	0.92±0.111 (1.85)^{c,f}	0.06±0.010 (0.12)^{e,f}	0.38±0.050 (0.76)^{b,f}	0.17±0.010 (0.34)^{e,f}
200	5	3.64	0.75±0.060 (1.90)^{c,f,g}	0.04±0.001 (0.10)^{a,b,d,e,f,g}	0.13±0.020 (0.34)^{e,g}	0.18±0.011 (0.47)^{g}
	10	3.94	1.05±0.011 (2.94)^{h}	0.14±0.010 (0.40)^{h}	0.46±0.041(1.29)^{h}	0.20±0.011 (0.57)^{h}
	15	4.12	1.38±0.050 (3.07)^{h,i}	0.22±0.010 (0.49)^{i}	0.83±0.081 (1.84)^{i}	0.24±0.001 (0.54)^{h,i}

Values of yields ± standard deviation of three replicates. Values in parenthesis represent the equivalent concentration in $g.L^{-1}$. DRM stands for dry raw material. The values in the columns for each compound having similar superscript letters do not differ between each other at a significant level of 0.05.



(A) PC based HMI/SCADA system control panel

(B) 19-L reactor

(C) Blow-tank (discharge tank)

(D) Condensate-collector tank

(E) Vent to the atmosphere

Figure 6-1: Steam explosion pretreatment unit (IAP GmbH) used for pretreatment of triticale straw.

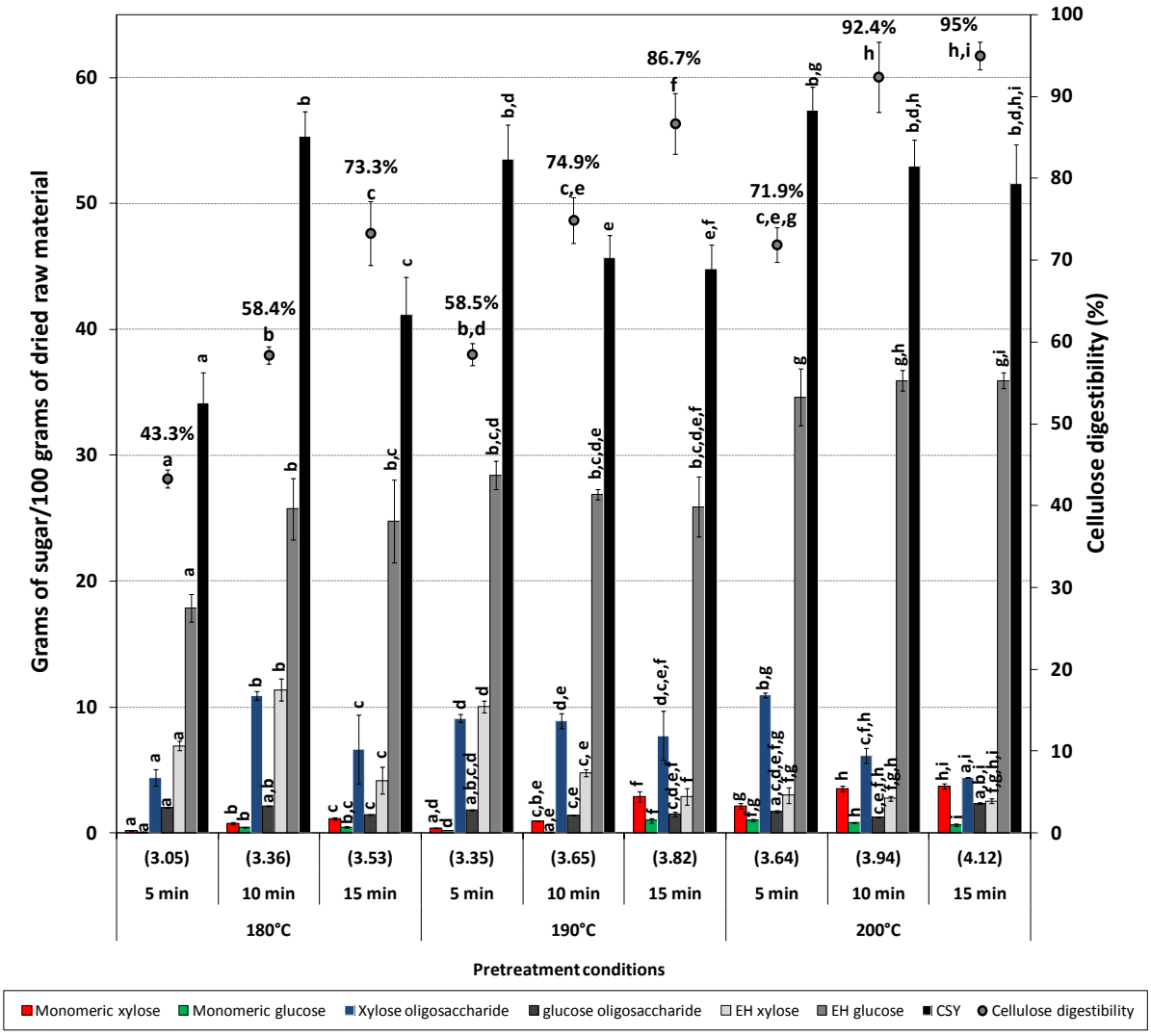


Figure 6-2: Yields, expressed as g/100 g raw material, of monomeric and oligomeric sugars in pretreatment liquor (xylose and arabinose, glucose), monomeric xylose and glucose from enzymatic hydrolysis (EH) and the combined sugars (CSY) of the whole process from pretreatment of triticale straw. Cellulose digestibility (%) of the pretreated solids is represented as comparison. Values in brackets represent the severity of the pretreatment condition. Letters on top of the block bars are given from Pairwise analysis. Comparisons for statistical significance should be done by comparing each yield with respect to the same yield at other pretreatment conditions. Yield bar having similar letters below do not differ between each other at a significant level of 0.05.

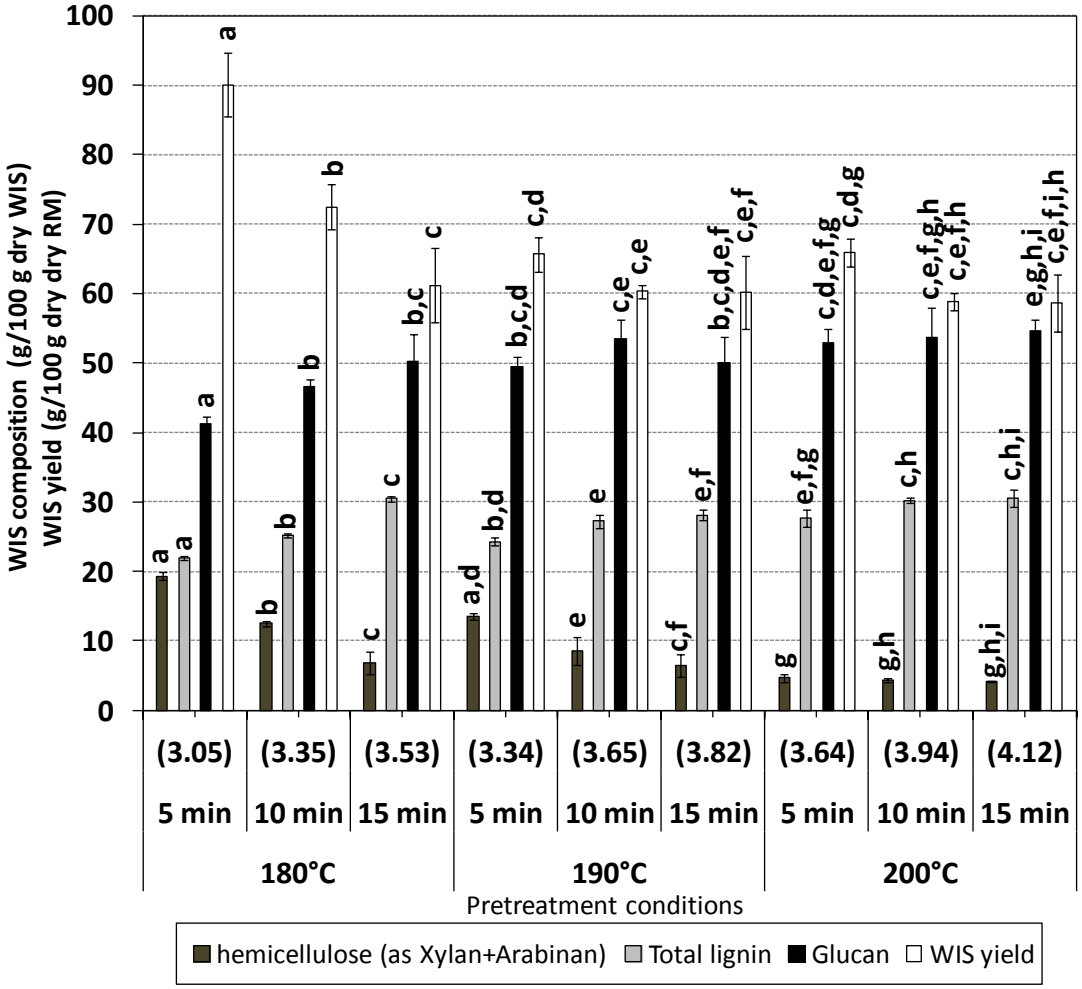


Figure 6-3: Insoluble solids recovery and composition of water insoluble solids (WIS) from SE of triticale straw. Values in brackets represent the severity of the pretreatment condition. Letters below the block bars are given from Pairwise analysis. Comparisons for statistical significance should be done by comparing each yield with respect to the same yield at other pretreatment conditions. Yield bar having similar letters below do not differ between each other at a significant level of 0.05.

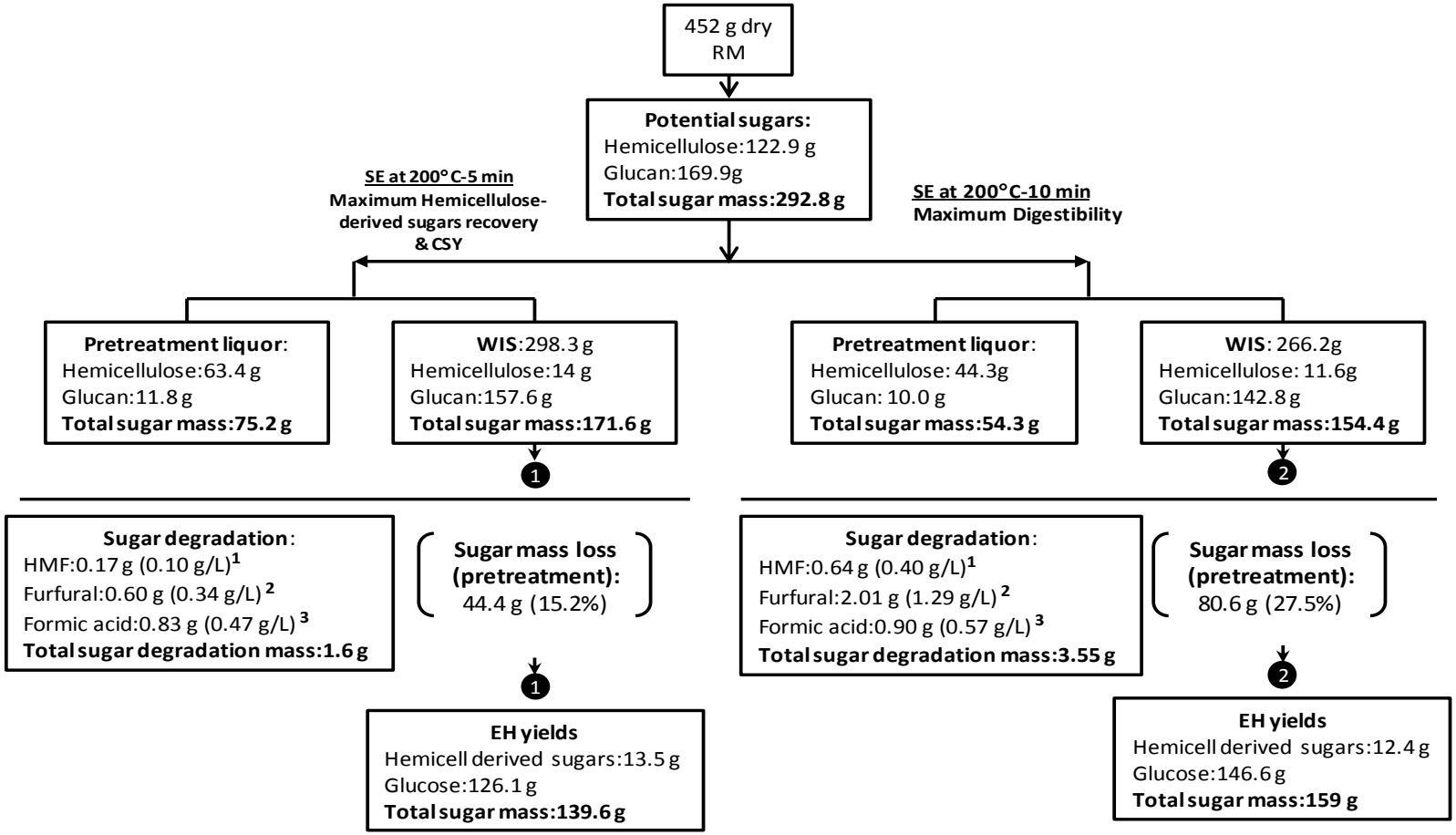


Figure 6-4: Mass balance of sugars and sugar degradation products (in grams) from uncatalyzed steam explosion pretreatment of triticale straw followed by enzymatic hydrolysis (EH) of the WIS. [1,2,3] correspond to the equivalent concentration (in grams per litre) of inhibitors of HMF, furfural and formic acid, respectively.

Chapter 7

7 Impact of cultivar selection and pilot-plant pretreatment optimisation on the combined sugars output of straw from triticale cultivars

The entire content of Chapter 7 was structured as two manuscripts to be submitted for publication to Fuel and Green Chemistry. The unified document of the submission versions is presented here.

Title:

"Optimization of steam explosion pretreatment for sugars production from triticale straw"

Authors: Roberto A. Agudelo, Maria del Prado Garcia-Aparicio, and Johann Görgens

Department of Process Engineering, Stellenbosch University, Private Bag X1, Matieland, 7602, Stellenbosch, South Africa.

Contribution to the study objectives

Chapter 7 addresses the **Objective 3**: Develop predictive models with statistical significance for improved combined sugars yield from steam explosion pretreatment at pilot plant scale and enzymatic hydrolysis of the top three (preferred) triticale straws.

The results systematically achieved in **chapters 4** and **5,** regarding the selection of the top 3 performer straws, as well as the results disclosed in **chapter 6** on the statistically identified areas of pretreatment conditions for sugar maximisation, were applied in the present chapter. Pilot-scale steam explosion was optimised with straw samples from each of the three preferred cultivars, to identify differences between cultivars in sugar yields from the combined pretreatment-hydrolysis process and study the impact of pretreatment optimisation on improved sugar yield. Optimisation was performed to maximise the combined sugar yields from pretreatment-hydrolysis, while limiting inhibitor concentrations to acceptable values. The outcomes of the present chapter were consequently applied in **Chapter 8,** where different strategies of solid loading were followed into the integrated configuration SSF to experimentally evaluate the ethanol yield from straw cultivars under optimised pretreatment conditions. Finally the impact of pretreatment optimisation on experimental ethanol yields was observed.

Summary of findings in present chapter

Uncatalysed and SO_2-catalysed steam explosion (SE) pretreatments at pilot-plant scale followed by enzymatic hydrolysis were optimised by Response Surface Methodology for maximum yield of combined sugars (CSY) constrained to low inhibitors production. Predictive models with statistical significance were developed to describe CSY as the primary pretreatment response, over the range of conditions tested for each straw and pretreatment mode. Models for glucose yield from enzymatic hydrolysis (EH glucose) and yield of hemicellulose derived sugars were also developed. The models were used to predict pretreatment conditions to maximise CSY, as well as to predict a common area of pretreatment conditions that could achieve near-to-maximum CSY (at 95% of confidence) for all of the straws samples, both for uncatalysed and SO2-catalysed SE pretreatment. The predicted optima for pretreatment conditions for each of the straw samples were validated experimentally. Optimised, uncatalysed and SO_2-catalysed SE could both achieve high combined sugars yields of between 50 - 59 and 53 - 64.4 g/100 g DRM, respectively. Experimental validation showed close agreement with predicted sugars and inhibitors yields (relative error < 6%). Thus, the developed predictive models were robust for prediction of combined sugars and inhibitors formation. Pretreatment optimisation enabled an improvement in CSY for uncatalysed (between 28 and 62%) and SO_2-catalysed SE (~33%) among straws. CSY for uncatalysed SE was maximised in the temperature range from 192-203°C and residence times of 4-9 min that would give severity factors between 3.66 - 3.81. For SO_2-catalysed SE, maximum CSYs were obtained at temperatures between 174 - 185°C in combination with residence times of 7-14 min, resulting in severity factors between 3.26 - 3.58. For uncatalysed SE, maximum experimental concentrations of 2.5 and 1.2 $g.L^{-1}$ were observed for acetic acid and furfural respectively, which was always higher than for catalysed pretreatment. Formic acid and HMF concentrations never went above 0.7 $g.L^{-1}$ at optimal uncatalysed SE conditions and were also always higher than for catalysed SE. Thus, pretreatment optimisation resulted in improved CSY at limited levels of toxicity for downstream biological sugar conversions. Improved combined sugar per gram of material is essential for superior ethanol yield from straw.

Candidate declaration

With regard to chapter 7 page numbers 173-219 of this dissertation, the nature and scope of my contribution were as follows

Name of contribution	**Extent of contribution (%)**
Planning of experiments	90
Executing experiments	100
Interpretation of results	70
Writing the chapter	100

The following co-authors have contributed to chapter 7 page numbers 173-219 of this dissertation.

Name	**e-mail address**	**Nature of contribution**	**Extent of contribution**
1. Maria del Prado Garcia-Aparicio	Garcia@sun.ac.za	- Planning of experiments - Providing inputs - Interpretation of results - Reviewing the chapter	10 60 10 70
2. Johann Görgens	jgorgens@sun.ac.za	- Interpretation of results - Providing inputs - Reviewing the chapter	10 20 25
3.Lidia Auret	lauret@sun.ac.za	- Contribution with Matlab - Providing inputs - Interpretation of results - Reviewing the chapter	100 20 10 5

Signature of candidate……

Date……

Declaration by co-authors

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 7 page numbers 173-219 in the dissertation,
2. No other authors contributed to chapter 7 page numbers 173-219 in the dissertation besides those specified above, and
3. Potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in to chapter 7 page numbers 173-219 of this dissertation.

Abstract

Triticale, a non-food based, low-cost and broadly adaptable crop with abundant straw yield, can potentially be used for bioethanol production. Ethanol production from straw is primarily determined by the response of the material to pretreatment and subsequent enzymatic hydrolysis, which impacts on the extraction of fermentable sugars. This study aimed at finding an optimum set of pretreatment conditions for straws from three preferred triticale cultivars grown in South Africa (selected in chapter 5), reflected in a pretreatment severity that maximises the combined sugar yields (CSY) from pretreatment-hydrolysis while limiting the formation of inhibitors. Uncatalysed and SO_2-catalysed steam explosion (SE) pretreatments followed by enzymatic hydrolysis were optimised by Response Surface Methodology, by varying the pretreatment time and temperature. Predictive models for the CSY for each straw and pretreatment mode were developed. Pretreatment severities that result in acceptable CSY values for all three straw samples were identified and experimentally validated. Uncatalysed SE primarily released oligomeric hemicellulose derived sugars and optimisation improved the CSY by 28-62%, for the different straws. SO_2-SE released mostly monomeric hemicellulose sugars and the CSY was improved by ~33% through optimisation. The optimum conditions for maximum CSY by uncatalysed SE (50-59 g/100 g DRM) were 192-203°C, with residence times of 4-9 min that would give severity factors between 3.66-3.81. The maximum CSYs for SO_2-catalysed SE (53-64.4 g/100 g RM) were obtained at temperatures between 174 and 185°C combined with residence times of 7-14 min, resulting in severity factors between 3.26 and 3.58. Inhibitors concentrations at optimal SE-conditions were in all cases below thresholds of inhibition for *S. cerevisiae*. Our results suggest that the variability in the responses to pretreatment-hydrolysis of straw samples from various triticale cultivars can be addressed effectively by selection of pretreatment conditions that are suitable for all straw samples. Thereby, a pretreatment process is provided that can deal with the inevitable variations in straw properties observed at industrial scale.

Keywords: Combined sugars; Inhibitors; Pretreatment optimisation; Steam explosion; Triticale straw.

7.1 Introduction

Second generation (2G) ethanol is considered an alternative to petroleum based fuels in the transport sector, with the potential to reduce global greenhouse gas (GHG) emissions, which is a primary motivation for their implementation through European and American directives [1]. Triticale is a promising energy crop due to several favourable traits, for instance the fact that it is a non-food crop that can be grown in marginal lands and presents superior straw yields (5.3-6.6 ton.ha^{-1}) compared to other cereals [2]. Lignocellulosic biomass such as triticale straw is converted to ethanol by firstly subjecting it to a pretreatment step. This increases carbohydrates accessibility to the

enzymatic hydrolysis for conversion of cellulose and hemicelluloses into monomeric fermentable sugars. It is well documented that steam explosion pretreatment (SE) is an effective method to reduce lignocellulose recalcitrance, while providing good overall sugar yield from feedstocks of different quality [3]. The application of steam pretreatment to material pre-soaked in water or acid further enhances both the hemicellulose sugar recovery and cellulose digestibility [4;5]. The "optimum" pretreatment conditions will be a compromise between sugar solubilisation in pretreatment liquor, cellulose digestibility and solids recovery. This requires the optimisation of the "combined" sugar yield (CSY), as the total of hemicellulose and cellulose sugars obtained from pretreatment and subsequent enzymatic hydrolysis [6]. Further consideration should be given to the inhibitors concentration in relation to the combined sugar concentrations, to ensure that these are within the limits accepted by the fermentative microorganism.

The pretreatment step has a substantial impact on the efficiency of bioconversion of straw into ethanol. Thus, there is an increasing interest in defining the optimum pretreatment conditions for each method and for different feedstocks [7], by taking into account the impact on subsequent hydrolysis and fermentation steps. Determination of pretreatment conditions that provide the maximum sugar yield from straw, as predicted by statistically significant models, is relevant for the prediction of sugar recoveries in industrial 2G plants. However, application of such predictive models for steam explosion (SE) pretreatment with triticale straw has not been performed before. Pre-soaking the material in water prior to SE pretreatment has been shown to increase the reactivity of the fibres [8]. Pre-soaking the material in water is also a requisite to facilitate sulphur dioxide diffusion into the solids, when SO_2-catalysed SE is performed [9]. While the impregnation with just water represents a simpler and cheaper option, the addition of a catalyst such as SO_2 may result in improved sugar yields [10].

The main objective of this work was to determine the optimum conditions to maximise CSY for water impregnated and SO_2-catalysed SE pretreatment of straw samples from three selected triticale cultivars, while limiting the toxicity (inhibitor concentration) of pretreated materials to within biologically acceptable ranges. Statistical predictive models were developed and used to build contour plots describing the pretreatment area for maximal combined sugars yield for the feedstocks. A region of optimum pretreatment conditions that was in common for all of the straws, giving proximity to the maximum observed CSYs, was identified, providing a pretreatment process with flexibility with regards to feedstock properties.

7.2 Materials and Methods

7.2.1 Raw material and sample preparation

Straw from three triticale cultivars cultivated in experimental fields located in the region Mariendahl, Western Cape, South Africa (latitude: 33.7166° N; longitude: 18.6333° E; elevation: 42 masl) was used as raw material in this study. The triticale cultivars corresponded to US2014 (CIMMYT, Mexico), 27thITYN39 (Barenbring, South Africa), and 98T376 (Pannar, South Africa), which were named M9, M13 and M14, respectively. The straw samples were collected after harvesting the grain in 2011. The moisture content (MC) of the feedstocks "as received" was 9.3% (w/w) on average. The material was then baled separately, labelled and stored in a temperature and moisture controlled room set at 20°C and relative humidity of 65% until needed. The total storage time of the samples did not exceed 6 months.

The straw material was prepared for pretreatment by coarsely grinding with a Condux-Werk type mill (Wolfgong bei Honou, Germany) and sieved to obtain particle size between 3.8 mm and 10 mm. The grinded material from each cultivar was then mixed until homogeneity and quarter-sampled for further analysis and pretreatment.

7.2.2 Steam explosion pretreatment

An experimental unit of 454 g of dry material (500 g of straw with moisture as received) was used for steam explosion (SE) pretreatment. The experimental unit was pre-soaked in 10 litres of distilled water, left overnight, and dewatered to a residual MC of approximately 65% by using a tumble drier. Water impregnation SE was performed with dewatered material. SO_2 pretreatment was carried out by application of 3% (w/w) of SO_2 (approx. 22.5 g of gas) on dewatered material following techniques described elsewhere [11].

The straw material (454 g of dry material) was pretreated in a batch pilot steam explosion unit (IAP GmBH, Graz, Austria) equipped with a 19-L reaction vessel and a blow tank constructed of stainless steel and a boiler capable of producing saturated steam of up to 40 bar. A control panel comprised of a PC based HMI/SCADA system with PLC's was used to control steam conditions, valves operation, as well as the processing temperature and residence time for each steam explosion run. Pressure monitoring inside the reactor was done by a redundant system comprising two Norgren type electronic pressure switches (33D-0863412) with a range of sensing of up to 40 bars (Norgren-GmbH Werk Fellbach. Stuttgart, Germany). The bottom of the vessel tapers down

gradually to a 78.5 mm line where a ball-type discharge valve is attached. This valve is capable of opening within less than 0.5 s and is automatically actuated. The pretreatment temperature in the reactor was controlled by supplying steam through automatic manipulation of two air-actuated needle control valves (Samson AG, Frankfurt, Germany). Saturated steam (around 30 bar of gauge pressure) was then injected into the reactor and the biomass heated up to the pre-set pretreatment temperature and residence time.

7.2.3 Enzymatic hydrolysis of the pretreated material

Two commercially available enzyme preparations, Spezyme CP (Genencor-Danisco, Brabrand, Denmark) and Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark), were used to perform enzymatic hydrolysis (EH) on water insoluble solids (WIS). Sodium azide at 0.02% (w/v) was added to the enzyme solution to prevent microbial contamination. The experiments were performed at 2% WIS in 0.05 M citrate buffer pH 5.0 with doses of cellulase and β-glucosidase per gram of WIS of 15 FPU and 15 IU, respectively. The flasks were incubated in a waterbath at 50°C with agitation of 90 rpm for 72 h. After 72 h the hydrolysate was removed from the mixture by centrifugation at 14000 rpm and prepared for sugars analysis by high-performance liquid chromatography (HPLC) as described below. The concentrations of glucose, xylose and arabinose were used to determine the yields of sugars as well as the digestibility of the pretreated solid.

7.2.4 Experimental design and optimization

Central composite design (CCD) under Response Surface Methodology (RSM) was applied to optimise SE conditions for the two types of pretreatment. Temperature and residence time were selected as independent variables. The yields of arabinose, xylose and glucose in monomeric and oligomeric form released after pretreatment, and the same sugars in monomeric form after enzymatic hydrolysis, were combined in a single output and referred to as combined sugar yield (CSY). Oligomeric sugars were converted to equivalent monomeric sugars for totalling up CSY. CSY and monomeric glucose (as major sugar released) from EH were considered as the main response variables. The residual acetyl groups in the WIS, and the concentrations of hydroxymethyl furfural (HMF), furfural, acetic acid and formic acid in the pretreatment liquor, were set as constraints for the optimisation process and consequently targeted simultaneously for minimisation.

A two level, two factor full factorial design [12] with four axial points and three replicates at the centrepoint was applied for each of the two types of pretreatment of straw, as well as enzymatic

hydrolysis of the pretreated material (WIS). The design led to a total number of eleven experiments in all cases. The matrixes of experimental design applied for water impregnated material were adapted to each of the feedstocks according to the results found in the preliminary studies performed with triticale straw and reported in Chapter 6 of this dissertation work. The range of conditions investigated for SO_2-impregnation was selected based on studies on wheat straw [13] and corn stover [14]. Table 7-1 provides the experimental conditions, pretreatment severities and resulting pH values after pretreatment for each type of SE pretreatment and feedstock. The pretreatment experiments were performed in a random order. The coded values for the axial, factorial and centrepoints were -1.4142 (at the lowest point) and +1.4142 (at the highest point), -1 and +1, and 0 (zero), respectively as shown in Table 7.1. The uncoded values were calculated according to equation 7-1.

$$X_i = X_{min} + \big((x_i + 1)/2\big).(X_{max} - X_{min}) \quad i = 1, 2, \dots, \quad n \tag{7-1}$$

Where X_i is the uncoded value of the independent variable i, X_{min} and X_{max} are the uncoded minimum and maximum values (corresponding to -1 and +1 coded values), and x_i is the code value to be translated. The second order polynomial model described by equation 7-2 was used for predicting the optimal pretreatment conditions.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + n \sum_{i=1}^{n} \beta_{ii} X_{ii}^2 + \sum_{i=1}^{n-1} \sum_{j=1}^{n} \beta_{ij} X_i X_j \tag{7-2}$$

Where Y is the estimated value of the response; n is the number of independent variables; β_0 is an intercept, β_i, β_{ii} and β_{ij} represent the regression coefficients for linear, quadratic and the interaction of two independent variables respectively; X_i, X^2_i and X_iX_j refers to linear, quadratic and two-way interaction effects, respectively.

Table 7-1: Matrix of the experimental designs, and resulting pH values of the slurry and severities for uncatalyzed and SO2 impregnation pretreatments of the feedstocks.

Uncatalyzed pretreatment	M9				M13				M14			
	Conditions				Conditions				Conditions			
Run	Temperature (°C)	Time (min)	pH	Log (R'o)	Temperature (°C)	Time (min)	pH	Log (R'o)	Temperature (°C)	Time (min)	pH	Log (R'o)
Factorial points												
1	180	5	4.4	3.1	180	2	4.4	2.7	190	5	4.2	3.35
2	200	5	3.9	3.6	200	2	4.0	3.2	210	5	3.7	3.94
3	180	15	4.1	3.5	180	8	4.2	3.3	190	15	3.9	3.83
4	200	15	3.5	4.1	200	8	3.5	3.8	210	15	3.5	4.41
Axial points												
5	175.86	10	4.3	3.2	175.86	5	4.4	2.9	185.86	10	4.2	3.53
6	204.14	10	3.5	4.1	204.14	5	3.7	3.8	214.14	10	3.3	4.36
7	190	2.93	4.3	3.1	190	0.76	4.5	2.5	200	2.93	4.1	3.41
8	190	17.07	3.7	3.9	190	9.24	3.9	3.6	200	17.07	3.6	4.18
Centered points												
9	190	10	4.0	3.6	190	5	4.1	3.3	200	10	3.7	3.94
10	190	10	4.0	3.6	190	5	4.1	3.3	200	10	3.7	3.94
11	190	10	3.9	3.6	190	5	4.1	3.3	200	10	3.7	3.94

SO_2-pretreatment	M9, M13 & M14			M9		M13		M14	
Run	Temperature (°C)	Time (min)	Log (R'o)	pH	CSF	pH	CSF	pH	CSF
Factorial points									
1	170	5	2.8	1.9	0.84	2.5	0.29	2.1	0.71
2	190	5	3.3	2.7	0.62	1.9	1.48	1.9	1.43
3	170	15	3.2	1.7	1.58	2.1	1.17	2.2	1.02
4	190	15	3.8	2.2	1.61	1.7	2.13	2.1	1.68
Axial points									
5	165.9	10	2.9	2.2	0.76	2.7	0.22	2.5	0.44
6	194.1	10	3.8	2.3	1.52	2.1	1.63	2.3	1.47
7	180	2.93	2.8	2.3	0.48	2.8	0.04	2.7	0.13
8	180	17.1	3.6	2.3	1.28	2.4	1.16	2.4	1.24
Centered points									
9	180	10	3.4	2.1	1.26	1.7	1.71	1.9	1.44
10	180	10	3.4	1.9	1.46	2.3	1.08	2.0	1.40
11	180	10	3.4	2.2	1.18	2.2	1.16	2.1	1.27

(*) The effects of the operational variables temperature and residence time for water-impregnation and SO_2-pretreatments were expressed in terms of the single parameter termed severity factor and combined severity factor, defined by the equations: $\mathrm{Log}\,(R'_0) = \mathrm{Log}\left(t \cdot \exp\left(\frac{T-100}{14.75}\right)\right)$ and $\mathrm{CSF} = \mathrm{Log}\,(R'_0) - \mathrm{pH}$, respectively.

Predictive models were developed for CSY. Inhibitors formation and CSY were subjected to simultaneous optimisation using a desirability function, whereby the responses were assigned with values from 0 (less desired output) to 1 (more desired output), according to determined benchmarks (in range of the values obtained in the study, maximise, minimise or target a specific value) and the weight allocated to them (0-100%) [15]. The maximum weight (95%) for the optimisation was given to maximise CSY. In addition, the yields of HMF, furfural, formic and acetic acids were totalled and set as threshold value for minimisation, with a weighting of 20%. A residual acetyl group content in the WIS, yielding acetate concentrations below 5 $g.L^{-1}$ during a SSF at 20% of total solids, was also preferred. Additionally, the fitted models were statistically validated by conducting five pretreatment experiments at the predicted optimum conditions.

Statistical analysis was performed by Design Expert, version 8.0.2 (State Ease Inc., Minneapolis, United States). ANOVA analysis was also performed by Design Expert to determine the statistical significance of the independent variables on the process responses. Predictive polynomial equations were developed to describe the experimental yield of combined sugars for all of the feedstocks by type of pretreatment, using Matlab® (Version R2013R). The equations for the CSYs were introduced as continuous functions to represent the contour plots described by the equations into the input range of the experimentation. An uncertainty of 5% (95% of statistical confidence) was set into the programming steps to reproduce graphical representations of at least 95% of the maximum value of the CSY response. Finally, pretreatment conditions in common for all three straw samples, incorporating conditions able to yield CSYs of at least 95% of the maxima observed for individual straw samples, were identified. These conditions were validated by running triplicate runs at severities in the overlapping pretreatment area, by using the M4 straw as control for validation.

7.2.5 Calculations

The effects of the operational variables temperature and residence time for water only pretreatment were expressed in terms of the single parameter termed severity factor [16], defined by the equation (7-3):

$$\mathrm{Log}\,(R'_0) = \mathrm{Log}\left(t \cdot \exp\left(\frac{T-100}{14.75}\right)\right) \qquad (7\text{-}3)$$

Where *t* is the residence time in min, and *T* is the pretreatment temperature in °C.

The effects of the temperature, time and acid concentration, in the case of SO_2-impregnation pretreatment, were unified in the combined severity factor (CSF) expression [16] calculated by the following expression:

$$\mathrm{CSF} = \mathrm{Log}\, R'_0 - \mathrm{pH} \qquad (7\text{-}4)$$

Where Log R'_0 is defined by the equation (7.3) and pH corresponds to the pH of the environment in which the pretreatment takes place, often measured in the whole slurry resulting from pretreatment.

The yields of monomeric xylose, glucose and arabinose in pretreatment liquor, as well as in the wash fraction from both types of pretreatment, were calculated on the basis of the amount of material fed into the reactor for comparisons of outputs among pretreatment conditions for each straw. This was expressed as gram of sugar per 100 grams of dry raw material (DRM) by the equation 7-5. The sugar yields in liquor and wash were finally totalled and reported as sugar yields from pretreatment liquor.

$$\text{Sugar yield } \left({}^{\mathrm{g}}/_{100\ \mathrm{g\ dry\ RM}}\right) = \frac{\text{Sugar concentration (g.L}-1)\times \text{ volume of pretreatment liquor (l)}}{\text{dry weight of raw material fed}} \qquad (7\text{-}5)$$

Where sugar yield represents the yield of xylose, glucose or arabinose expressed in gram per 100 grams DRM. Sugar concentration is the concentration of the sugar from HPLC analysis ($g.L^{-1}$), volume of pretreatment liquor is the total volume obtained after removing the liquor from the whole slurry after pretreatment corrected with the residual MC left in the solids after liquid separation (L), and the denominator of the expression corresponds to the dry mass of the raw material fed into the steam gun unit.

Additionally, the yields were compared to the measured contents of each sugar in the raw material. This was expressed as percentage recovery of the maximum potential sugars present in the feedstock, using equation 7-6, to facilitate comparisons of results among types of pretreatments for each straw. All sugar-oligomer yields were converted to the respective equivalent monomeric sugar using conversion factors of 1.136 and 1.111 for pentoses and hexoses, respectively.

$$\text{Sugar recovery } (\%) = \frac{\text{Sugar yield } \left({}^{\mathrm{g}}/_{100\ \mathrm{g\ dry\ RM}}\right)}{\text{Sugar equivalents in raw material } \left({}^{\mathrm{g}}/_{100\ \mathrm{g\ dry\ RM}}\right)} \times 100 \qquad (7\text{-}6)$$

The sugar yield is given by equation 5 and the denominator represents the measured sugar content in the raw material, in grams of sugar per 100 grams DRM. In the same way the concentrations of the inhibitors HMF, furfural, formic acid and acetic acid, determined in the pretreatment liquor, were expressed as yields in gram per 100 grams DRM, using the equation 7-5. However, the sugar concentration term was substituted for inhibitor concentration in the same units.

7.2.6 Characterization of raw material and pretreated materials

The chemical compositions of the raw and pretreated materials were determined according to the laboratory analytical procedures (LAPs) provided by the National Renewable Energy Laboratory (NREL), USA. The SE pretreated material, so-called slurry, was firstly characterised in terms of total solids, water insoluble solids (WIS) and water soluble solids (WSS) contents according to the NREL procedure [17].

Ash content was determined directly on the materials according to Sluiter *et al*. [18], while the structural components (cellulose, hemicelluloses and lignin) analysis was performed on extractive-free samples of raw material or directly in the WIS [19;20]. Additionally, the ability of triticale straw to provide buffering to maintain an almost constant pH in an aqueous environment, was determined by measuring the acid buffer capacity according to Han *et al*.,2010 [21].

The pretreatment liquors were analysed for main sugars in monomeric and oligomeric form, as well as fermentation inhibitors (HMF, furfural, acetic acid and formic acid) by HPLC as described below. The pretreatment liquor was also subjected to acid hydrolysis according to the method specified in [22], to convert oligomeric or polymeric sugars into monomeric equivalents, and include the latter in the measurement of the total sugars content.

7.2.7 HPLC Analysis

The composition of the main sugars (xylose, arabinose, cellobiose and glucose) and fermentation inhibitors (HMF, furfural, acetic acid and formic acid) in pretreatment liquor were analysed by HPLC. The concentration of monomeric sugars, acetic acid, formic acid, ethanol and glycerol was determined by an HPLC system equipped with an Aminex HPX-87H Column and a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). The concentration of HMF and furfural were analyzed on a Phenomenex Luna C18 (2) reversed phase column equipped with a Phenomenex Luna

C18(2) precolumn (Separations, Johannesburg, South Africa) with the same characteristics and specifications as described elsewhere [23].

7.3 Results and Discussion

7.3.1 Native triticale straw composition

The compositional analyses of the straw samples from different triticale cultivars are summarised in Table 7-2. Glucan, xylan and arabinan, representing the holocellulose fraction, varied from 54.3 to 58.2% (w/w) in the straw samples. Total soluble and insoluble lignin content ranged between 17.5 and 18.9% (w/w), with the acid-insoluble lignin content significantly lower in the straw M13 compared to other samples. *O*-acetyl groups, which give an indication of the degree of acetylation of the hemicellulose in the biomass, were around 2.1% (w/w) in all the feedstocks. The content of water-extractives varied between 7.4 and 9.4% of the chemical composition, while ethanol-extractives remained stable at 1.9 % (w/w). Ash content was below 3% in all the feedstocks.

Table 7-2: Chemical composition of the triticale straw feedstocks

Component	M9	M13	M14
Carbohydrate			
Glucan	38.8 (0.63)^{A}	35.6 (1.0)^{B}	37.2 (1.0)^{B}
Xylan	17.3 (0.22)^{A}	17.0 (0.63)^{A}	17.8 (0.7)^{A}
Arabinan	2.1 (0.07)^{A}	1.7 (0.1)^{B}	1.9 (0.2)^{B}
Lignin			
Acid insoluble	17.0 (0.7)^{A}	15.6 (0.4)^{B}	16.4 (0.6)^{A}
Acid soluble	1.9 (0.3)^{A}	1.9 (0.1)^{A}	1.8 (0.1)^{A}
O-acetyl groups	2.1 (0.1)^{A}	2.3 (0.4)^{A}	2.2 (0.1)^{A}
Extractives			
Water	8.5 (0.4)^{A}	7.4 (0.3)^{B}	9.4 (0.5)^{C}
EtoH	1.8 (0.1)^{A}	2.0 (0.2)^{A}	1.9 (0.1)^{A}
Ash	2.4 (0.1)^{A}	2.6 (0.1)^{B}	2.8 (0.1)^{B}
Acid buffer capacity*	3.24 (0.3)^{A}	4.12 (0.4)^{B}	4.23 (0.3)^{B}

Composition is given in % of dry matter. Values in parenthesis show the standard deviation of three replicates. Values on the same row with different superscript letters are significant different ($p \leq 0.05$). * Expressed in mili-equivalents (mEq) acid per 100 grams of oven dry sample.

Several works have demonstrated that differences in feedstock properties lead to different pretreatment requirements, even for related biomasses or varieties of the same species [23]. According to ANOVA the straw from cultivar M9 showed higher glucan and arabinan and reduced

ash content, whilst the contents of these components in straws M13 and M14 were similar (Table 7-2). Lignin content was significantly lower in straw M13, but comparable between straws M9 and M14, while the content of water-extractives showed significant differences between samples (Table 7-2). The measured glucan contents were in close agreement with reported values for triticale straw, while xylan contents were lower than reported values [24]. This observation may be explained by the fact that other hemicellulose sugars, such as mannan and galactan which are commonly found in triticale straw, were not measured in the present study. Lignin composition agreed with typical contents of around 19% (w/w) reported for triticale straw [24]. Although there are limited examples of triticale composition reported in literature, ash contents of the studied straw samples were considerably lower than typically reported for triticale straw (7.5-8.2-% w/w) [24;25] and wheat straw (around 4.7% w/w) [26]. Ash and lignin contents have been found negatively correlated to sugars released from straw in wheat [27]. Differences in lignin and ash contents in the triticale straw samples under study were not generalised but specifically differentiated straw M13 as the lowest lignin content sample and M9 as the sample with the lowest ash content between straws. As discussed in chapter 4, straws M9 and M13 were found to give the highest total fermentable sugars after dilute-acid pretreatment optimisation (bench-scale) compared to the straws (M14, O19 and S7). Such good performance was then attributed to lower ash content, especially in straw M9. The influence of ash content as an important quality feature for pretreatment processability (sugar release and pretreatment requirements) of the straws is further discussed in this section.

The buffering capacity of the straw samples is also given in Table 7-2. Straw M9 showed the lowest buffering capacity between samples of 3.24 mEq acid/100 g of straw, which was significantly lower ($P < 0.05$) than other samples (Table 7-2). The lower buffering observed for straw M9 may be explained by its significantly lower ash content, as well as the presence of other organic acids apart from acetic (acetyl content was similar among straws), such as methylglucuronic acid, which was not quantified. A link between ash content, buffering capacity and release of sugars after pretreatment has been suggested for other lignocellulosic materials such as switchgrass and corn stover [28]. This association was also observed for straw from triticale cultivars. Thus, variability in ash content between cultivars will necessarily be reflected in variations in pretreatment response between straws as discussed later in the next section.

7.3.2 Pretreatment

The effects of temperature and time on hemicellulose and inhibitors yields in the pretreatment liquor, and sugar release from subsequent EH of WIS, were evaluated separately. Pretreatment

conditions were optimised with the aim of maximising the combined sugar yield (CSY) from pretreatment and subsequent EH, while limiting inhibitors formation to acceptable levels.

7.3.2.1 *Pretreatment liquor*

Yield of hemicellulose derived sugars from pretreatment

A high yield of hemicellulose derived sugars, mainly xylan and arabinan in the case of triticale straw, is essential to maximise the CSY. Figure 7-1 illustrates the yields of all hemicellulose derived sugars (xylose and arabinose both in monomeric and oligomeric form) from water-only and SO_2-impregnation pretreatments of the straws M9, M13 and M14. Water-only SE of triticale straws increased the portion of hemicellulose derived sugars in oligomeric form, compared to SO_2-impregnated SE. The release of monomeric hemicellulose sugars by water impregnation SE varied from 0.49 g/100 g DRM (Figure 7-1 B, run 1) to 4.4 g/100 g DRM (Figure 7-1 A, run 6), whilst yields of sugars in oligomeric form ranged from 1.2 g/100 g DRM (Figure 7-1 C, Run 4) to 11.8 g/100 g DRM (Figure 7-1 A, Run 10). The total hemicellulose sugars recovery in the pretreatment liquor (monomers plus oligomers) ranged from 17.6 to 69.3% of measured content in straw samples. Pretreatment severities that maximised the yields of hemicellulose derived sugars in the water-SE pretreatment liquor were similar between some straw samples, reaching the highest yields of 13.7 and 11.4 g/100g DRM (69.3 and 59.5% of hemicellulose recovery) for M9 and M13 straw samples, respectively, at severities of approximately 3.65 (Figure 7-1, Inserts A and B). The highest hemicellulose sugars yield of 11.3 g/100 g DRM (55.7% of hemicellulose recovery; Figure 7-1 C) during water-only SE of straw M14 was obtained at a severity of 3.83 (run 3). Further increases in the severities, beyond indicated maximum, substantially decreased sugars yields and recoveries, apparently due to sugar degradation.

SO_2-catalysed SE (effectively H_2SO_3-catalysed by SO_2 solubilisation in the moisture present in straw samples; thus acid-catalysed) was more effective in releasing hemicellulose sugars in pretreatment liquor, and in converting the solubilised sugars to monomeric sugars, compared to water-only SE (Figure 7-1). Monomeric hemicellulose sugars yields from SO_2-SE varied between 3.3 g/100 g DRM (Figure 7-1 E, Run 7) and 12.4 g/100 g DRM (Figure 7-1 F, Run 2), whereas the oligomeric sugars yields ranged from 1.6 g/100 g DRM (Figure 7-1 F, Run 2) to 10.0 g/100 g DRM (Figure 7-1 D, Run 2). These total sugar yields represented hemicellulose sugars recoveries of between 43.8 and 86.3% of the measured content in the straw samples.

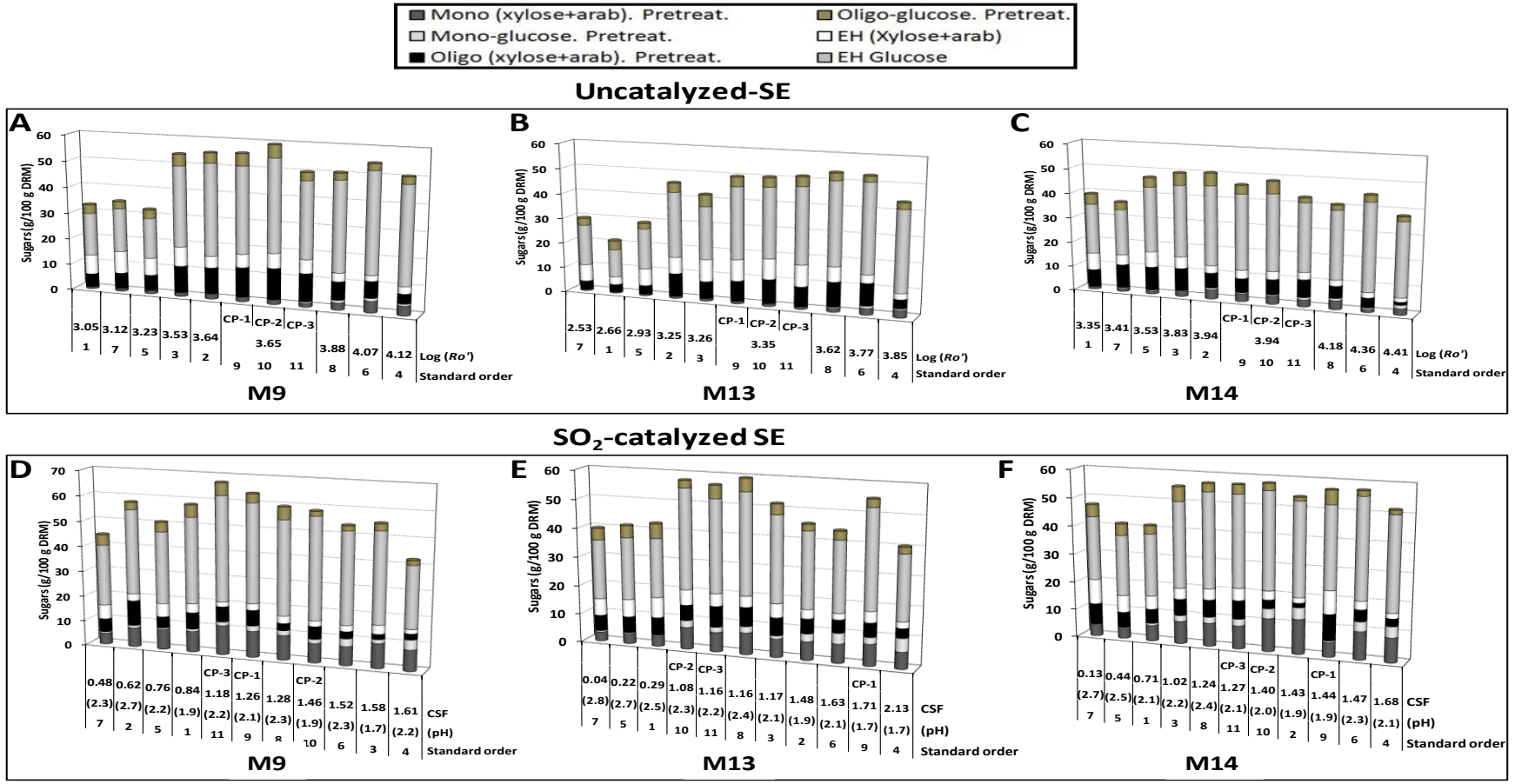


Figure 7-1: Yield of sugars as monomers and oligomers in pretreatment liquor and after enzymatic hydrolysis (EH) from uncatalyzed and SO2-catalyzed SE pretreatments of the feedstocks M9 (A and D), M13 (B and D) and M14 (C and F). CP-1, CP-2 and CP-3 correspond to the replicates of the centrepoint conditions. The yields of combined sugars are given as the sum of all the sugars at each pretreatment condition. Yields expressed in gram of sugar per 100 gram of dry raw material (DRM). Log (Ro') and CSF refer to the pretreatment severities according to the equations (3) and (4), for uncatalyzed-SE and SO2-SE, respectively. pH values are given for SO2-SE to show differences in severities within centrepoint conditions. Standard order denotes the order of the run into the matrix of experimental designs for both types of pretreatments (Table7-1).

The highest recovery of hemicellulose derived sugars (86.3%) from SO_2-SE was obtained with straw M9 (Figure 7-1 A, run 2), while maximum recoveries that were comparable and nearly 18% lower than M9, were obtained with straws M14 and M13. Consistently high hemicellulose sugars yields (recoveries greater than 79%) were observed at severities (CSF values) of 0.62, 1.26 and 1.46 for straw M9 (Figure 7-1 D). On the other hand, straw M14 required a CSF of 1.27 to achieve the highest hemicellulose sugar yield of 14.8 g/100 g DRM (Figure 7-1 F, run 11), representing a sugar recovery of 70%. A comparable maximum recovery was obtained for M13 straw at CSF 1.16 (Figure 7-1 E, run 8). Straw M9 was clearly shown to perform better in releasing hemicellulose derived sugars from both uncatalysed and SO_2-catalysed pretreatment. Straw samples M13 and M14 gave comparable yields of hemicellulose derived sugars but lower than that for straw M9. This was true for both uncatalyzed and SO_2-catalyzed SE. It can be inferred that low ash content in straw had a positive impact on the release of sugars from pretreatment, as observed with straw M9 at small-scale pretreatment (Chapter 5). Similarly, the ash content in straws M13 and M14 (Table 7-1) may also confirm this hypothesis by showing comparable yields, especially under SO_2 when the buffering capacity of the straw becomes more relevant (acid environment). Recovery of hemicellulose derived sugars after pretreatment showed a clear differentiation between pretreatment modes in favour of SO_2-pretreatment. SE under impregnation with SO_2 has been shown to be more beneficial in removing hemicellulose from biomass such as corn stover [29] and wheat straw [30].

Fermentation inhibitors production from pretreatment

For the maximisation of the combined sugars yield, pretreatment conditions should be selected to maximise both the solubilisation and recovery of hemicellulose derived sugars. Thus, lower severities to minimise sugar degradation are preferred [31]. However, ensuring sufficient digestibility of pretreated solids, which is also necessary to obtain acceptable glucose yield from subsequent enzymatic hydrolysis of the WIS fraction, typically requires higher severities [31]. Mild pretreatment conditions may also result in WIS with increased content of residual acetyl groups, resulting in acetic acid release during subsequent hydrolysis and fermentation. Thus, maximisation of CSY involves a compromise between the lower severities required for maximum hemicellulose sugars recovery, and the higher severities required for cellulose digestibility, while still limiting the formation of inhibitors to acceptable values. Figure 7-2 shows the yields of furan and weak acids in pretreatment liquor of water-impregnation and SO_2-SE of the feedstocks. As expected, there was a proportional increase in the formation of inhibitors in response to an increase in the pretreatment severity [32;33], for all of the straw samples.

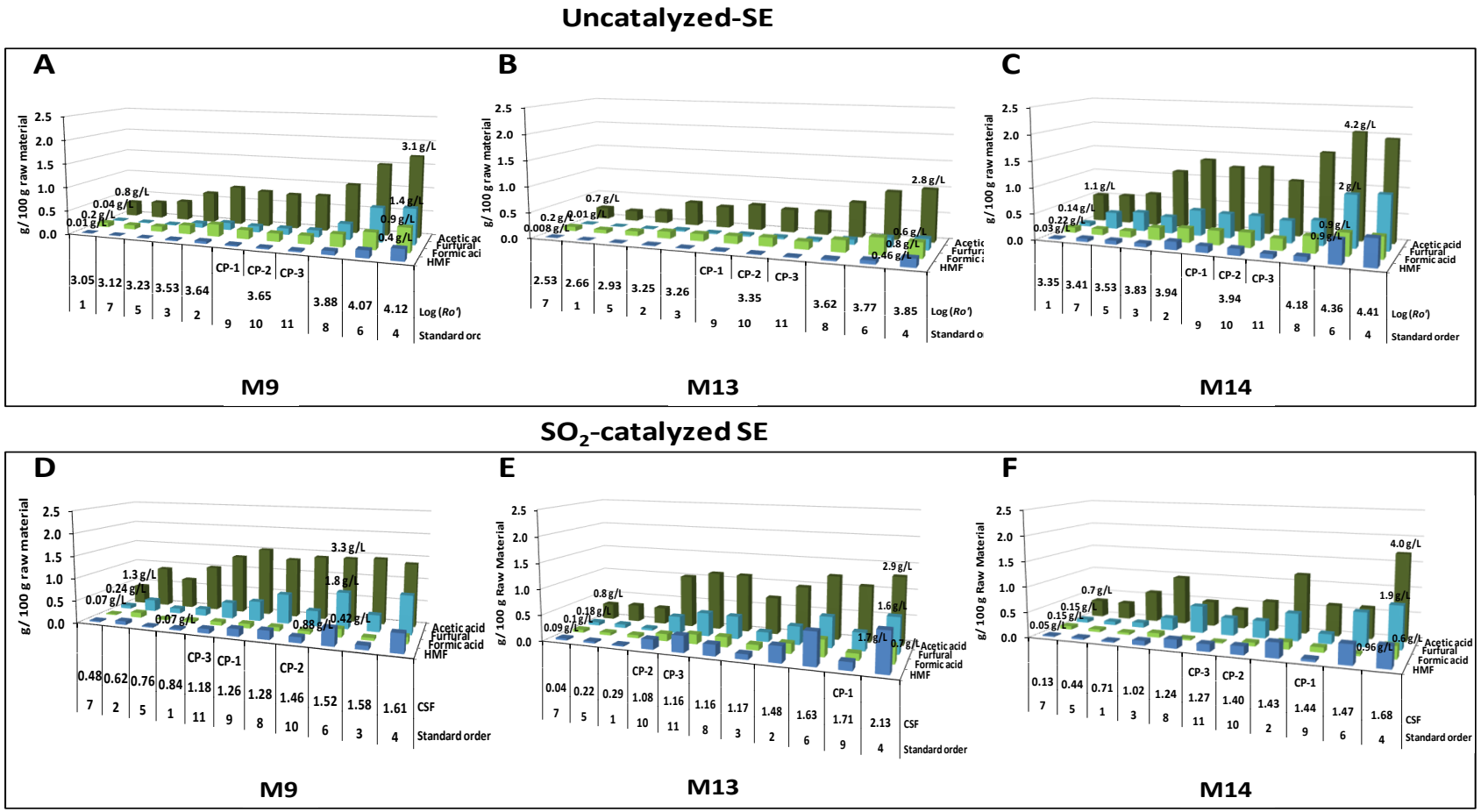


Figure 7-2: Yields of sugar degradation by-products (HMF, formic acid and furfural) and acetic acid in pretreatment liquor generated from uncatalyzed- and SO2-catalyzed SE for the straws M9 (A and D), M13 (B and E) and M14 (C and F). The yields were plotted as a function of the severity Log (*Ro'*) and CSF for uncatalyzed and acid catalyzed pretreatments, respectively. The yields are expressed in grams per 100 grams of dry raw material. CP-1, CP-2 and CP-3 correspond to the replicates of the centrepoint conditions (runs 9, 10 and 11). Standard order denotes the order of the run into the matrix of experimental design for the feedstock. The corresponding concentrations in grams of HMF, formic acid, furfural, and acetic acid per litter of pretreatment liquor are given next to the respective bars for the minimum and maximum values into the experimental designs for both types of pretreatments.

The highest yields of furfural from all the conditions tested with water-only SE, were about 0.7, 0.2 and 1.0 g/100 g DRM at severities of 4.12, 3.85 and 4.41 for the feedstocks M9, M13 and M14, respectively (Figure 7-2. Inserts A, B and C, run 4). A maximum furfural yield of about 0.7-0.8 g/100 g DRM was observed for SO_2-SE at CSF values of 1.6-2.13 (Figure 7-2 Inserts A, B and C, run 4). The yield of HMF, a degradation product of hexoses, was generally lower than furfural for both types of pretreatments. The only exception to this rule was for severities 1.63 and 2.13 using SO_2-SE of straw M13 (Figure 7-2. Insert E, runs 4 and 6), where HMF yields were comparable to the yields of furfural at 0.6 and 0.7 g/100 g, respectively for the two severities. The highest HMF yield for water-only SE of straws M9 and M13 was comparable (0.2 g/100 g DRM), whereas a 2.5-fold higher yield was obtained for M14 at the highest severity (Figure 7-2. Inserts A, B and C). Maximum HMF yields of 0.4 and 0.7 g/100 g DRM were found for SO_2-SE of feedstocks M9 and M13 (Figure 7-2. Inserts D and E, run 4), respectively. This was considerably higher than obtained for the water-SE pretreatment of these feedstocks. Conversely, water-SE of sample M14 resulted in a 0.51 g/100 g DRM yield, which represents a 22% increase in HMF yield compared to that of SO_2-SE. In summary, furfural yields were similar between water-only and SO_2-SE, while HMF yields from SO_2-SE where higher than those from water-only SE in most instances.

Furans can degrade further into formic acid during pretreatment [34], and thus formic acid concentration may be a good indication of the severity of the pretreatment. The maximum formic acid yield from water-SE pretreatment was obtained with straw M9 at 0.50 g/100 g DRM at the harshest severity of 4.12 (Figure 7-2 A, run 4). Although much higher severities were applied on the CCD for water-SE pretreatment of M14, formic acid did not exceed 0.43 g/100 g DRM which was found at run 6 (Figure 7-2 C). The range of conditions applied for SO_2-SE pretreatment gave a maximum formic acid yield of 0.32 g/100 g DRM for all the samples across the CCDs (Figure 7-2 E, Run 6); thus, significantly lower than that for water-only SE (0.50 g/100 g DRM). Significant acetic acid release was observed during both types of pretreatment for all straw samples. The highest yields of acetic acid were 2.0 g/100 g DRM (water-only) and 1.7 g/100 g DRM (SO_2-SE), found at the harshest severities applied to straw M14 (Figure 7-2 C, run 6 and Figure 7-2 F, run 4). The resulting fibres from these pretreatment conditions presented limited residual acetyl content, thus limiting the release of acetic acid during subsequent enzymatic hydrolysis or SSF.

Inhibitors production was directly associated with pretreatment severity and pretreatment mode, although interaction between pretreatment and straw properties was also observed. The more severe conditions at both pretreatment modes showed the highest concentrations of inhibitors [32].

Differences in total inhibitors production between types of pretreatment were larger than such differences between straw samples. The results demonstrated that water-only pretreatment gives lower inhibitor formation than SO_2-SE at the range of conditions tested. Thus, the inclusion of acid as catalyst (increased severity of pretreatment) increased the toxicity of sugars streams from SE. The effect of straw properties on inhibitors production could help to explain the higher maximum total inhibitors production (at the harshest severities) for the feedstocks M9 and M13 compared to M14. Better processability of M9 and M13 caused more effective hemicellulose deacetylation, since up to 53 and 67% of the total inhibitors yield from water and SO_2-pretreatment, respectively consisted of acetic acid. Higher inhibitors concentrations were also observed for straw samples with higher pretreatment requirements for improved combined sugars yield (i.e. straw M14 under water-only SE), since severity greatly influences inhibitors concentration [32]. Little has been investigated on the effect of chemical composition features such as ash content in triticale, and its relatives such as wheat and rye, on inhibitors production from pretreatment. However, the results from this study suggest the existence of a negative association between ash content (with resulting buffering capacity variation) of the straw and total inhibitors production from SE pretreatment.

The acidity (pH) of the slurry is an important parameter that will have an impact on the downstream processing, due to impacts on the toxicity of other compounds such as acetic acid in the pretreatment liquor [35]. For example, acetic acid concentrations between 2 and 6 $g.L^{-1}$ has been shown to completely inhibit yeast growth at pH between 3 and 3.5 (enzymatic hydrolysis and fermentation are steps normally conducted at pH 5-5.5), while these concentrations will promote glucose consumption and ethanol production at pH values around 5. As expected, SO_2 impregnation resulted in slurries with lower pH than water impregnation (1.7-2.8 versus 3.3-4.5), but slight differences were observed between the different cultivars at similar severity (Table 7-3).

7.3.3 Slurry properties and glucose from EH of the water insoluble solids

The selection of pretreatment conditions is determined by the properties of the pretreated material or slurry, such as yields of total solids (soluble and insoluble), WIS and water soluble solids (WSS), slurry pH and the total inhibitors concentration (HMF + furfural + formic acid + acetic acid) of pretreatment liquor, thereby impacting on process optimisation. The properties of the slurry from both types of pretreatment are summarised in Table 7-3. It can be observed that the percentage of total solids varied among all of the varieties between 11.8 and 25.6% (Table 7-3, SO_2-impregnation

Table 7-3: Properties of pretreated materials (slurry) for uncatalyzed and SO2-catalyzed pretreatments of the feedstocks.

Feedstocks																						
M9									M13								M14					
Uncatalyzed					SO2-catalyzed				Uncatalyzed				SO2-catalyzed				Uncatalyzed				SO2-catalyzed			
Run	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)	TS (%)	WIS (%)	WSS (%)	Inh (g.L-1)
1	19.0	19.9	3.1	1.1	18.6	13.7	5.0	2.4	22.3	19.1	3.2	0.8	25.6	21.8	3.8	1.6	17.4	13.1	4.3	1.6	20.2	15.5	4.8	2.3
2	18.6	13.1	5.4	3.3	19.6	11.2	8.3	2.8	19.9	14.3	3.2	2.1	20.8	15.3	5.5	6.2	14.2	9.7	4.4	4.2	19.2	12.6	6.6	6.0
3	15.3	12.0	3.3	1.8	17.4	12.0	5.4	4.0	20.1	16.2	3.9	1.4	13.3	9.5	3.8	1.9	14.7	10.9	3.9	3.3	18.1	12.4	5.7	3.7
4	13.1	10.3	2.8	5.9	13.9	9.2	4.7	5.2	17.0	13.0	4.0	4.7	13.2	8.7	4.5	6.9	9.1	7.3	1.8	5.4	15.8	11.5	4.3	7.6
5	18.9	16.3	2.5	1.4	22.4	15.2	7.2	2.5	22.5	20.0	2.6	0.9	17.9	12.9	5.0	1.1	17.0	11.3	5.7	2.6	22.4	15.2	7.2	1.1
6	13.0	9.3	3.8	4.5	16.6	10.8	5.8	6.5	18.9	12.6	6.3	3.9	11.8	6.9	4.9	4.9	13.6	10.9	2.7	8.1	16.6	10.8	5.8	3.3
7	19.9	15.7	4.3	1.2	22.8	16.0	6.8	1.8	23.2	20.7	2.5	1.0	19.7	14.6	5.1	1.3	17.6	12.4	5.2	2.7	22.8	16.0	6.8	1.1
8	15.1	10.8	4.3	3.4	16.2	11.0	5.2	4.9	18.9	13.6	5.3	2.7	15.9	11.8	4.1	2.9	13.1	8.5	4.7	4.3	19.2	13.6	5.6	2.4
9	17.2	12.5	4.7	2.6	19.8	13.5	6.3	4.0	22.0	15.1	6.9	1.8	16.1	11.9	4.3	5.4	14.9	10.8	4.1	4.4	19.2	14.7	4.6	2.3
10	17.3	12.1	5.2	2.1	17.9	11.8	6.0	4.3	19.9	14.7	5.2	1.6	14.7	11.0	3.7	4.5	14.5	9.9	4.6	4.2	17.9	12.7	5.2	1.9
11	17.6	12.4	5.2	2.0	17.8	11.5	6.3	4.1	18.4	13.8	4.6	1.7	12.4	10.5	4.0	4.1	13.8	9.4	4.4	3.4	17.1	11.5	6.3	1.8

Run stands for the standard order into the matrix of experimental design. TS: total solids, expressed in percentage. WIS: water-insoluble solids, expressed in percentage. WSS: water-soluble solids, expressed in percentage. Inh: total fermentation inhibitors (HMF + furfural + formic acid + acetic acid), given in grams per litre.

sample M13, runs 6 and 1, respectively). Total solids in the slurries were higher than 12%, except for water-impregnated SE with M14 in run 4 (Table 7-3). In general, SO_2-SE solubilised a larger portion of the straw samples, as indicated by higher values of WSS across pretreatment conditions compared to water impregnation, with only a few exceptions for water impregnated SE of M13 (Table 7-3, runs 6, 8-11).

The impact of pretreatment conditions on the accessibility of pretreated solids to the enzymes, as primary aim of pretreatment, was determined by enzymatic hydrolysis of the WIS fraction at 2% (w/w) solid loading. Higher solid loadings (5-30%) are preferred for enzymatic conversion, due to increases in the final sugar concentrations. Thereby productivity is increased and energy and water input is lowered [36]. However, lower solids loading of 2% (w/w) are more representative of typical conditions during Simultaneous Saccharification and Fermentation (SSF) processes, in terms of reduced end product inhibition and improved enzyme adsorption, compared to higher solids loadings. The pretreatment severities that yielded the highest values of glucose from EH of WIS, corresponding to the highest digestibilities of pretreated solids, were therefore identified in this study.

The yields of glucose after EH (EH-glucose) from water- and SO_2-SE pretreatments are illustrated in Figure 7-1 for all of the straw samples. Summation of the yields of xylose and arabinose as minor sugars from EH is presented as EH (xylose + arabinose) in Figure 7-1. Minimum EH-glucose yields of 15.4 (run 5), 11.2 (run 1) and 18.6 g/100 g DRM (run 7) were observed for water-SE for straws M9, M13 and M14 (Figure 7-1, Inserts A, B and C), respectively. As a general trend for all of the feedstocks, EH-glucose yields for straw M9 improved as severity increased, until reaching a maximum value of 38.2 g/100 g DRM at severity 4.07 (run 6), representing a recovery of 88.6% of the measured glucose content in the straw. Comparable maximum yields of 35 g/100 g DRM at severities 3.77 and 4.36 (run 6) were determined for straws M13 and M14 (Figure 7-1, Inserts D, E and F), corresponding to glucose recoveries of 89 and 85%, respectively. The harshest pretreatment condition in each CCD (run 4) decreased the glucose yields by nearly 3, 10 and 16%, compared to maximum values found for M9, M13 and M14, respectively (Figure 7-1, Inserts A, B and C). Digestibility of the glucan for both types of pretreatment is given in Table 7-4. Digestibilities from water-SE pretreatment varied between 38.4 – 87.6, 25.5 – 81.6 and 47.4 – 91% for the pretreated straws M9, M13 and M14, respectively (Table 7-4).

Table 7-4: Recovery of total sugars from the theoretical sugars in the raw material from pretreatment (uncatalyzed and SO2-catalyzed) followed by enzymatic hydrolysis and enzymatic digestibility of WIS for the triticale straw samples M9, M13 and M14.

	Feedstock											
	M9				M13				M14			
Run	Uncatalyzed		SO_2-catalyzed		Uncatalyzed		SO_2-catalyzed		Uncatalyzed		SO_2-catalyzed	
	Sugar recovery[1]	Digesti-bility[2]	Sugar recovery[1]	Digesti-bility[2]	Sugar recovery[1]	Digesti-bility[2]	Sugar recovery[1]	Digesti-bility[2]	Sugar recovery[1]	Digesti-bility[2]	Sugar recovery[1]	Digesti-bility[2]
1	52.3	39.9 (0.00)	88.4	65.1 (0.02)	36.6	25.5 (0.01)	72.5	45.0 (0.01)	64.0	47.4 (0.01)	66.4	56.6 (0.07)
2	87.7	83.6 (0.04)	92.0	81.4 (0.03)	78.6	62.0 (0.03)	77.0	82.2 (0.07)	81.3	78.2 (0.05)	85.7	88.4 (0.04)
3	86.2	66.5 (0.01)	84.2	73.0 (0.05)	71.9	41.4 (0.02)	87.3	70.8 (0.04)	80.8	66.6 (0.04)	89.2	79.0 (0.01)
4	79.0	84.1 (0.07)	65.4	68.3 (0.05)	73.6	80.7 (0.05)	67.7	82.6 (0.06)	60.5	80.3 (0.07)	82.1	86.1 (0.01)
5	51.9	38.4 (0.02)	79.0	69.3 (0.06)	49.7	31.9 (0.02)	70.0	52.7 (0.02)	77.0	70.4 (0.03)	66.8	52.7 (0.02)
6	85.8	86.7 (0.05)	83.8	92.1 (0.06)	89.8	81.6 (0.02)	74.4	90.6 (0.04)	72.8	91.0 (0.02)	91.4	84.0 (0.01)
7	55.4	45.0 (0.05)	67.5	55.7 (0.04)	50.3	34.0 (0.02)	67.4	55.6 (0.03)	60.2	52.6 (0.04)	76.9	53.5 (0.06)
8	79.7	84.5 (0.03)	89.7	79.8 (0.04)	90.9	76.9 (0.08)	100.3	86.0 (0.02)	65.8	73.7 (0.04)	91.5	79.1 (0.03)
9	88.7	81.7 (0.04)	101.5	83.4 (0.03)	84.7	62.5 (0.03)	92.5	75.6 (0.05)	74.7	77.3 (0.06)	92.5	79.2 (0.03)
10	94.7	82.8 (0.03)	97.9	83.5 (0.02)	85.7	64.6 (0.08)	96.7	79.4 (0.03)	78.6	80.4 (0.03)	92.6	84.2 (0.02)
11	79.8	81.8 (0.05)	92.8	83.4 (0.06)	87.1	74.0 (0.04)	95.9	83.6 (0.02)	69.4	77.9 (0.04)	91.8	79.3 (0.01)

[1]Expressed in percentage. [2] Digestibility of the WIS, in percentage. Values in parenthesis represent the standard deviation of three replicates. Run refers to the standard order into the respective matrix of experimental design for each feedstock and type of pretreatment.

EH-glucose yield by SO_2-SE pretreatment could be improved only for feedstock M9 with 41.4 g/100 g dry DRM at severity 1.26 (Figure 7-1.D), 8% more glucose recovered with respect to water-SE. On the other hand, EH-glucose yields of 35.4 (severity 1.44) and 35 g/100 g dry DRM (severity 1.16) (Figure 7-1, Inserts E and F) from SO_2-SE of straws M13 and M14 represented glucose recoveries comparable to water-SE pretreatment. The highest EH xylose yields from water-SE were 8.4, 8.2 and 6.8 g/100 g DRM, while SO_2-SE gave yields of 5.6, 6.9 and 8.9 g/100 g DRM, for the straws M9, M13 and M14, respectively. Under pretreatment conditions that resulted in the highest EH glucose yields for straws M9, M13 and M14, contributions of the EH-xylose to the total sugar yields were nearly 5.4, 8.5 and 5.8% for water-SE, and 4.4, 9.9 and 7.6% for SO2-SE pretreatment, respectively. Contributions of up to 33% from EH-xylose to the total EH sugars yield was observed for all the straws at the mildest pretreatment conditions, with significantly lower solids digestibility and hemicellulose sugars removal from solids.

Compared to water-only, acid-catalysed pretreatment slightly improved the digestibilities of pretreated solids for the M9 and M13 straws, while a marginal reduction in the digestibility was found for M14. Thus water-only and SO_2-SE are promising methods for improved digestibily although the acid-catalysed pretreatment offers additional enhancement. Improvements of 7% to 82% in glucan digestibility by inclusion of acid catalyst has been reported for SE of herbaceous feedstocks such as wheat straw [37] and switchgrass [38]. The highest digestibilities of pretreated solids of 92.1, 90.6 and 88.5% for SO_2-SE were found at CSF of 1.63 for the M9, M13 and M14 straws, respectively. Thus improvement in digestibility of the straw samples followed similar association with high pretreatment conditions as generally found for herbaceous feedstocks, such as wheat and switchgrass, and differentiation between straws was little. Similar glucan conversions of 93.1 and 95% have been reported for steam explosion of uncatalysed (CSF of 0.17) and acid-soaked (CSF of 1.05) rice straw [39], as well as wheat straw (94% at 700 KPa for 40 minutes) [40], switchgrass (95% at 195°C for 7.5 minutes with 3% of SO_2 w/w) and sugarcane bagasse (94% at 205°C for 10 minutes with 3% of SO_2 w/w) [5].

7.3.4 Combined sugars yield

Although EH glucose is the primary sugar product from pretreatment-hydrolysis of straw, the maximisation of the combined sugars yields (CSYs: glucose + xylose + arabinose) was the primary goal of the pretreatment process optimisation. The combined sugars yield is a summation of sugars released into the pretreatment liquor (monomeric and oligomeric) and monomeric sugars released during subsequent enzymatic hydrolysis. Recent developments with xylose fermenting

microorganisms [41;42], has justified the inclusion of fermentable hemicellulose sugars for the integrated optimisation of pretreatment with subsequent hydrolysis. Additionally, the selected pretreatment optimisation approach also limited inhibitors formation to the thresholds of inhibition of the fermentative yeast *S. cerevisiae*, commonly used in downstream fermentations. The yield of combined sugars from water- and SO_2-SE pretreatment is given in Figure 7-1 for all of the feedstocks and types of pretreatment. The yields were normalized to the sugar content of the untreated straw samples and reported as percentage of the maximum potential sugar recovery (See Table 7-4, facilitating the comparison of pretreatment efficiencies between feedstocks.

Uncatalyzed SE pretreatment conditions for maximum yield of combined sugars after pretreatment followed by enzymatic hydrolysis were found for a particular triticale straw (Mariendahl-originated) in Chapter 6. Severities (log (Ro)) around 3.65 were identified best to able to locate an appropriate centrepoint in the experimental design for pretreatment optimization in the present chapter.

For the uncatalysed pretreatment, straw M9 gave maximum CSY of 61.7 g/100 g DRM (95% of combined sugars recovery) at SF = 3.65. This severity is in close agreement with previous results for maximum CSY by uncatalyzed SE found in Chapter 6. It is worth noticing that straw sample tested by uncatalyzed SE in Chapter 6 presented a low ash content (1.5±0.4%) fairly comparable to straw M9 studied in the present chapter (2.4±0.1%) whereas straws M13 and M14 displayed higher ash content than M9 (> 2.6%) which could help to explain similarities in severities. On the other hand, straw M14 yielded 57.1 g/100 g DRM (91% of sugars recovery) at SF = 3.62, and straw M13 gave maximum CSY of 55.2 g/100 g DRM (81.3% sugars recovery) at SF = 3.94. The SO_2-catalysed pretreatment led to increase in the CSY for all straw samples, compared to the water-only pretreatment. Straw M9 gave maximum CSY of 66.1 g/100 g DRM at CSF = 1.26 and straw M13 resulted in maximum CSY of 61.9 g/100 g DRM at CSF = 1.16; both yields representing virtually complete combined sugars recovery based on original sugars content in the straws. In the case of straw M14, a maximum CSY of 59.1 g/100 g DRM (92.6% of combined sugars recovery) was obtained at CSF = 1.40. Severity of 3.65 (190°C and 10 min) has been found to result in maximum overall sugars yield with 100% of sugars recovery for SO_2-SE of wheat straw [43]. These literature findings are however higher than those found for experimental measurements at 180°C and 12 min (severity of 3.43) in this study for triticale straw.

Differences in CSY were also related to quality features of the straws. M9 was found to be the best performer straw with regards to CSY under the studied dilute acid pretreatment (Chapter 5) and water-only/SO_2 pretreatments (Chapter 7), which was attributed to its low ash content. Also, higher CSY for SO_2-pretreatment and lower CSY for water-only pretreatment were observed for straw M13, when compared to those for straw M14. Although no statistical differences in ash content between these straws were found, the numerical difference of ash contents between straws 2.6% (M13) and 2.8% (M14) could probably account for the better performance of M13 under the SO_2 pretreatment conditions. On the other hand, straw M14 required higher severities to reach maximum CSY, suggesting straw M14 to be the most recalcitrant sample. This could probably be due to its material properties such as higher neutralising capacity (Table 7-2) and other factors (i.e. different cell-wall structure) compared to M9 and M13.

The yields of sugars found in this study for water-only SE pretreatment are generally similar to those of other genetically related feedstocks such as wheat straw, provided that the material is pre-soaked in water prior to pretreatment. Pre-soaking the material in water prior to pretreatment has been proven to increase the reactivity of the fibres for the same pretreatment conditions [4]. In the present study, the material was pre-soaked in water and pretreated with initial moisture of 65%. Similar yields of hemicellulose (~60%) and glucan digestibility (80%) to those obtained in this study have been reported for steam explosion (SF 3.94) of wheat straw with initial moisture of 80% [44]. More severe pretreatment conditions have been applied to achieve glucan conversions of 82% for steam exploded rye straw (SF of 4.25) [45] and 90% for pretreated wheat straw (SF ≥ 4.24) [46] with 10% of initial moisture in the materials.

7.3.5 Development of predictive models for optimisation of CSY

Predictive statistical models were developed to study the effect of the pretreatment conditions on the yields of EH-glucose (as major sugar to optimise CSY) and combined sugars, as well as predict maximum yields from the straws by RSM analysis. Second-order regression models were fitted to describe the experimental data of both yield responses for water-SE as well as EH-glucose yield for the straws M13 and M14 under SO_2-SE pretreatment.

Experimental data for EH-glucose yield of straw M9 and CSY under SO_2-SE could only be described with statistical significance by third order polynomial expressions. This entailed the inclusion of two additional terms into the expressions representing the second-order interactions of temperature and residence time (T^2t). To focus the attention on the CSY as the core of the present

study, the predictive models developed for EH-glucose yield for all of the feedstocks at both types of pretreatment and the model based contour plots representing EH-glucose as a function of temperature and residence time, are provided as Annexe at the end of this chapter (equations A1-A6 and Figure A-1. Also included in this information are the results of the analysis of variance (ANOVA) of the CSY models for water- and SO_2-SE pretreatments for the feedstocks (Table A-1 and Table A2.).

7.3.5.1 *Combined sugars yield*

The polynomial expressions from ANOVA describing the experimental data for the response CSY from the water-SE pretreatment of the feedstocks M9, M13 and M14 are given by equations 7-7 to 7-9, followed by the respective coefficient of determination (R^2), respectively. These polynomial equations depict the interaction effects of the independent parameters *Temperature* and *residence time* on maximising the response CSY. Contours describing the response CSY as a function of temperature and residence time are given in Figure 7-3.

$$CSY = 57.16+6.21T+4.84t-6.93Tt-4.85T^2-5.25t^2 \quad (R^2\ 0.88) \qquad (7\text{-}7)$$

$$CSY = 50.44+7.49T+6.54t-5.77Tt-5.32T^2-5.12t^2 \quad (R^2\ 0.95) \qquad (7\text{-}8)$$

$$CSY = 47.29+0.72T+0.31t-6.00Tt+0.65T^2-3.15t^2 \quad (R^2\ 0.88) \qquad (7\text{-}9)$$

Similarly, model expressions for the CSY from SO_2-SE of the straws M9, M13 and M14 correspond to equations 7-10 to 7-12, respectively.

$$CSY = 63.45+1.12T+5.11t-3.65Tt-4.81T^2-5.71t^2-10.13T^2t-3.59Tt^2 \quad (R^2\ 0.80) \qquad (7\text{-}10)$$

$$CSY = 57.77+0.94T+7.08t-3.66Tt-7.23T^2-3.69t^2-6.23T^2t-3.24Tt^2 \quad (R^2\ 0.91) \qquad (7\text{-}11)$$

$$CSY = 58.83+5.55T+3.28t-4.20Tt-4.34T^2-2.72t^2-0.22T^2t-3.60Tt^2 \quad (R^2\ 0.87) \qquad (7\text{-}12)$$

The *p* value for the lack of fit and for the models themselves were respectively over 0.05 and below 0.05, indicating that the models developed for the CSY response were statistically significant for all the feedstocks and both types of pretreatment. The coefficients of determination had values of 0.88-0.99 for the developed models of glucose yield from enzymatic hydrolysis (Annexe) and CSY, as shown above. This implies that only 1-12% of the variation of the response could not be explained by the models, and indicates relatively good agreement between experimental and predicted values.

The *p* values for the linear (T: temperature; t: time) and quadratic (T^2 and t^2) model terms as well as their interaction Tt, were less than 0.05 for water impregnation SE of the cultivar M13 (Table A 1).

Statistical significance at 95% confidence interval was found for the linear model terms and their linear interaction for water-only pretreatment of the straw M9, although the quadratic terms showed significance at 90% of confidence (Table A 1). The CSY from SO_2-pretreatment of cultivar M13 showed statistical significance of the second order interaction between T and t variables (T^2t) as indicated by a *p* value of 0.0092 (Table A 1). In the case of M14, only the quadratic term time (t^2; *P* 0.0298) and the linear interaction temperature-time (Tt; *P* 0.0048) were statistically significant and had a significant influence on the CSY response at the 95% confidence interval (able A 2. Thus, the straw samples presented different types of interactions between pretreatment parameters. This is a direct indication of their specific pretreatment demands for CSY maximization, as found for varieties of sugarcane bagasse [47].

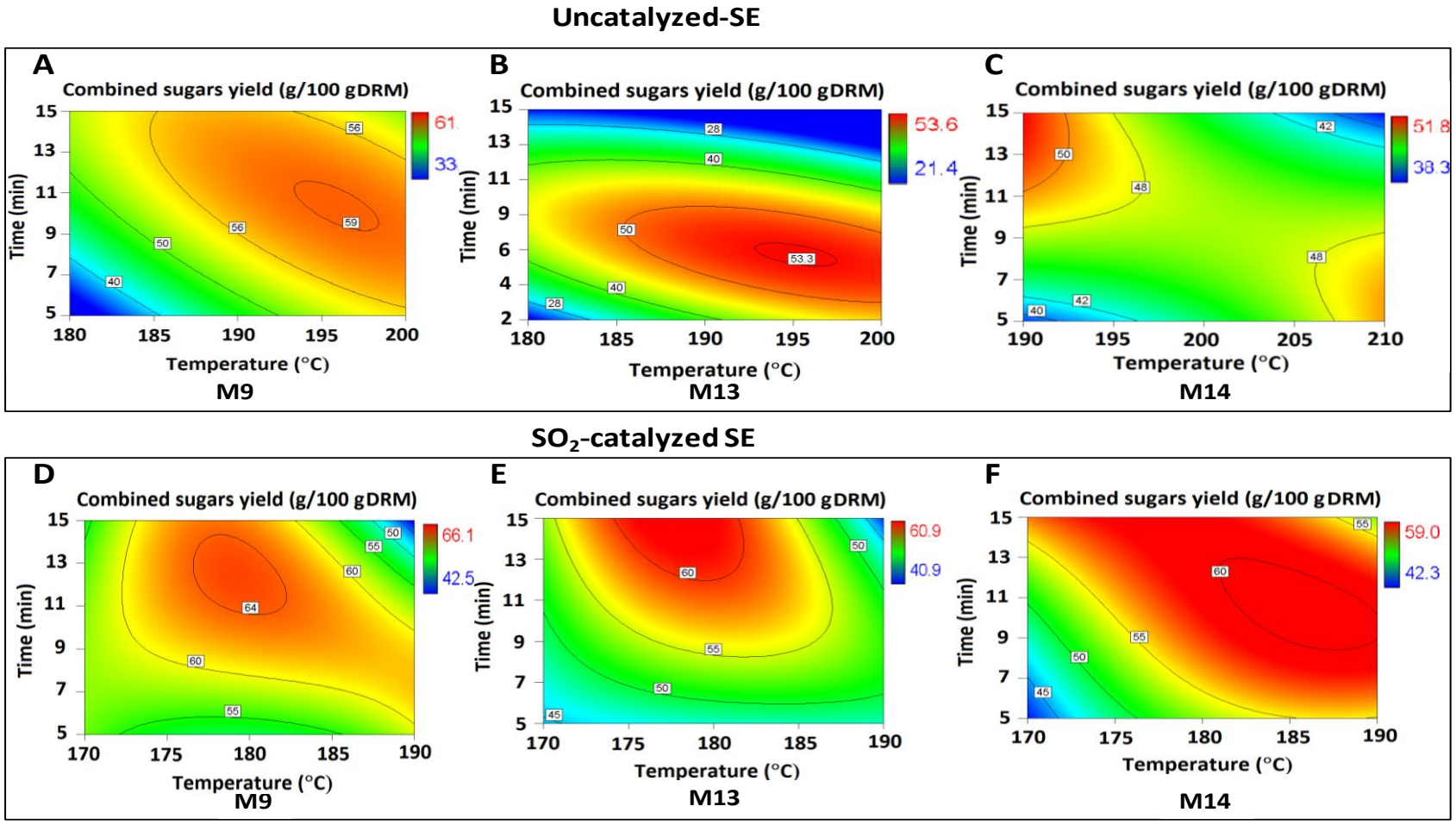


Figure 7-3: Contour plots for the response combined sugars yield from pretreatment followed by enzymatic hydrolysis of the WIS resulting from (A, B, C) uncatalyzed and (D, E, F) SO_2-catalyzed pretreatments of triticale straw. (A) and (D) feedstock M9, (B) and (E) feedstock M13, and (C) and (F) feedstock M14. The response is plotted as function of pretreatment temperature and residence time for both types of pretreatment. For SO_2 pretreatment, the acid concentration was kept constant at 3% (w/w). Yields are expressed as grams per 100 grams of dry raw material (DRM) straw for all of the feedstocks.

7.3.6 CSY Optimisation and models validation

The predictive models developed for the CSY response for both pretreatments and various straw samples were subjected to simultaneous optimisation by the desirability function, to maximise CSY and constrain inhibitors formation, as stated in section 7.2.4: Experimental design and optimisation. The production of inhibitors during pretreatment was limited according to yeast tolerance thresholds during fermentation as described previously. Concentrations of furans and derivatives of higher than 2 and 0.5 $g.L^{-1}$, respectively, have been reported to inhibit enzymatic hydrolysis and fermentation [48]. In the case of weak acids, the inhibitory effect depends mainly on the pH in the fermentation broth. However, concentrations higher than 1 $g.L^{-1}$ and 2-6 $g.L^{-1}$ of formic acid and acetic acid, respectively, have been reported to decrease ethanol production by *S. cerevisiae* [14;26]

Experimental validation of the predicted optimum conditions was performed with the straw samples under pretreatment at each pretreatment mode. The optimum pretreatment conditions, desirability of the response and the predicted/experimental CSY and inhibitors yields, for each feedstock and pretreatment mode under the established criteria, are listed in Table7-5. As indicated by relatively high desirability values (0.77 – 0.91), these pretreatment conditions are within the optimal regions for maximisation of CSY (see Figure 7-3), while limiting inhibitors formation. Higher CSY was predicted for straw M9 by water and SO_2 pretreatments with yields of 59.1 and 64.4 g/100 g DRM, (91 and 98.8% total sugars recovered), respectively (Table 7-5), compared to other straws. Straws M13 and M14 gave a comparable prediction of maximum CSY with values of 52.2 and 50.4 g/100 g DRM (sugars recovery of 88.6 and 82%), respectively, by water-SE and fairly similar yield of around 61 g/100 g DRM by SO_2-SE, close to the maximum potential sugars recovery (Table 7.2) Some of the predicted optima were associated with acetic acid concentrations that were marginally above the set limits of inhibition of 2-6 $g.L^{-1}$ (Table 7-5). Residual acetyl group content in the WIS fraction at the predicted optimum conditions ranged from 1.6 – 2.3 $g.L^{-1}$ (Table 7-6). In all cases there was less than 6% variation between predicted responses and measurements obtained by validation after statistical optimisation (Table 7-5).

The feedstocks required different ranges of pretreatment conditions for the optimisation of the CSY by water-SE pretreatment (Table 7-5). Straw M13 presented lower water-SE pretreatment requirements for optimum CSY (192.6°C and 5.6 min, see Table 7-5) as a result of the high influence of pretreatment temperature and time in linear and quadratic fashion for this specific straw (Table A1). For M9 (197°C and 10 min, see Table 7-4) the yield was influenced by only the linear model terms of temperature and time (Table A-1). For M14 straw (190°C and 11.7 min) the yield was

influenced by the linear interaction temperature-time and the quadratic term of time. However, straw M9 gave close to 9% more CSY compared to M13 and M14, which resulted in comparable validated yield (Table 7-5). Thus, straw quality features may be responsible for its observed specific pretreatment needs to maximise the CSY.

The SO_2 and water-only pretreatments proved to be adequate for providing highly digestible solids. Thus, both pretreatment methods show promising applications with regards to improving digestibility. Similar trends were also observed for wheat straw [30]. With regards to combined sugars yield/recovery, SO_2 pretreatment was the most effective method, yielding mostly monomeric sugars for all the straw samples, although at an expense of higher inhibitors production (~2.47-fold total inhibitors when compared to water-only pretreatment). In addition, the SO_2-catalysed method reduced the pretreatment temperature demands by 8-16°C when compared to the water-only method (Table 7-5). The above observations could be primarily attributed to features of SO_2-pretreatment which improves digestibility, mainly by facilitating high hemicellulose solubilisation [30]. On the other hand, addition of external catalyst was not required to achieve high combined sugars recoveries (81-95%) for water-only pretreatment, which is relevant from a process point of view.

Improvements between 4.8 and 8.6% were realized after pretreatment optimisation at pilot-scale when the results of the pretreatment optimisations at bench- and pilot-plant scale were compared. Pretreatment performance observed at bench-scale regarding optimum pretreatment conditions for maximum yields of sugars after pretreatment-enzymatic hydrolysis matched well to what was observed after pilot-plant SO_2-SE optimization. Optimum conditions found in Chapter 5 for maximum sugars yield (straw M9. Entry 01T43) were 182°C-0.39% (w/w) H_2SO_4-15.5 min. Optimum SO_2-SE pretreatment conditions for maximal CSY for straw samples M9, M13 and M14 in the present chapter showed to be in the range 178-182°C, 3% (w/w) SO_2 and 11.6-14 min. Differences observed in acid required under pilo-plant SE may be explained by the fact that homogeneus and effective absorption of gaseous SO_2 inside the material is difficult prior pretreatment besides part of the acid is lost during feeding. Impregnation with aqueous H_2SO_4 could be also more effective in penetrating the pores of the straw structure and consequently less acid may be required. The inclusion of SO_2 as catalyst for SE resulted in about 5.6-11.3% additional improvements in combined sugars yield among straws. Therefore, improvements in estimate 2G ethanol yield per ton of straw of similar magnitudes are envisaged after pretreatment optimisation.

Pretreatment process optimisation also included the identification of a set of preferred pretreatment conditions. These could be applied to any of the triticale straw samples, to achieve at least 95% of the maximum CSY predicted by optimisation, while maintaining inhibitor concentrations below acceptable thresholds. Contour plots, obtained from continuous functions that describe the predictive model equations, bounded by an input data from the experimentation, were obtained for each type of SE pretreatment (See Figure 7.4). An overlapping range of preferred pretreatment conditions in terms of temperatures and residence times were identified for both water-only and SO_2-impregnation pretreatments (at the 95% confidence interval), representing an area of pretreatment conditions in common for any of the triticale straw samples. Water-only pretreatment at temperatures between 190 and 205°C and residence times between 4 and 9 min, corresponding to severities of 3.35-3.79, will achieve at least 95% of the maximum CSY observed for each of the straw samples (Figure 7-4). SO_2-impregnated pretreatment at temperatures between 173 and 187°C combined with residence times of 7-14 min, corresponding to severity factors of 3.30-3.41, will achieve at least 95% of the maximum CSY from any of the straws (Figure 7-4).

The common pretreatment conditions (area of intersection in Figure 7-4) that maximise CSY of the studied straw samples were also evaluated with an additional straw (sample M4 with technical entry US2007, season 2010). This sample showed the poorest response to pretreatment in terms of sugars yield in the performed screening selection of cultivars (Chapter 4). This additional evaluation sought to confirm that pretreatment severities in common for maximum CSY, of the straws used for model generation, are also relevant for other triticale cultivars. Water- and SO_2-pretreatments were carried out by triplicate runs at the pretreatment severities Log (*R'o*) of 3.78 and 3.30 which corresponded to pretreatment severities within the overlapped areas for both pretreatments, respectively. Straw M4 showed glucose and xylose contents of 36.0±0.9 and 23.5±0.6 g/100 g DRM respectively, while ash content was 1.3±0.1 g/100 g DRM. Thus straw M4 had potential combined sugars recovery (xylose + glucose) of 60 g/100 g DRM. As observed in Table 7-5, sample M4 yielded combined sugars of 60.5 g/100 g DRM (water-only) and 50.9 g/100 g DRM (SO_2-SE), representing recoveries of 98 and 82% of combined sugars respectively. Straw M4 showed even higher recoveries by uncatalysed pretreatment than those obtained for straw samples M9, M13 and M14 ($\leq$ 95%) and relatively high CSY recovery by acid-catalysed pretreatment. Straw M4 also yielded lower inhibitor concentration compared to the other studied straw samples, although its residual acetyl groups in WIS reached 2.5 $g.L^{-1}$, which was slightly higher than that for straw M9 (Table 7-5).

Table 7-5: RSM based predictive models of the combined sugars yield (CSY) and model validation from uncatalyzed and SO_2-catalyzed SE pretreatment followed by enzymatic hydrolysis for the feedstocks M9, M13 and M14. Temperature (T) and time (t) are given in coded form. The desirability of the optimization is given in values between 0 and 1. Straw M4 was included for validation (tested at selected conditions for water and SO_2 SE pretreatment).

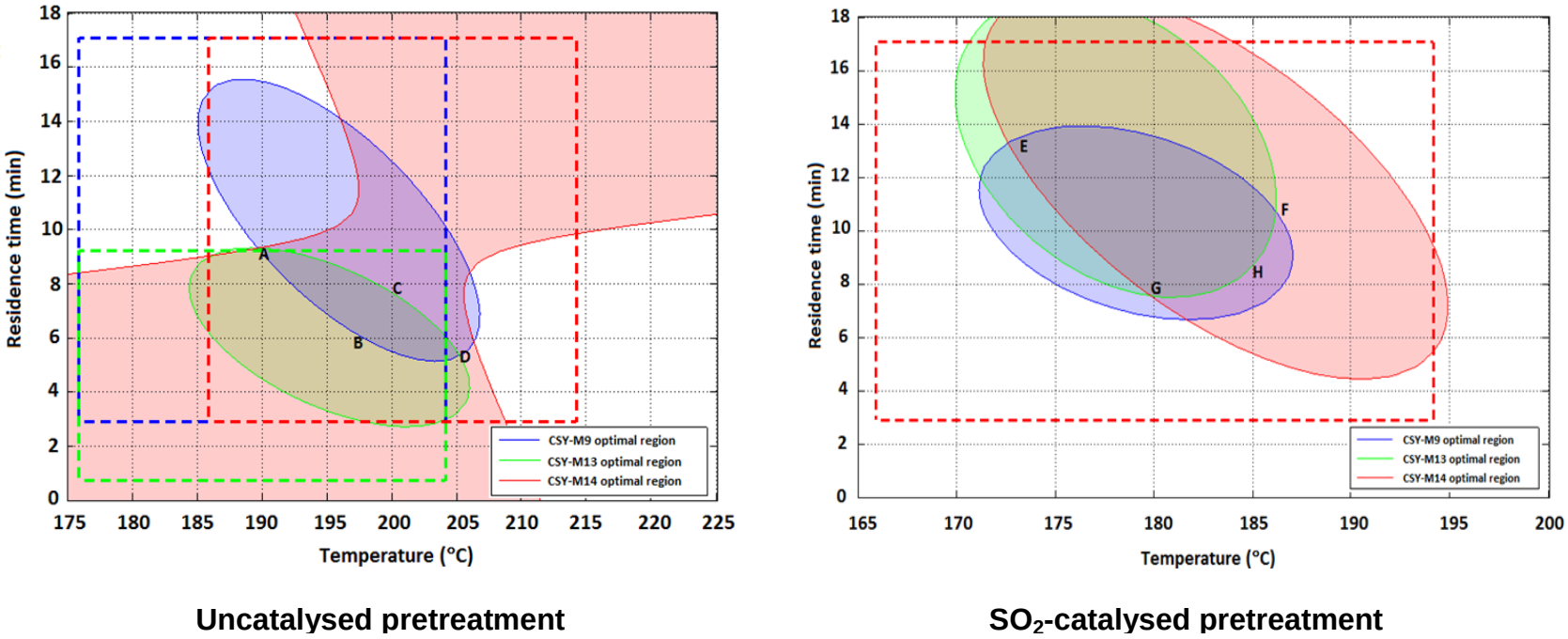
Water-Only Pretreatment										
Optimum Conditions			Predicted and experimental combined sugars yield (CSY)[1]							
Feedstock	Temperature (°C)	Time (min)	CSY (g/100 g DRM	HMF ($g.L^{-1}$)	Furfural ($g.L^{-1}$)	Formic Acid ($g.L^{-1}$)	Acetic Acid ($g.L^{-1}$)	Acetyl Groups[2] ($g.L^{-1}$)	Desirability	Relative Error[3]
M9	196.9	9.9	59.1 (58.9±2.2)	0.15 (0.16±0.01)	0.67 (0.93±0.06)	0.52 (0.67±0.03)	1.95 (2.54±0.07)	1.55 (1.50±0.05)	0.81	-0.33
M13	192.6	5.1	52.2 (54.2±1.8)	0.88 (0.66±0.01)	0.18 (0.19±0.01)	0.46 (0.49±0.01)	1.30 (1.50±0.03)	2.62 (2.1±0.03)	0.77	3.83
M14	190	11.7	50.4 (53.4±1.8)	0.06 (0.22±0.02)	0.47 (1.2±0.08)	0.30 (0.61±0.01)	1.46 (2.43±0.09)	2.11 (1.64±0.06)	0.81	5.95
M4 (validation)	194.5	10.0	60.5	0.03±0.01	0.20±0.01	0.29±0.01	1.23±0.03	1.55±0.07	-	-
SO_2-Impregnation Pretreatment										
Optimum Conditions			Predicted and experimental combined sugars yield (CSY)[1]							
Feedstock	Temperature (°C)	Time (min)	CSY (g/100 g DRM	HMF ($g.L^{-1}$)	Furfural ($g.L^{-1}$)	Formic Acid ($g.L^{-1}$)	Acetic Acid ($g.L^{-1}$)	Acetyl Groups[2] ($g.L^{-1}$)	Desirability	Relative Error[3]
M9	180.7	11.6	64.4 (62.4±1.6)	0.38 (0.08±0.01)	1.14 (0.36±0.01)	0.21 (0.17±0.01)	3.08 (1.47±0.03)	1.62 (2.34±0.03)	0.80	-3.11
M13	177.6	13.6	61.1 (57.8±0.2)	0.77 (0.26±0.01)	1.03 (0.59±0.01)	0.47 (0.27±0.01)	2.93 (2.22±0.01)	1.86 (2.1±0.17)	0.91	-5.40
M14	181.8	11.7	60.2 (57.4±0.7)	0.53 (0.15±0.01)	1.06 (0.64±0.02)	0.21 (0.19±0.01)	2.30 (1.91±0.09)	1.57 (1.92±0.07)	0.87	-4.65
M4 (validation)	178	10.0	50.9	0.04±0.01	0.25±0.01	0.15±0.01	1.18±0.01	2.54±0.06	-	-

(1). Predicted yields. Values in parenthesis indicate the averaged experimental value for the response yield. Combined sugars yield (CSY) is expressed in grams per 100 grams of dry raw material (DRM).Yields of inhibitors and acetyl groups are given in $g.L^{-1}$.

(2). Represent the potential acetic acid, expressed in $g.L^{-1}$ that could be released from the residual acetyl groups in the WIS during SSF conducted at 20% w/v of solid loading.

(3). Refers to the divergence between the experimental and predicted CSY as optimized response (+/-) signs indicate high/low experimental value with respect to the expected.

R^2 corresponds to the coefficient of determination of the model equation.



Uncatalysed pretreatment **SO_2-catalysed pretreatment**

Figure 7-4: Contour plots representing the overlapping of pretreatment conditions (temperature and time) that results in not lesser than 95% of the maximum yield of combined sugars from either uncatalysed (left graph) or SO_2-catalysed (right graph) steam pretreatment of all the feedstock. The overlapped areas are enclosed by A, B, C and D (water-only) and E, F, G and H (SO_2-impregnation). Combined sugars yield (CSY) is expressed in grams per 100 grams of dry raw material. The continuous and dotted coloured lines represent the area of conditions that maximize CSY and the input range of the independent variables into the experimental design, respectively.

The much lower ash content in straw M4 in comparison to the other studied straw samples (lower by margins of 1.1-1.5), could be responsible for enhancement of the performance of straw M4 at the established optimum pretreatment conditions. Thus the ash contents of triticale straw impacts the sugar release negatively; which follows similar trends found for wheat straws [27] and switchgrass [28]. These results demonstrated that pretreatment optimisation is essential to improve the yields of combined sugars per gram of straw based on the selection of cultivars with low ash content in straw. It also demonstrated the suitability of using the statistically derived common pretreatment area to predict high combined sugars recovery from other straws of different triticale cultivars grown in South Africa.

7.4 Conclusions

The study evaluated optimisation of SO_2- and water-only pretreatment of triticale straw for maximum CSY at pilot scale. This was accomplished by determining the optimum conditions through the development of robust models to maximise CSY, while limiting toxicity of pretreated materials from straws with demonstrated cultivar variability.

Triticale cultivars varied in their response to pretreatment. This had a large impact on the CSY under the studied pretreatment methods. Response to SE pretreatment (processability) by cultivars is directly related to ash content of straw (chemical composition feature). The impact of the ash content was significant and comparable irrespective of the scale of pretreatment, since methods of pretreatment at the studied pilot scale displayed fundamental similarities (effective hemicellulose solubilisation) to those previously used in a small scale study (Chapter 5). Pretreatment with SO_2 is more effective in the recovery of combined sugars with an achieved improvement of 8 -16% compared to water-only pretreatment. However, higher inhibitor concentrations (acetic acid as a major proportion) stand as a detriment in the SO_2 method.

Through pretreatment optimisation at pilot-plant scale, improvement of up to 14% in the yields of combined sugars compared to the previous bench-scale stage was achieved. Pretreatment optimisation was demonstrated to effectively address the impact of cultivar variability of triticale straw on pretreatment response, while also limiting inhibitor production.

Of the three studied regions, triticale straws from Mariendahl exhibited the lowest ash content, which suggests that straws from Mariendahl could be good candidates for 2G ethanol production. The yields of ethanol was successfully maximised through the use of robust pretreatment

optimisation models, which could be a relevant tool to fulfil a feedstock predictability requirement for industrial 2G ethanol production.

Acknowledgements

The authors gratefully acknowledge Dr. Willem Botes from Department of Genetics, Stellenbosch University, for performing the field trials and providing the triticale straw material used as feedstocks in this study. Manda Rossouw and Levine Simmers at Dept. of Process Engineering, University of Stellenbosch are greatly acknowledged for laborious HPLC analysis during the experimentation. The Technology Innovation Agency (TIA), an entity of the Department of Science and Technology (DST) of the Republic of South Africa, is acknowledged for funding support of this study and its authors.

Competing interests

The authors declare have no competing interests to declare.

Authors' contributions

RA performed all the experimental work, data interpretation and drafted the manuscript. MGA provided guidance for experimental work, participated in coordination of the study, and critically reviewed the manuscript. JFG conceived the study, provided critical inputs into the study in terms of experimental approaches, participated in its design and coordination and reviewed the manuscript. All authors read and approved the final manuscript.

Abbreviations

1G: First generation; 2G: Second generation; ANOVA: Analysis of variance; CCD: Central composite design; CSF: Combined severity factor; CSY: Combined sugar yield; EH: Enzymatic hydrolysis; FPU: Filter paper unit; GHG: Greenhouse gas; ha: Hectare; HMF: Hydroxymethylfurfural; HPLC: High-performance liquid chromatography; IU: International unit; LAPs: Laboratory analytical procedures; MC: Moisture content; DRM: Dry raw material; RSM: Response surface methodology; SE: Steam explosion; SF: Severity factor; SSF: Simultaneous saccharification and fermentation; WIS: Water- insoluble solids; WSS: Water Soluble Solids.

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Annexe of chapter 7

Glucose yield from enzymatic hydrolysis for uncatalyzed- and SO_2- catalyzed SE of the feedstocks M9, M13 and M14 was subjected to optimisation and predictive models to describe the experimental yields were developed.

EH-glucose optimisation

The predictive models developed for EH-glucose yield for all of the feedstocks for water- and SO_2-pretreatments of the straws M9, M13 and M14 are given by the Equations A1 – A3 and A4 – A6, respectively.

Water-pretreatment:

M9: G_Y= 32.46+7. 16T+5.15t-3.08Tt-1.90T^2-2.61t^2 (R^2 0.92) (A1)

M13: G_Y = 28.83+6.57T+4.90t-0.94Tt-*2*.21T^2-2.81t^2 (R^2 0.96) (A2)

M14: G_Y = 30.46+3.13T+2.32t-2.73Tt+0.26T^2-3.56t^2 (R^2 0.97) (A3)

SO_2-pretreatment:

M9: G_Y = 40.26+2.74T+4.60t-2.74Tt-3.84T^2-4.66t^2-6.44T^2t-5.76Tt^2 (R^2 0.99) (A4)

M13: G_Y = 33.55+0.28T+3.21t-3.30Tt-5.47T^2-3.04t^2 (R^2 0.89) (A5)

M14: G_Y = 32.00+4.13T+3.03t-2.08Tt-1.44T^2-1.58t^2 (R^2 0.89) (A6)

Contour plots describing graphically the EH-glucose yield as function of temperature and time were represented based on the predictive models (Figure A-1)). The highest EH glucose yields of 38.1 and 41.4 g/100 g DRM were predicted for the straw M9 from water-SE and SO_2-impregnation pretreatments, respectively (Figure A-1) and Figure A-D), respectively). Higher demand in pretreatment temperature for the straw M14 to achieve maxima EH glucose yield from both types of pretreatment (35 g/100 g DRM) compared to M9 and M13 was observed. These observations are in line with the experimental trends presented in Figure 7-1.

The effect of water- and SO_2-pretreatment conditions on maximising EH glucose yield for all of the feedstocks is shown in Figure A-1. As observed, higher temperatures (> 200°C) were required to reach maximum glucose yields by water-SE by the feedstocks M9 and M13 but even much higher by M14 (Figure A-1 below). It was also found that requirements in residence time for maximum yield differed more notoriously among cultivars; required times ranges of 9 - 14 min, 6 – 8 min, and 7 -11

min would result in maximum EH-glucose yield for the straws M9, M13 and M14, respectively (Figure A-1, Inserts A, B and C). If temperatures of 200°C for straws M9 and M13 and 210°C for straw M14 are assumed together with the respective referred ranges of residence time, severities (Log (*R'o*)) for maximal EH-glucose yield for water-SE from the straws M9, M13 and M14 would correspond to ranges of 3.9 – 4.09, 3.72 – 3.85, and 4.08 – 4.28, respectively. These in line with severities found for maximal experimental glucose yield.

Small differentiation in temperature for maximal EH-glucose between the feedstock M9 and M13 (around 180°C) but larger against M14 (> 190°C) was found coupled with little differences in residence time (around 12 min) for SO_2-SE pretreatment among feedstocks. Thus severities (CSF) for maximizing EH-glucose yield by SO_2-SE, if assumed pH values of 2.2 and 1.7 respectively during pretreatment of straws M9 and M13 at 180°C – 12 min, and 2.2 for pretreatment of M14 at 190°C – 12 min (based on trends of pH as observed in Table 7-1), would correspond to 1.23, 1.73 and 1.53. Severities also in agreement with severities for maximum measured EH-glucose yields (Figure 7-1).

Table A-1: Summary of the ANOVA analysis for the combined sugars yield from uncatalyzed and SO2-catalyzed SE of straw samples M9 and M13.

Source	M9-CSY										M13-CSY									
	uncatalyzed					SO2-catalyzed					uncatalyzed					SO2-catalyzed				
	SS	df	MS	F value	p-value	SS	df	MS	F value	p-value	SS	df	MS	F value	p-value	SS	df	MS	F value	p-value
Model	911.1	5	182.2	7.1	0.0255	533.1	7	76.2	10.6	0.0394	1161.8	5	232.4	21.0	0.0023	592.3	7	84.6	39.3	0.0060
T-Temp	308.4	1	308.4	12.0	0.0180	5.0	1	5.0	0.7	0.4658	448.2	1	448.2	40.6	0.0014	3.6	1	3.6	1.7	0.2885
t-time	187.6	1	187.6	7.3	0.0428	104.5	1	104.5	14.5	0.0318	342.4	1	342.4	31.0	0.0026	200.7	1	200.7	93.2	0.0024
Tt	191.8	1	191.8	7.5	0.0412	53.4	1	53.4	7.4	0.0725	133.1	1	133.1	12.0	0.0178	53.7	1	53.7	24.9	0.0154
T^2	132.6	1	132.6	5.2	0.0724	130.5	1	130.5	18.1	0.0238	159.8	1	159.8	14.5	0.0126	295.4	1	295.4	137.3	0.0013
t^2	155.9	1	155.9	6.1	0.0571	184.4	1	184.4	25.6	0.0149	148.3	1	148.3	13.4	0.0145	76.8	1	76.8	35.7	0.0094
T^2t	-	-	-	-	-	205.3	1	205.3	28.5	0.0129	-	-	-	-	-	77.6	1	77.6	36.1	0.0092
Tt^2	-	-	-	-	-	25.8	1	25.8	3.6	0.1548	-	-	-	-	-	20.9	1	20.9	9.7	0.0525
Residu-al	128.6	5	25.7			21.6	3	7.2			55.2	5	11.0			6.5	3	2.2		
Lack-of-Fit	80.9	3	27.0	1.1	0.5013	5.2	1	5.2	0.6	0.5084	52.9	3	17.6	15.5	0.0612	2.7	1	2.7	1.43	0.3546
Pure Error	47.7	2	23.9			16.4	2	8.2			2.3	2	1.1			3.8	2	1.9		
Cor Total	1039.7	10				554.7	10				1217.0	10				598.7	10			

SS: Sum of squares. Df: degree of freedom. MS; Mean square. P-value (Prob > F)

Table A-2: Summary of the ANOVA analysis for the combined sugars yield from uncatalyzed and SO2-catalyzed SE of straw M14.

Source	M14-CSY									
	uncatalyzed					SO2-catalyzed				
	SS	df	MS	F value	*p-value*	SS	df	MS	F value	*p-value*
Model	219.9	5	44.0	7.1	0.0252	408.5	7	58.4	267.9	0.0003
T-Temp	4.1	1	4.1	0.7	0.4527	123.1	1	123.1	565.1	0.0002
t-time	0.8	1	0.8	0.1	0.7407	43.0	1	43.0	197.2	0.0008
Tt	143.8	1	143.8	23.3	0.0048	70.6	1	70.6	324.3	0.0004
T^2	2.4	1	2.4	0.4	0.5623	106.4	1	106.4	488.2	0.0002
t^2	55.9	1	55.9	9.0	0.0298	41.7	1	41.7	191.3	0.0008
T^2t	-	-	-	-	-	0.1	1	0.1	0.4	0.5565
Tt^2	-	-	-	-	-	25.9	1	25.9	119.1	0.0016
Residu-al	30.9	5	6.2			0.7	3	0.2		
Lack-of-Fit	13.6	3	4.5	0.5	0.7086	0.5	1	0.5	5.9	0.1358
Pure Error	17.3	2	8.7			0.2	2	0.1		
Cor Total	250.8	10				409.1	10			

SS: Sum of squares. Df: degree of freedom. MS; Mean square. *P*-value (Prob > F)

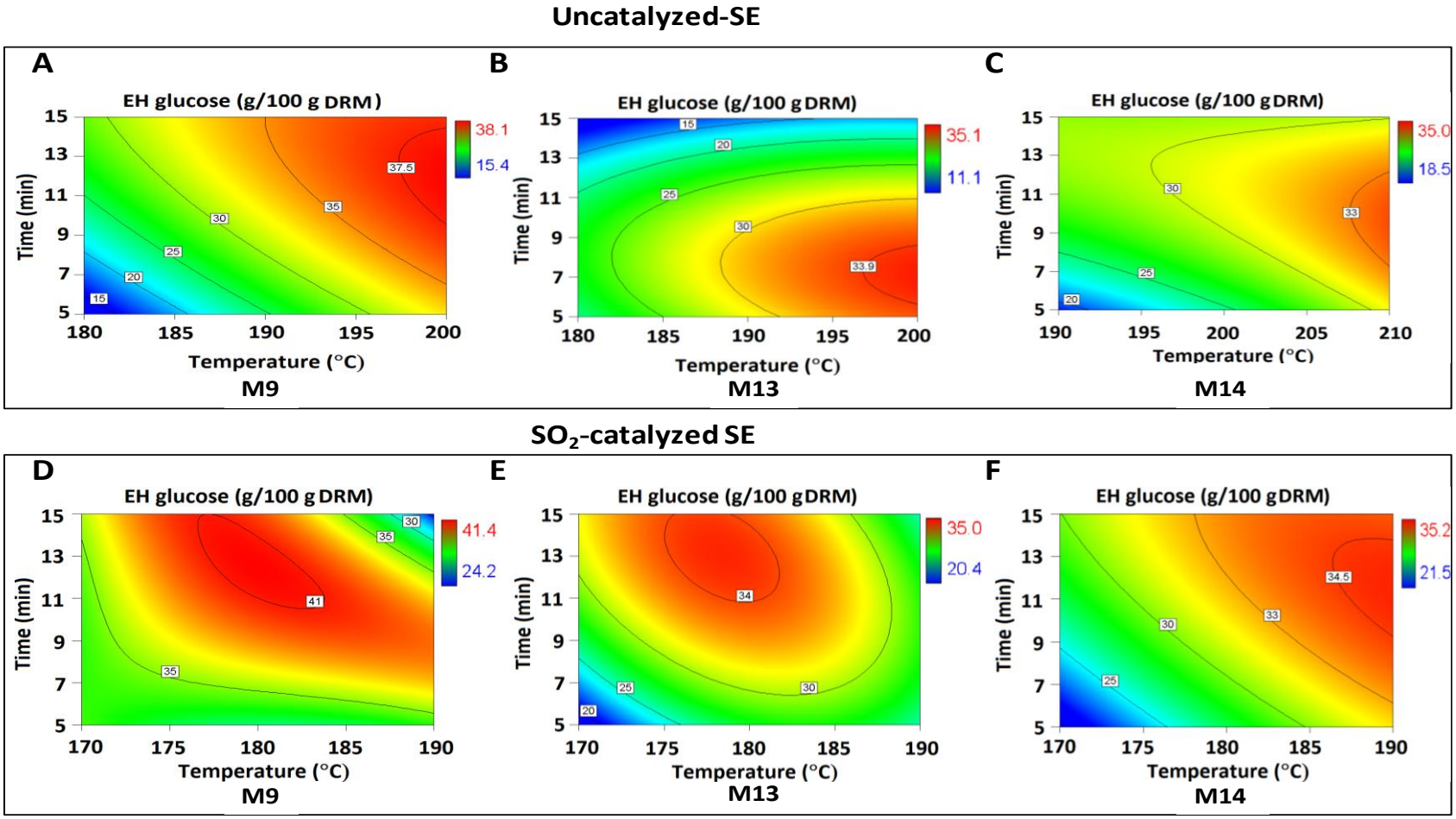

Figure A-1: Contour plots for the response EH-glucose yield of the WIS resulting from (A, B, C) uncatalyzed and (D, E, F) SO2-catalyzed pretreatments of triticale straw. (A) and (D) feedstock M9, (B) and (E) feedstock M13, and (C) and (F) feedstock M14. The response is plotted as function of pretreatment temperature and residence time for both types of pretreatment. For SO_2 pretreatment, the acid concentration was kept constant at 3% (w/w). Yields are expressed as grams per 100 grams of dry raw material (DRM) straw for all of the feedstocks.

Chapter 8

8 Impact of cultivar selection and further pretreatment optimisations on experimental ethanol yield of straw from triticale cultivars in the integrated configuration SSF

Chapter 8 was structured here as a draft of a manuscript for submission to a journal with the entire experimental data and results on the study performed to experimentally observe the impact of cultivar selection and pretreatment optimization on the SSF configuration.

Objective of dissertation

Chapter 8 addresses the **Objective 4**: Assess the effects of cultivar selection and subsequent pretreatment optimisations on the experimental ethanol yield of the preferred straws cultivars in the integrated configuration SSF.

In this chapter, the results of the pretreatment optimisations, described in Chapter 7, were evaluated by an integrated process configuration for sugars-to-ethanol conversion. The impact of cultivar selection and subsequent pretreatment optimisations for maximised combined sugars yields on the ethanol output from straws was experimentally assessed. Fermentation strategies applying high solid loadings and a fed-batch approach were investigated with the aim to study the fermentability of the pretreated materials at optimal pretreatment conditions. Additionally, the experimentation was conducted in order to obtain ethanol concentrations at benchmark levels close to 4% (v/v), as required at large-scale operation.

Summary of findings in present chapter

In order to achieve high yields of fermentable sugars and subsequently high ethanol concentrations from lignocellulosic biomass, the material must first be pretreated. Pretreatment is primarily intended to enhance enzymatic digestibility of the biomass which is subsequently subjected to hydrolysis-fermentation. After completion of pretreatment, pretreated solids (highly digestible) and a liquid phase (containing all the soluble sugars, inhibitors and other soluble matter in water) results in a liquid-solid mixture which is commonly referred to as “whole slurry”. Industrial cellulosic ethanol production requires a hydrolysis-fermentation process with final ethanol concentrations of at least 4% (v/v) to be suitable for distillation. It is generally recognized that the

required increase in ethanol concentrations is accomplished by increasing the concentration of fermentable sugars in the process. This can be achieved by increasing the substrate loading above 10% which has been shown to obtainment of the required fermentable sugar and ethanol yield targets. The use of the whole slurry with no further processing as a substrate in hydrolysis-fermentation represents an option. However, this approach comes with associated drawbacks such as mixing problems, end product inhibition, and larger amounts of inhibitors present in the mixture and mass transfer limitations generally taking place at above 20% solids loading concentrations. The use of the water insoluble solids, which is obtained by separating the liquid fraction from the whole slurry (commonly by press-filtration), so called unwashed WIS, substantially reduces most of these potential problems and especially those related to the presence of inhibitors in the substrate for hydrolysis-fermentation. However, some studies have shown that washing the WIS (resulting in the so called washed-WIS) can alleviate problems of low enzymatic activity caused by enzyme inhibiting compounds, such as furaldehydes and lignin derivatives. These inhibiting compounds usually remain adhered to the fibres when unwashed WIS is used as a substrate. Thus, solids loading concentrations of between 10 and 20%, in combination with the use of washed WIS, are promising options to be evaluated on pretreated triticale straw with the aim to reach ethanol concentrations close to 4% (v/v). Preliminary fed-batch SSF with pressed slurry at 20% (w/w) solids loading, to evaluate the maximum solid loading operational for the yeast, resulted in maximal ethanol concentrations not higher than 28 $g.L^{-1}$. This is less than 30% of the theoretical ethanol yield of 0.51 g $ethanol.g^{-1}$ glucose. Solid loading of 13% (w/w) using water insoluble solids (WIS) was found to reduce the effects of inhibitors in the sugar stream. SSF tests at 13% solids using pretreated solids from uncatalysed-SE reached maximum ethanol concentrations of 25.8, 24.5 and 30.8 $g.L^{-1}$, corresponding to ethanol yields of 75, 63.3 and 75.5% with respect to the theoretical yield (0.51 g ethanol/g glucose) for straws 13, 9 and 14, respectively. For SSF at 13% WIS (w/w), maximum values of ethanol concentration of 21.8 and 38.3 $g.L^{-1}$, corresponding to ethanol yields of 0.27 and 0.51 g ethanol/g glucose consumed (53.2 and 99.9% of respective theoretical yields) were found for SO_2-WIS (washed) straw 9 and SO_2-WIS (intensively washed) straw 14, respectively. This corresponded to ethanol productivity of 0.38 and 0.8 gram ethanol/L/h (at 24 h). The experimental results of SSF with intensively washed WIS from catalysed pretreatment of straw M14 showed a maximum ethanol concentration of 38.3 $g.L^{-1}$, which is only 4.2% lower than 40 $g.L^{-1}$ (considered as benchmark for distillation). Feedstock selection combined with high solid loadings was therefore required for acceptable ethanol process performance. Pretreatment is responsible for both sugars availability and inhibitor concentrations - both of which have a massive impact on ethanol production. Ethanol production as a biological process is therefore greatly influenced by the properties of pretreated

materials, making pretreatment performance (straw response to pretreatment) a critical bottleneck in ethanol production from straw. In the experimental work done for this chapter, cultivar selection and further pretreatment optimisation were demonstrated to have a positive impact on the maximisation of the experimental ethanol yield from triticale straw in the process configuration SSF.

Candidate declaration

With regard to chapter 8 page numbers 220-246 of this dissertation, the nature and scope of my contribution were as follows

Name of contribution	**Extent of contribution (%)**
Planning of experiments	40
Executing experiments	90
Interpretation of results	60
Writing the chapter	90

The following co-authors have contributed to chapter 8 page numbers 220-246 of this dissertation.

Name	**e-mail address**	**Nature of contribution**	**Extent of contribution**
1. Maria del Prado Garcia-Aparicio	Garcia@sun.ac.za	- Planning of experiments - Executing experiments - Providing inputs - Interpretation of results - Writing the chapter - Reviewing the chapter	40 10 75 30 10 80
2. Johann Görgens	jgorgens@sun.ac.za	- Planning of experiments - Interpretation of results - Providing inputs - Reviewing the chapter	20 10 25 20

Signature of candidate……

Date……

Declaration by co-authors

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 8 page numbers 220-246 in the dissertation,

2. No other authors contributed to chapter 8 page numbers 220-246 in the dissertation besides those specified above, and

3. Potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in to chapter 8 page numbers 220-246 of this dissertation.

Abstract

High solid loadings during enzymatic hydrolysis and/or fermentation of pretreated lignocelluloses are essential to reach the desired final ethanol concentration of at least 4% (v/v) as required for industrial distillation. The use of whole slurry is an alternative to ensure high solids loadings that provides higher sugar concentration in the fermentation broth. However, with this approach problems associated with homogenisation and fermentation inhibitors are commonly observed due to high solids loadings. These problems can however be reduced by using the pressed pretreated material and fed-batch feeding during the SSF. In this study, solids loadings of 13% (total solids, TS) of steam exploded material were applied in a fed-batch mode of the simultaneous saccharification and fermentation (SSF) process. Solid feedings of 1 and 2% TS were done daily to reach the final 13% TS in the broth. The pretreated materials were obtained from optimised uncatalysed and SO_2-catalysed steam explosion conditions (described in **Chapters 6** and **7**) of straws from the preferred triticale cultivars 9, 13 and 14 of Mariendahl origin (selected in **Chapter 5**). The whole slurry material was press-filtered to approximately 40% of total solids, separated from the liquid and used in SSF. In addition to fermentation testing with pressed-slurry, washing and filtration of SO_2-SE pretreated materials from straws 9 and 14 were also performed, to obtain WIS as feedstock for SSF, and to evaluate the possible negative effects of inhibitors in pressed-slurries on ethanol production. An optimised enzyme mixture, which is highly inhibitor tolerant and contains similar dosages of cellulase and hemi-cellulase of 0.1 ml/g TS, was added at time zero of the SSF together with an inoculum of *S.cerevisiae* strain MH1000 at 5 $g.L^{-1}$. Preliminary SSF tests at 20% solid loading reached maximal ethanol concentrations not higher than 28 $g.L^{-1}$ (less than 30% of theoretical ethanol yields based on 0.51 g ethanol/g glucose) from pressed slurries from both types of pretreatments. SSF tests at 13% solid loading from uncatalysed pressed slurries reached maximum ethanol concentrations of 25.8, 24.5 and 30.8 $g.L^{-1}$, corresponding to ethanol yields of 75, 63.3 and 75.5% of the theoretical yield (0.51 g ethanol/g glucose) for straws 13, 9 and 14, respectively. When SSF was repeated using WIS at a solids loading of 13% WIS (w/w), final ethanol concentrations of 21.8 and 38.3 $g.L^{-1}$ were found for SO_2-WIS (washed) straw 9 and SO_2-WIS (intensively washed) straw 14, respectively. This demonstrated that extensive washing is required to overcome the effect of inhibitors present in pretreated solids. These ethanol concentrations corresponded to ethanol yields of 0.27 and 0.51 g ethanol/g glucose consumed (53.2 and 99.9% of theoretical yields), respectively. Experimental ethanol yield from the studied straws ranged between 159 - 181 L/ton when pressed slurries were utilised for SSF. This increased to 224.2 $L.ton^{-1}$ with nearly complete conversion of sugars in WIS to ethanol, when intensive washing was applied to SO_2-SE pretreated solids from straw 14 for recovery

of WIS. The highest ethanol yield with unwashed WIS, when using uncatalysed SE pretreatment solids, was obtained at 13% solid loading (181 $L{\cdot}ton^{-1}$). Process performance of triticale straw, regarding pretreatment conditions used in pretreatment and ethanol yield from SSF, seemed to be better than for wheat straw. Pretreated triticale straws from severities between 3.43 and 3.85 resulted in ethanol yields between 158 and 182, while WIS from pretreatment severities 3.64 and 3.94 for wheat straw were reported at 142.5 and 157.4 $L.ton^{-1}$. Although experimental ethanol concentrations from the preferred straws did not reach the desired level of 4% (v/v), values above 3% (v/v) could be achieved with straw 14, reaching close to 4% (v/v) with WIS. Feedstock selection combined with high solid loadings was therefore required for acceptable ethanol process performance. Pretreatment is responsible for both sugars availability and inhibitor concentrations - both of which have a massive impact on ethanol production. Ethanol production as a biological process is therefore greatly influenced by the properties of pretreated materials, making pretreatment performance (straw response to pretreatment) a critical bottleneck in ethanol production from straw.

8.1 Background

The production of cellulose derived ethanol at industrial scale requires a hydrolysis-fermentation process that can yield a final ethanol concentration in the fermentation product of at least 4% (v/v) in order to be suitable for distillation [1]. However, some reports indicate that values of 3% (v/v) could be acceptable. Solid concentrations of at least 12% in hydrolysis and/or fermentation are typically required to provide fermentable sugars at a concentration that would achieve the benchmark of 4% (v/v). Carrying out the hydrolysis and/or fermentation using the whole pretreated material (whole slurry) at high solid loadings can provide higher sugar concentration and consequently increase the final ethanol concentration. However, few microorganisms are robust enough to cope with the harsh conditions when all of the inhibitors generated in pretreatment are included in the fermentation, while also being able to ferment both glucose and xylose efficiently. During steam explosion solubilisation takes place with the formation of compounds that are toxic to the fermentative microorganism - mainly furans, acetic acid, and formic acid [2]. The furans, degradation products of pentoses and hexoses, result in long lag phases during fermentation. Acetic acid originates from solubilisation of acetyl groups of the hemicelluloses, while formic acid is a degradation product of the furans. These weak acids have been shown to reduce biomass formation, thereby reducing fermentation efficiency [3]. The whole slurry from pretreatment can be processed to remove some of the inhibitors prior to hydrolysis/fermentation, by filtration/pressing to generate two fractions for separate hydrolysis-fermentation. One of these fractions is the liquid that contain

mainly the hemicelluloses (xylose) solubilised during pretreatment (water soluble solids, WSS), and the other is a solid fraction enriched in accessible cellulose (glucose). The resulting “pressed slurry” will contain significantly lower amounts of inhibitors than the whole slurry from pretreatment. This can be further washed/filtered to remove residual inhibitors, generating what is called water insoluble solids (WIS). This processing, however, will add cost and require additional waste water treatment.

In addition to the concentrations of inhibitors introduced via the liquid portion of the pretreatment slurry, Simultaneous Saccharification and Fermentation (SFF) is also limited by difficult mixing. These mixing problems are due to high fibre content associated with the high solids loadings, which are required to achieve the desired final ethanol concentrations [4]. In addition to inhibitor tolerance, the SSF process based on pressed slurry or whole slurry also requires efficient co-fermentation of glucose and xylose to maximise ethanol yields. One of the strategies in order to alleviate problems with mixing and inhibitors is fed-batch SSF [5], which consists of the sequential addition of the substrate during the SSF so that the polysaccharides are gradually hydrolysed and fermented [5]. This strategy not only reduces the mixing limitations of high solids loadings, but also allows for a steady adaptation and improved tolerance of the fermentative organism to inhibitors. The SSF strategy also facilitates the uptake of xylose by the yeast by keeping glucose levels low, which reduces the competition between xylose and glucose for the same transporter in the yeast. However, xylose uptake will only benefit yeasts capable of xylose conversion to ethanol.

The aim of this chapter was to assess the effects of cultivar selection and subsequent pretreatment optimisations on the experimental ethanol yield from the preferred straw cultivars in the integrated configuration SSF. The industrial strain *S. cerevisiae* MH1000 used is a hexose (C6) sugar consuming microorganism which is not able to ferment xylose. Processing of the whole slurry obtained from pretreatment prior to hydrolysis-fermentation, through solids-liquid separation and washing, would impact on ethanol production and was therefore evaluated first. The preferred straw samples M9, M13 and M14 were subjected to pretreatment optimisations (uncatalyzed and SO_2-catalyzed pretreatments). Optimum pretreatment conditions were determined which provided the maximum yield of combined sugars at limited inhibitors concentrations for each particular straw sample and pretreatment mode (Chapter 7). Pretreated materials, resulting from pretreatments of the straw samples under optimum conditions using each type of pretreatment mode, were used as substrate to perform SSF experiments. These SSF experiments were performed with a single enzyme dosage which had also previously been optimised. A preliminary test was done using pressed slurry

from uncatalysed and SO_2-catalysed SE of straw M13 as substrate. This was a compromise approach between the use of the whole slurry and the washed WIS. This test was performed by fed-batch SSF at 20% solids loading in order to establish the maximum solid loading operational for the yeast. For fed-batch SSF experimentation after the preliminary evaluation, the whole slurry material from uncatalysed-SE of straws M9, M13 and M14 were press-filtered to approximately 40% of total solids, separated from the liquid and used in SSF at 13% TS (w/w). In addition to SSF testing with pressed slurry, washing and filtration of SO_2-SE pretreated materials (pressed-washed slurry referred to as washed WIS) from straw M9 and pressed slurry with intensive washing (referred to as intensive-washed WIS) from SO_2-SE of straw M14 were also performed. This was done to obtain WIS as feedstock for SSF, and to evaluate the possible negative effects of inhibitors in pressed slurries on ethanol production. Fed-batch SSF of the optimised straws were performed for 150 h. The ethanol yield ($L.ton^{-1}$ of straw) obtained from the experimental conditions was determined and compared with the theoretical ethanol yield of 0.44 g ethanol/g glucose or g sugar (glucose+xylose) from pretreatment and enzymatic hydrolysis (presented in **Chapter 7**). SSF experiments were conducted to evaluate the fermentability of the pretreated materials from optimum conditions as well as to obtain ethanol concentrations close to 40 g/l and finally to calculate the overall experimental ethanol yield ($L.ha^{-1}$) from straw. The overall ethanol yield per hectare facilitated the identification of preferred cultivars for bio-ethanol production.

8.2 Materials and Methods

8.2.1 Substrate and preparation

Pretreated material (slurry) from uncatalyzed and SO_2-catalyzed SE pretreatment of straw from Mariendahl originated sprint triticale cultivars M9, M13 and M14 (x *Triticosecale Wittmack*), of the 2011 crop, was used as substrate (conditions stated in **Chapter 7**). The slurry materials (with moisture contents between 79.9 and 87.5%) were pressed to final moisture content of about 40% by using a 50-ton hydraulic press with gauge model TDR NO. 55002 (Northern Tool and Equipment Company, USA) set at 5 MPa. The substrate was subsequently gamma radiated (High Energy Processing, Cape Town, South Africa) at a dosage 5kGy min as a means of sterilisation prior to SSF tests.

8.2.2 Yeast and culture medium

Strain. The Fermentative microorganism *S. cerevisiae* strain MH1000 (industrial distillery yeast; Stellenbosch University, South Africa) kept at −80˚C in 30% glycerol solution was used for SSF experiments.

Cultivation. Yeast cells were grown in 250-ml Erlenmeyer flasks at 30°C in a shaker at 150 rpm in 50 ml of mineral media (20 $g.L^{-1}$ yeast extract, 3.4 $g.L^{-1}$ KH_2PO_4, 7.5 $g.L^{-1}$ $(NH_4)_2SO_4$, 0.8 $g.L^{-1}$ $MgSO_4.7H_2O$, 1 ml trace element solution, 0.05 $g.L^{-1}$ $CaCl_2.H_2O$, 20 $g.L^{-1}$ glucose and 0.5 $g.L^{-1}$ citric acid) [6] for 24 h. The yeast was transferred to two 1-L Erlenmeyer flasks containing 300 ml of preconditioning medium in each flask. This preconditioning medium consisted of mineral media (previously autoclaved at 121°C for 20 min) with 20% (v/v) pretreatment liquor from uncatalysed or SO_2-catalysed SE which had been filter-sterilised using 0.22 µm Stericup filters (Millipore, Billerica, MA). The preconditioning media flasks were incubated at 30˚C under agitation at 150 rpm for approximately 16 - 18 h until optical density of the liquid reached values between 4.5 and 5.5.

Cell harvest. The yeast cells were harvested from the preconditioning media by centrifugation at 8000 rpm for 5 min (centrifuge model Z366, Hermle Labortechnik GmbH, Wehingen, Germany) to separate cells from the supernatant. The yeast cells were washed with phosphate buffered saline (PBS) solution (containing 0.2 $g.L^{-1}$ KCl, 8.01 $g.L^{-1}$ NaCl, 1.78 $g.L^{-1}$ $Na_2HPO_4.2H_2O$ and 0.27 $g.L^{-1}$ KH_2PO_4 with pH adjusted to 7.4). The suspension of cells was washed twice to ensure no media traces in the cells.

Inoculum. The inoculum for SSF assays consisted of 5 grams of wet cells per litre of SSF broth (~1.34 $g.L^{-1}$ of dry cells).

8.2.3 Simultaneous saccharification and Fermentation (SSF)

SSF runs were conducted in batch regimen in 250-ml Erlenmeyer flasks with a final working weight of 200 g. Pressed slurry, obtained by uncatalysed and SO_2-catalysed steam explosion pretreatment at optimum conditions, from of straw of cultivars M9, M13 and M14 (Mariendahl) was used as substrate.

8.2.3.1 *Enzymes*

A previously optimised enzyme dosage, consisting of a mixture of cellulase Cellic Ctec2 of 0.1 ml/g TS (total solids) and endoxylanase Cellic Htec2 at 0.1 ml/g TS with high inhibitor tolerance, provided by Novozymes (A/S, Denmark) was used according to a previous study [7].

8.2.3.2 *Solid loadings*

8.2.3.3 *Preliminary SSF experimentation*

Pressed slurry from uncatalyzed and SO_2-catalysed SE pretreatments of straw 13 (Mariendahl) at a solid loading of 20% (w/w) was evaluated. SSF with two daily solid feedings of 1 and 2% (weight/total weight) were carried with 8 hour intervals until the target of 20% of total solids in the flask was reached (about 144 h). Enzyme dosage corresponded to cellulase 0.1 ml/g TS and endoxylanase 0.1 ml/g TS added once-off at the beginning of the SSF, together with the inoculum which was added at a fixed concentration of 5 $g.L^{-1}$ (wet cells).

The pressed slurry was supplemented with mineral media without glucose and the pH was adjusted to 5.0 by adding 3 M KOH. After pH adjustment, the enzymes Cellic Ctec2 and Cellic Htec2 were added at two loadings. The initial dosage was 0.15 ml of Cellic Ctec2/g pretreated material (dry basis) and 0.0167 ml of Cellic Htec2/g pretreated material. The final enzyme dosage consisted of Cellic Ctec2 and Cellic Htec2 at 0.15 ml/g pretreated material and 0.213 ml/g pretreated material, respectively. After the enzymes were added the mixture was left for 1h for pre-saccharification at 35°C and thereafter the inoculum was added.

8.2.3.4 *SSF experimentation*

After the preliminary experimentation, pressed slurry from uncatalysed and SO_2-catalysed SE pretreatments of straws M9, M13 and M14 at a solid loading of 13% (w/w) were evaluated by SSF. Whole slurry from SO_2-SE pretreatment of straw M9 was press-filtered and washed with distilled water using a 10-fold volume of water with respect to the slurry weight; thereby, generating a washed WIS material for SSF. Whole slurry from SO_2-SE pretreatment of straw M14 was press-filtered and washed with distilled water using a 20-fold volume of water with respect to the slurry weight and thereby resulting in intensive-washed WIS. These washed WIS and intensive-washed WIS substrates were evaluated, together with pressed slurry from straw 13, for any negative effects of inhibitors in the slurries on ethanol productivity. Except for the type of substrate as stated above, the SSF assays were performed similar to the preliminary runs with two daily solid loadings.

8.2.4 HPLC analysis

The content of sugars in the WIS was determined by the laboratory analytical procedures (LAPs) proposed by the National Renewable Energy Laboratory (NREL) [8]. Concentrations of main sugars in pretreatment liquor (glucose, xylose and arabinose), collected after pressing of the slurry, were analysed by HPLC. Oligomeric sugars were calculated after subtraction of total sugars measured by post-hydrolysis [9] and the sugars in liquid quantified previously. Monomeric sugars, acetic acid, formic acid, ethanol and glycerol were determined by a High Performance Liquid Chromatography (HPLC) system equipped with an Aminex HPX-87H Column and a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). The column was set to a temperature of 65 °C with a mobile phase of 5 mM sulphuric acid and a flow rate of 0.6 ml/min. The concentrations were measured with an RI detector (Shodex, RI-101) operated at 45 °C. Xylose and arabinose were analysed using an XbridgeTM Amide column (4.6 x 250 mm, 3.5 µm particle size) and an XbridgeTM Amide precolumn (Waters) set at 30 °C using 0.05% ammoniumhydroxide (AH) in water (A) and 0.05% AH in 90% acetonitrile (B) as mobile phases with a flow rate of 0.7 ml/min. The concentration of HMF and furfural in the pretreated liquor were analysed on a Phenomenex Luna C18(2) reversed phase column equipped with a Phenomenex Luna C18(2) pre-column (Separations, Johannesburg, South Africa) with column temperature set to 25 °C and a flow rate of 0.7 ml/min. The mobile phases used for elution were 5 mM trifluoroacetic acid (TA) in water (A) and 5mM TA in acetonitrile (B). HMF and furfural concentrations were measured with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm [10].

8.3 Results and discussion

Pressed slurry from uncatalysed and SO_2-catalysed pretreatments, at optimum conditions, of straw samples M9, M13 and M14 (Pretreatment optimisations discussed in Chapter 7) was used as substrate to carry out fed-batch SSF. The SSF experiments were conducted to evaluate the fermentability of the pretreated materials. Thereby the impact of inhibitors between pretreatment modes/optimised straws on SSF could be observed. Additionally, the experimentation allowed the identification of cultivars yielding ethanol concentrations from straw close to the benchmark of 40 g/l. These were identified as preferred cultivars with high potential for bioethanol production.

8.3.1 Chemical compositions of pretreated materials

The properties of the whole slurries from the uncatalysed-SE and SO_2-SE pretreatments of straws M9, M13 and M14 are presented in Table 8-1. The contents of fermentable sugars, total solids, WSS, WIS and inhibitors in the pretreated materials will have an influence on the performance and configuration of hydrolysis and fermentation steps. As observed in Table 8-1, total solids of the pretreated materials varied between 12.5 and 20.1%.

The % of total solids varied in the range 12.53 to 20.09% between all the conditions, as shown in Table 8-1. This means that most of the optimised conditions would theoretically provide a sufficient concentration of fermentable sugars (between 78.47 and 115 g/L) without additional processing (such as pressing or filtering). If all of the sugars in these slurries could be efficiently fermented to ethanol without additional dilution, pressing or filtration, the final ethanol concentrations (at the theoretical maximum yield of ethanol on sugars) would be in excess of the desired 40 g/l. As found in Chapter 7, the SO_2 impregnation of the material prior to steam explosion favoured the solubilisation of the sugars more than the uncatalyzed (water-only impregnation). These resulted in higher values of water soluble solids (WSS) and consequently in just slightly lower insoluble solid recovery, as shown in Table 8-1. This fact can be explained by the lower severity of the pretreatment, mainly the temperature (differences of 12 to 16 °C) applied when using SO_2 as catalyst Table 8-1). However, the WIS content was generally higher for all varieties when the material was SO_2-impregnated.

The acidity (pH) and the inhibitors concentration are other important characteristics of the slurry that will impact its fermentability. SSF processes are usually conducted at pH 5 - 5.5 [11], and it has been shown that the pH has an impact on the toxicity of other compounds such as acetic acid [12]. For example, low acetic acid concentrations of 3 $g.L^{-1}$ completely inhibit yeast growth at pH 3-3.5, while it promotes glucose consumption rate and ethanol yield at a pH of about 5 [13]. As expected, SO_2 impregnation resulted in slurries with lower pH than water impregnation (2.12 - 2.94 vs 3.63 - 3.8), although slight differences were observed between the straws.

Table 8-1: Properties of the slurry from pretreatment at optimum steam explosion conditions determined for straws M9, M13 and M14 (Mariendahl).

Pretreatment Conditions	**Triticale cultivars (straw)**					
	M9		**M13**		**M14**	
Impregnation	**Uncatalysed**	**SO_2**	**Uncatalysed**	**SO_2**	**Uncatalysed**	**SO_2**
Temperature (˚C)	196.9	180.7	192.6	177.6	190.0	181.8
Time (min)	9.9	11.6	5.1	13.6	10.3	11.7
Slurry properties	**M9**		**M13**		**M14**	
	Water	**SO_2**	**Water**	**SO_2**	**Water**	**SO_2**
pH liquid fraction	3.64	2.74	3.80	2.12	3.63	2.94
% Total solids	14.1	18.6	18.7	20.1	12.5	18.4
% WIS	10.6	13.0	14.5	14.3	9.5	12.5
% WSS	3.5	5.6	4.3	5.8	3.0	5.9
Insoluble solids recovery %	66.5	63.5	67.9	64.0	60.6	60.0
Fermentable sugars ($g.L^{-1}$)*	**M9**		**M13**		**M14**	
	Uncatalysed	**SO_2**	**Uncatalysed**	**SO_2**	**Uncatalysed**	**SO_2**
Glucose in solids&liquids	65.8	85.5	80.5	88.3	62.8	78.5
Xylose in solids&liquids	17.8	29.6	22.7	22.3	15.7	27.4
Total (Glucose + Xylose)	83.6	115.2	103.2	110.6	78.5	105.9
Inhibitors in the liquid fraction ($g.L^{-1}$)						
HMF	0.134	0.060	0.047	0.178	0.183	0.100
Furfural	0.667	0.246	0.133	0.407	0.864	0.427
Formic acid	0.516	0.115	0.355	0.189	0.470	0.131
Acetic acid in liquid	1.897	1.007	1.081	1.544	1.804	1.262
Acetic acid residual in WIS	5.23	5.84	5.63	4.77	4.46	4.95

* Represent the theoretical concentration of fermentable sugars in the fermentation broth.

In terms of inhibitors, the liquid fraction was analysed for furans (hydroxylmethyl-furfural and furfural), formic acid and acetic acid. Additionally, the acetic acid that could be released from the residual acetyl groups in the WIS was also determined. The concentrations of these compounds in the slurry are presented in Table 8-1. The concentrations of these four components in the liquid portion of the slurries were generally higher for uncatalysed-SE pretreatment, except for the straw

13, where the SO_2-impregnation generated a higher concentration of furans and acetic acid. It is worth noting that the pretreatment conditions applied for straw 13 were milder than those for straws 9 and 14, with a corresponding reduction in inhibitors concentration (Chapter 7). The pretreated materials presented residual acetyl groups in the WIS that should be taken into account since they could be liberated during enzymatic hydrolysis and have a negative impact on yeast performance.

Maximum values of glucose and xylose content in WIS from uncatalysed and SO_2-catalysed SE pretreatments were calculated based on experimental data of solid recovery and the amount of glucose and xylose in the pretreatment liquor (Table 8-2).

Table 8-2: Sugar composition of WIS from optimal steam explosion conditions.

Component (% WIS, max. value)	Uncatalysed			SO_2-catalysed		
	9	13	14	9	13	14
Glucose	61.4	54.0	56.1	64.5	56.2	57.4
Xylose	17.4	19.5	17.3	17.1	16.9	13.1

Glucose content in WIS of pretreated straws M9, M13 and M14 varied between 54 and 64.5% for all the WIS materials used. SO_2-catalysed SE resulted in WIS more enriched in glucose compared to those from uncatalysed pretreatment. Xylose content varied from 13.1 to 19.5% between samples and pretreatment modes (Table 8-2). Higher potential glucose in WIS for conversion by the C6-sugar consuming yeast was measured for samples M9 followed by sample M14 and much lower in straw M13 (composition between 54-56.2%) at both modes of pretreatment. As observed, SO_2- WIS materials yielded more potential glucose for conversion to ethanol than WIS material from uncatalysed pretreatment, if only glucose content is considered.

8.3.2 Preliminary SSF tests at 20% (w/w)

Preliminary fed-batch SSF experiments were carried out at a solids loading of 20% (w/w) using pressed slurry from water-SE and SO_2-SE pretreatment of straw M13. The time-courses for sugars, ethanol, glycerol and weak acids yields from the preliminary SSF tests are illustrated in Figure 8-1.

It can be observed that ethanol production during SSF took place until the feeding of the TS reached a total of approximately 13 and 9 %TS (w/total weight) for the water-SE and SO_2-SE substrates, respectively (Figure 8-1). At these time-points of approximately 95 and 55 hours after the

start of the SSF runt, for the water-SE and SO_2-SE substrates, respectively, ethanol production ceased while glucose from enzymatic hydrolysis started to accumulate (Figure 8-1). The maximum ethanol concentrations at these time-points were 28 and 25.6 $g.L^{-1}$, respectively, corresponding to less than 30% of the theoretical ethanol yields (based on assumed theoretical ethanol yield of 0.44 g $ethanol.g^{-1}$ glucose present in the pressed-slurry [B]). The accumulation of glucose continued until the culture was stopped at 200 hours, indicating that enzymatic hydrolysis was not limiting ethanol production, but rather the yeast performance. At the time-points at which ethanol production ceased, the concentration of acetic acid in the fermentation broth was approximately 5 g/l, for both types of pretreated solids. The cessation of glucose conversion to ethanol may therefore be linked to the accumulation of inhibitory compounds, either added to the SSF run in the liquid components present in pressed slurry, or released during the hydrolysis of WIS (acetic acid only) (Table 8-1).

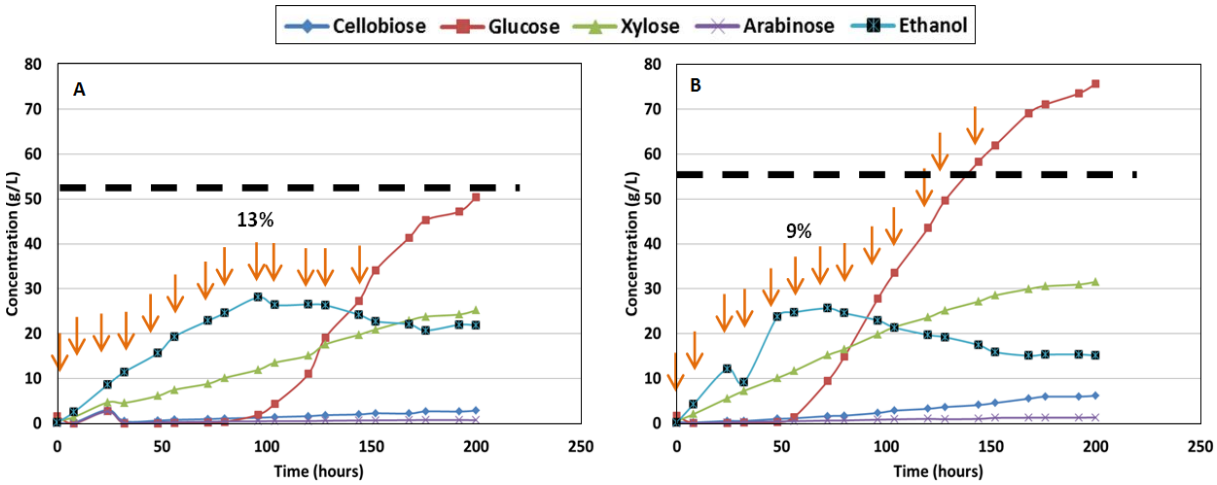

Figure 8-1: Time course of sugars (cellobiose, glucose, xylose and arabinose) and ethanol production during fed-batch SSF of pressed slurry from uncatalysed (A) and SO_2-catalysed (B) steam explosion of triticale straw M13. The orange arrows represent the time of feedings and the discontinuous black line represents the maximum ethanol concentration that could be reached for 20% w/w TS.

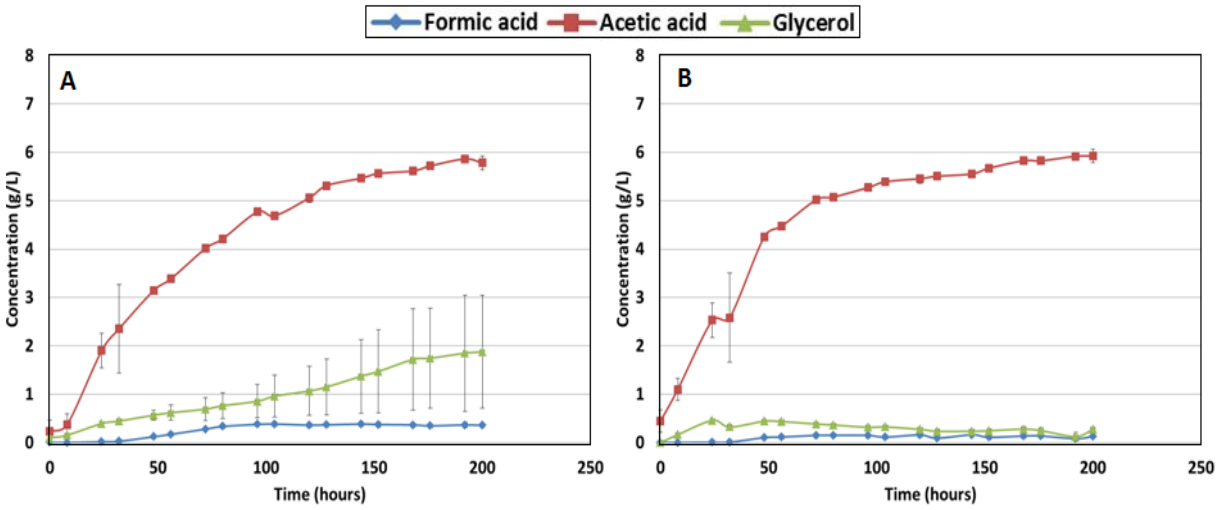

Figure 8-2: Time course of glycerol and weak acids (acetic and formic acids) production during fed-batch SSF of pressed slurry from uncatalysed- (A) and SO_2-catalysed SE (B) of triticale straw M13.

Maximum glucose concentrations reached at the end of the SSF runs were 50.4 and 75.6 $g.L^{-1}$, corresponding to 50 and 67% of the glucose in the WIS, when materials from water-SE and SO_2-SE pretreatments, respectively, were fed up to a final solids loading of 20%. Xylose accumulation was also higher for materials from SO_2-SE pretreatment, compared to water-SE pretreatment (25.2 and 31.5 $g.L^{-1}$, respectively). The hydrolysis of pretreated materials using the optimised enzyme cocktail was therefore more effective with the SO_2-SE pretreated materials. Although there was acetic acid in the liquid component of the pressed slurry fed into the SSF process, most of the measured acetic acid in SSF was released from the WIS fraction by enzymatic hydrolysis (see Table 8-1), which infers acetyl-esterase activity in the enzyme cocktail.

Based on the observations from SSF runs at 20% solids loading, subsequent runs were performed at a total solids loading of 13% (w/w), aiming to reduce the inhibitory effect of components in pretreated materials and thus avoid glucose accumulation. Thereby the complete conversion of a sufficient amount of glucose could be enabled to achieve a final ethanol concentration close to 4% (v/v). Thus, all further SSF work was done with WIS only with different levels of washing. Washed WIS was used in the SSF experimentation, rather than pressed slurry, due to yeast inhibition observed at solids loadings higher than 9% w/w TS with SO_2-SE substrates.

8.3.3 SSF runs with WIS at 13% (w/w)

The same feeding scheme as in the preliminary test was applied: Twice daily feedings of pressed slurry at 1 and 2% TS (w/total weight), respectively, until the target of 13% TS was reached (96 h). The optimum enzyme combination was dosed once-off at the beginning of the SSF together with the inoculum (5 $g.L^{-1}$ wet cells). The time course for the production of sugars (cellobiose, glucose, xylose and arabinose), ethanol, glycerol, acetic acid and formic acid during fed-batch SSF of the pressed slurry is represented in Figure 8-3.

Generally similar trends were observed for SSF of WIS from straws M13 and M9. The maximum ethanol concentrations were 25.8, 24.5 and 30.8 $g.L^{-1}$ for WIS from pretreated straws M9, M13 and M14 respectively (Figure 8-3), which corresponded to ethanol yields of 75, 63.3 and 75.5% with respect to the theoretical yields (0.44 g ethanol.g^{-1} glucose), respectively. SSF of WIS from pretreated straws M9 and M13 ceased ethanol production at 124 h, while ethanol production for straw M14 stopped 44 h earlier (Figure 8-3). Glucose accumulation was only observed for SSF with WIS from pretreated straw M14 from this point onwards. Xylose concentration increased gradually during SSF for the WIS from all three pretreated straws, although WIS from straw M13 presented the highest values at the end of SSF runs (17.6 $g.L^{-1}$ vs 5 - 6.5 $g.L^{-1}$).

Ethanol production stopped earlier for uncatalysed-SE WIS from straw 14, in spite of the fact that lower concentrations of acetic acid were determined during the SSF (less than 3 $g.L^{-1}$). Nevertheless, WIS from straw 14 resulted in high values of potential fermentable sugars (accounted as glucose + xylose), as well as the highest ethanol concentration, ethanol yield, and ethanol productivity during fed-batch SSF (see Table 8-3). As observed in Figure 8-3 (A and D), hydrolysis of sample M9 still took place during the time-course between the last feeding point and 120 h, although the acetic acid released from WIS seemed to be the cause of the cessation in ethanol production for sample M9 and apparently also for straw M14 .

The values of maximum ethanol concentration, ethanol yield and ethanol productivity during fed-batch SSF at 13% WIS (w/w) of the pretreated samples M9, M13 and M14 are given in Table 8-3. As stated previously, pressed slurry from uncatalysed pretreatments of straws M9, M13 and M14 was used as substrates for SSF. In the case of SO_2-catalysed pretreatment, only straws M9 and M14 were evaluated but using WIS at two levels of washing: washing with 10-fold volume of water in the case of M9 and with 20-fold volume of water for M14 (as stated previously in Section SSF Experimentation).

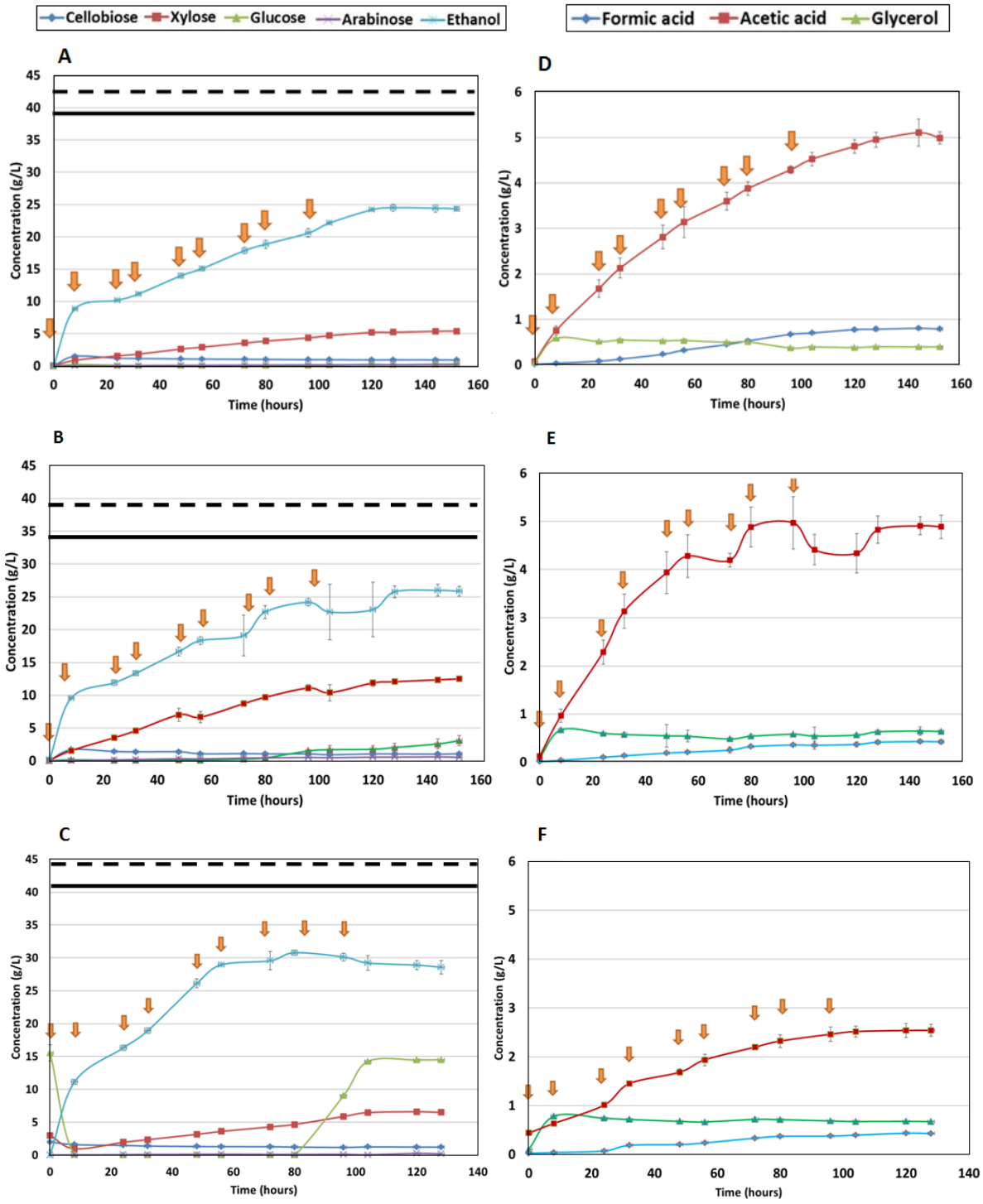

Figure 8-3: Time course (left graphs) for sugars (cellobiose, glucose, xylose and arabinose) and ethanol and (right graphs) glycerol and weak acids (acetic and formic acids) production during fed-batch SSF of pressed slurry from uncatalysed steam explosion of triticale straw from different cultivars: M9 (A and D), M13 (B and E) and M14 (C and F). The orange arrows represent the time of feedings, the black lines represent the maximum ethanol concentration that could be reached for 13% TS from glucose (discontinuous line) and glucose+xylose (continuous line).

In all the referred SSF experimentations a solid loading concentration of 13% WIS (w/w) was used. Catalysed SO_2-SE pretreatment seemed not to favour the fermentation process, in spite of the fact that the pressed slurry was subjected to a washing step. The ethanol yield was reduced by 10% compared to the SSF using pressed slurry from water impregnation, as shown in Table 8-3. The values of acetic acid in the fermentation broth with sample M9 from SO_2-SE (6.6 $g.L^{-1}$ in Table 8-3) were higher than those achieved in fed-batch SSF of uncatalysed-SE for the same straw (~5 $g.L^{-1}$ as shown in Figure 8-3D). The highest sugar-to-ethanol convertibility of 99.5% (Table 8-3) was achieved with intensive-washing WIS of straw 14 from acid-catalysed SE pretreatment. Improvement of 22% in the ethanol yield from intensive-washing WIS of straw M14 compared to that obtained from the pressed slurry of uncatalysed-SE (Table 8-3) was observed. This improvement in ethanol yield for the straw M14 led to a maximum ethanol concentration of 38.3 $g.L^{-1}$, only 4% below the benchmark of 40 $g.L^{-1}$ for industrial distillation of cellulosic ethanol. Further experiments need to be conducted to establish the most efficient washing method to be applied to all cultivars for comparison purposes.

Table 8-3: Maximum ethanol concentration, ethanol yield and productivity during fed-batch SSF of WIS from uncatalysed pretreatment of straws 9, 13 and 14 and SO_2-catalysed WIS from straws 9 and 14. Straw 14 was subjected to a more intense washing step. (Solid loading of 13% w/w).

Parameter	Uncatalysed-SE pretreatment (Using pressed slurry)			SO_2-SE pretreatment (Using washed WIS)	
	9	**13**	**14**	**9**[1]	**14**[2]
Maximum ethanol concentration ($g.L^{-1}$)	24.5	25.8	30.8	21.8	38.3
Y (g ethanol.g^{-1} glucose consumed)	0.32 (63.3)[3]	0.38 (75.0)[3]	0.39 (75.6)[3]	0.27 (53.2)[3]	0.50 (99.5)[3]
Q ethanol 24 h ($g.L^{-1}.h^{-1}$)	0.42	0.50	0.68	0.38	0.80
Q ethanol 80 h ($g.L^{-1}.h^{-1}$)	0.24	0.28	0.38	0.20	0.48
Q ethanol 128 h ($g.L^{-1}.h^{-1}$)	0.19	0.20	0.24	0.17 (6.6)[4]	0.30 (2.1)[4]

[1]washed WIS material.
[2]intensive-washing WIS.
[3]values in parenthesis correspond to ethanol yield as a % of the theoretical maximum.
[4]values in parenthesis represent the maximum acetic acid concentration in $g.L^{-1}$.

8.3.4 Effect of cultivar selection on combined sugars, processability and measured ethanol yield

Straw performance across the stages of the study was compared between cultivars to observe the highest 2G ethanol outputs estimated per hectare. Agronomic traits of grain and straw and overall performance were predominantly influential for improved total (1G + 2G) ethanol yield estimate in the screening selection stage. Figure 8-5 summarises the estimated ethanol yield per

hectare based on combined sugars from pretreatment (measured CSY at theoretical conversions (0.44 g ethanol/g sugar consumed) at each stage of the study (screening selection of cultivars, dilute-acid pretreatment optimisation at bench-scale and pilot-plant optimisation (SO_2-catalysed steam explosion as the most promising pretreatment mode).

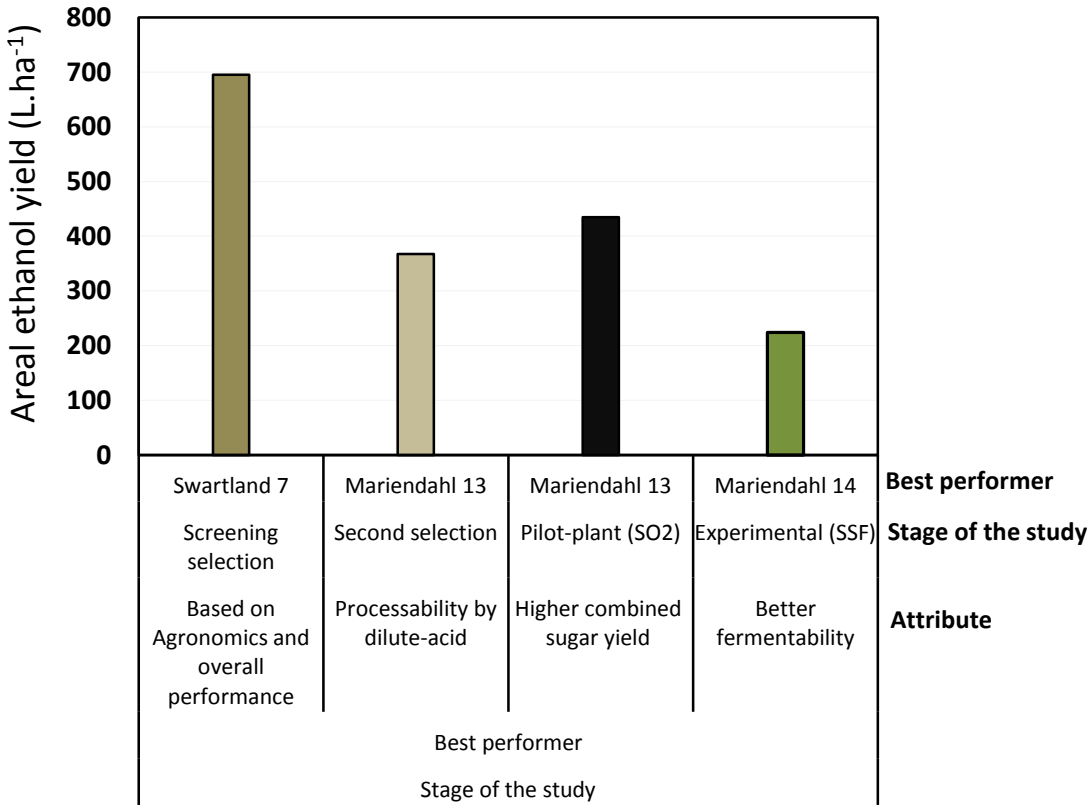

Figure 8-5: Estimated ethanol yield per hectare based on experimental total sugars yields at theoretical conversions (0.44 g ethanol/g sugar consumed) and agronomic data of straw yield at each stage of the study for the preferred straw cultivars (Mariendahl site), except for straw 7 (Swartland site). Screen: Screening selection; bench-scale; Dilute-acid pretreatment optimisation at bench-scale; Pilot-plant: SO_2-catalysed steam explosion optimization at pilot-plant.

Swartland site was identified as the preferred location for maximum total ethanol estimate per hectare and cultivar 7 from this site as the cultivar with the highest yield of 2G ethanol (with no compromise in 1G ethanol yield) was the top performer in the selection (See Figure 8-5). Superior processability for maximised total fermentable sugars was displayed by straw from cultivar 13 (Mariendahl) at the second screening selection compared to other screened straw samples (51.6 g.100 g^{-1} DRM against $\leq$ 50.6 g.100 g^{-1} DRM). Regardless of the higher combined sugar yield achieved by straw from cultivar 9 (Mariendahl) after pretreatment optimisation at pilot-plant scale, higher ethanol yield estimate per hectare was obtained by straw from cultivar 13 (Mariendahl)

(Figure 8-5), which also displayed higher straw yield (1.20 ton.ha^{-1} vs 0.95 ton.ha^{-1}). Pilot-plant optimisation enabled nearly 15% improvement in CSY in this straw and differences in straw yield compared to straws 9 and 14 resulted in 2G ethanol yield estimate per hectare of around 430 L.ha^{-1} (See Figure 8-5). Better fermentability of straw 14 achieved at 13% solid loading (intensive washing WIS) led to the realization of experimental ethanol yield estimate per hectare of above 200 L.ha^{-1} (See Figure 8-5).

The effect of cultivar selection and pretreatment optimisation on theoretical and experimental ethanol yields is represented in Figure 8-4. It was observed that an effective way to substantially improve ethanol output was by considering the contribution of hemicellulose derived sugars to the process, as followed in this dissertation. Increments of between 29.6 and 46.3% in theoretical ethanol yield among straws was found by considering the glucose and xylose streams from the combined process compared to the ethanol from only glucose (Figure 8-4). Differences in theoretical ethanol yield potential based on sugars yield from pretreatment-enzymatic hydrolysis varied between 298 and 328 L.ton^{-1} and between 320 and 339 L.ton^{-1} for uncatalysed and SO_2-SE straws.

Pretreatment with SO_2 seems to be potentially more conducive to reach the highest ethanol yields per ton of triticale straw (~220 L.ton^{-1} with straw M14). Additionally, straw M14 was shown to result in the highest ethanol concentrations in SSF experiments with pressed slurry and intensive-washing WIS (30.8 – 38.3 g.L^{-1}) compared to other samples (≥25.8 g.L^{-1}). Performance of pretreated materials under SSF was greatly influenced by solids loading concentration in first instance but also to large extent by conditioning of the pretreated solids when the solid loadings was fixed to 13% TS. Intensive washing of the WIS (in the case of the straw M14) resulted in the highest ethanol yield at an ethanol concentration of 38.3 g.L^{-1}, which is close to the theoretical ethanol yield. SSF performance is directly related to the conditioning of the pretreated solids by washing, since these conditioning wash steps remove inhibitors, such as furfural, HMF and acetic acid, which negatively affect yeast metabolism [7].

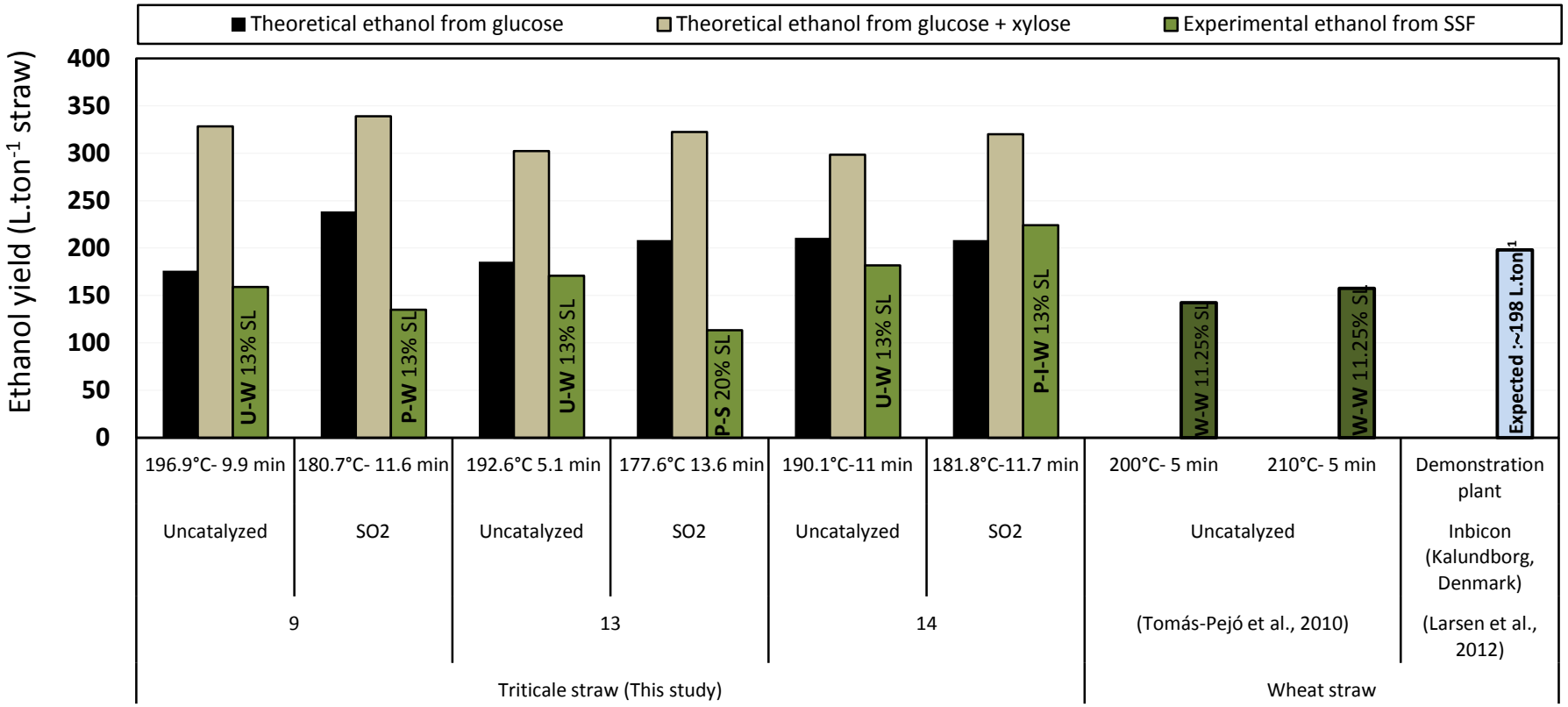

Figure 8-4: Ethanol yield expressed in L.ton^{-1} straw (dry weight) obtained from: Black bars: experimental glucose yields from pretreatment (PT) + enzymatic hydrolysis (EH) at theoretical sugar-to-ethanol (EtOH) conversion (0.44 g EtOH. g^{-1} glucose consumed). Tanned bars: experimental (glucose + xylose) yields from PT + (glucose + xylose) yields from EH at theoretical sugars-to-ethanol (EtOH) conversion (0.44 g EtOH. g^{-1} total sugars consumed). Light green bars: experimental (glucose + xylose) yields from PT + (glucose + xylose) yields from EH + experimental SSF. Dark green bars: experimental (glucose + xylose) yields from PT + (glucose + xylose) yields from EH + experimental fed-batch SSF performed with washed WIS (W-W) from two pretreatment conditions at 11.25% solid loading [5], included for comparison purposes. Blue bar: represents the expected yield of ethanol from wheat straw as feedstock in the commercial plant Inbicon already in operation in Denmark [18]. Numbers 9, 13 and 14 stand for straw from triticale cultivars 9, 13 and 14 (Mariendahl origin) subjected to steam explosion pretreatment optimisation, respectively. Bar inner legend: U-W: Unwashed WIS; P-W: Pressed-washed material; W-W: Washed-WIS; P-S: Pressed-slurry; P-I-W: Pressed-Intensive washed slurry. SL: Solid loading in % (w/w dry material).

Solids loadings of about 13% (w/w) with washed WIS showed better ethanol yield than higher solids loadings of 20% (w/w) when using pressed slurry. Ethanol production by SSF at 20% solid loadings was limited by accumulation of glucose due to poor yeast performance rather than enzyme inhibition. Therefore, common SSF limitations related to high inhibitors concentrations in the whole slurry when experiments are run at high solid loadings were also demonstrated in this study [7].

Process performance of triticale straw, regarding pretreatment conditions used in pretreatment and ethanol yield from SSF, seemed to be better than for wheat straw. Pretreated triticale straw from severities between 3.43 and 3.85 resulted in ethanol yields of between 158 and 182 $L.ton^{-1}$, while WIS from pretreatment severities 3.64 and 3.94, reported for wheat straw, result in 142.5 and 157.4 $L.ton^{-1}$ (See Figure 8-4). Performance of pretreated triticale straw from optimum pretreatment conditions is greatly influenced by its conditioning prior to SSF and SO_2-SE WIS was identified as the preferred substrate. Substantial improvement in ethanol yield (~22%) can be accomplished from SSF by washing the WIS as an effective method of inhibitors removal. Pretreatment optimisation was demonstrated to have a positive impact on SSF of triticale straw in order to reach ethanol concentrations close to the benchmark of 40 $g.L^{-1}$. Based on the results of previous chapters, the maximisation of ethanol yield per ton of straw is highly dependent on the performance of straws throughout pretreatment – SSF. This is due to the significant effects of processability and fermentability of the pretreated straw solids on ethanol yield per ton of straw basis. On the other hand, the maximisation of ethanol yield (from straw) per hectare of triticale cultivated is highly reliant on straw yield (straw resourced per hectare), while processability and fermentability may be considerably overshadowed.

SO_2-catalysed SE shows advantages in sugar yields and fermentability of pretreated solids over uncatalysed pretreatment. Acetic acid released from pretreatment together with residual acetic acid in the pretreated solids (as acetyl groups) [16] can be considered as a limiting factor in reaching high ethanol concentrations in the SSF configuration with triticale straw. The production of acetic acid cannot be prevented, since it is an inherent product resulting from hemicellulose hydrolysis during pretreatment [17]. However, acetic acid was shown to negatively affect fermentability of the pretreated solids to a large extent and can be seen as a determinant of the fermentability of pretreated triticale straw. Effective methods of washing the WIS, as a means of substrate conditioning, may substantially alleviate the negative effects on fermentability brought about by acetic acid released during pretreatment. Another challenge remains in the form of the potential acetic acid in WIS (acetyl groups) which is believed to reduce fermentability and decline ethanol

concentrations during SSF. In this study, the highest sugar-to-ethanol convertibility of 99.5% (Table 8-3) was achieved with pressed slurry subjected to intensive washing of straw 14 from acid-catalysed SE pretreatment. This observation suggests that regardless of the bottleneck of poor yeast robustness in coping with the observed levels of inhibitors present in the treated material, pretreatment effectively resulted in highly digestible fibres for improved feedstock performance.

Differences in the ethanol yield among the preferred triticale cultivars M9, M13 and M14 ranged from 159-170.7 L.ton^{-1} straw for straws using uncatalysed-SE and comparable washing methods, based on this study's experimentally obtained data. The differences in ethanol yield for the cultivars under SO_2-pretreatment ranged from 113-224 L.ton^{-1} straw although with differences in the WIS conditioning prior to SSF. In terms of experimental performance in fed-batch SSF, triticale straw from optimised uncatalysed pretreatment showed better performance than reported values for wheat straw (142.5-157.4 L/ton straw). The potential of cultivar selection and pretreatment optimisation of triticale to increase the feasibility of triticale to comply with expected industrial ethanol outputs, was estimated by comparing potential and experimental ethanol yields found in this study with expected yields per ton of straw (wheat) set by Inbicon plant (2G-ethanol plant fully operational since 2013; See Figure 8-4). Straw M14, under optimum SO_2- steam explosion conditions, represents the best option to maximise ethanol yield from straw to ~220 L per hectare of triticale. The measured ethanol concentration of 25.8 g.L^{-1} from straw M13 under optimum uncatalysed-SE pretreatment is low compared to the benchmark of 40 g.L^{-1}. However, this straw still displays high potential for reaching the industrial goal of nearly 200 L.ton^{-1} [18] at only 18 L short per ton of straw (Figure 8-4), as a result of its high straw yield. Thus, improved experimental productivity of sugars through cultivar selection and pretreatment optimisation resulted in triticale reaching the technical margin for industrial application of the lignocellulosic biomass comparable with wheat straw, as observed in Figure 8-4. Nevertheless, further improvements in the process of sugars-to-ethanol downstream steps are required if uncatalysed or SO_2-catalysed SE is the pretreatment of choice. The potential ethanol realizable from glucose + xylose from optimised pretreatments is highly promising for the triticale cultivars selected in this study. Pretreatment efficiencies of between 82 and 91% in sugars recovery are anticipated for uncatalysed-SE for straws 9, 13 and 14 (Chapter 7, Subsection 7.36: CSY Optimisation and models validation). Therefore, potential ethanol yield, at theoretical conversions (0.44 L.g^{-1} sugars consumed) of sugars from pretreatment, would run over ~244 and ~300 L.ton^{-1} straw, however with a wide range of feasible feedstocks.

8.4 Conclusions

This study substantiated the fact that pretreatment optimisation results in the provision of highly digestible material for further sugars-to-ethanol conversion. The positive impact of cultivar selection and subsequent pretreatment optimisation results in an integrated configuration for ethanol production from triticale straw, which is however highly dependent on solids loadings and conditioning of pretreated material.

Fermentability of pretreated triticale straw from optimal pretreatment conditions is dictated by the type of pretreatment and pretreatment itself. The observed high fermentability of the intensive-washing WIS from SO_2-SE suggests that highly digestible fibres are formed from optimum pretreatment conditions, even though high levels of acetic acid and other inhibiting compounds are also produced. In the context of rising fermentability at reduced costs of conditioning, options such as high initial cell density fermentations and detoxification of the pretreated material may alternatively lessen the still high inhibitory effects of the optimised pretreatments.

Triticale in South Africa, if subjected to cultivar selection and pretreatment optimisation for improved combined sugars yield, can potentially reach ethanol concentrations close to the industrial benchmark for distillation of 40 $g.L^{-1}$ and result in ethanol yields of over ~200 $L.ton^{-1}$ straw.

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Chapter 9

9 Summary of findings, conclusions, delimitations and recommendations

This dissertation studied triticale cultivars in South Africa through novel process development in a systematic and integrated manner: Selection of cultivars with improved features and subsequent pretreatment optimization at pilot-scale of industrial relevance for improved areal ethanol yield with no compromise with grain ethanol. Original contribution to knowledge was the establishment of a systematic approach based on selection of cultivars with superior traits in straw and preferred locations for improved processibility and sugars yields **(Chapters 4** and **5)**, and further development of predictive models with statistical significance for industrial application that maximize sugars yields through pretreatment optimization for maximum sugars at low toxicity levels yield (**Chapters 6** and **7**). Thus improved yield of fermentable sugars per hectare is achieved, translated into an improvement in the estimated ethanol yield per hectare of preferred triticale cultivars. Finally, experimental evaluation of the results from pretreatment optimization in an integrated configuration (**Chapters 8**) was investigated to determine the maximum experimental ethanol yield realizable and the fermentability of the treated material as concluding stage of the approach.

This section of the dissertation summarizes the significant empirical findings realized through the development of the study, which were important for decision-making criteria at later stages, provided evidences of concepts presented previously or led to key conclusions. Additionally, the conclusive implications of the empirical findings of this dissertation will follow the above direction showing how these conclusions address the proposed objectives and converge into the general aim of the study. Finally, delimitations imposed in the study and opportunities for future work are discussed in the final section of this chapter.

9.1 Summary of findings

9.1.1 Variability in agronomic yield

Grain and straw yields were found highly variable in triticale. Variability in grain yield related to cultivar ranged 2.8 - 4.3 $Mg.ha^{-1}$ although higher variability between 0.43 and 3.76 $Mg.ha^{-1}$ was found in straw yield. Site–related variabilities were also important in determining agronomic yields. Grain yield-dependency on site was lessen accentuated than straw yield. Figure 9-1 shows how both grain and straw yields in several cultivars were negative or positively site-influenced. Overberg

clearly showed less straw variability at inconsistently high grain yield conversely to what was observed for Swartland (dotted lines on Overberg and Swartland sites (Figure 9-1). Mariendahl was found as the site with the significant lowest straw output between locations with 0.99 (±0.353) Mg.ha^{-1}, nearly 50 and 70% inferior yield than Overberg and Swartland, respectively.

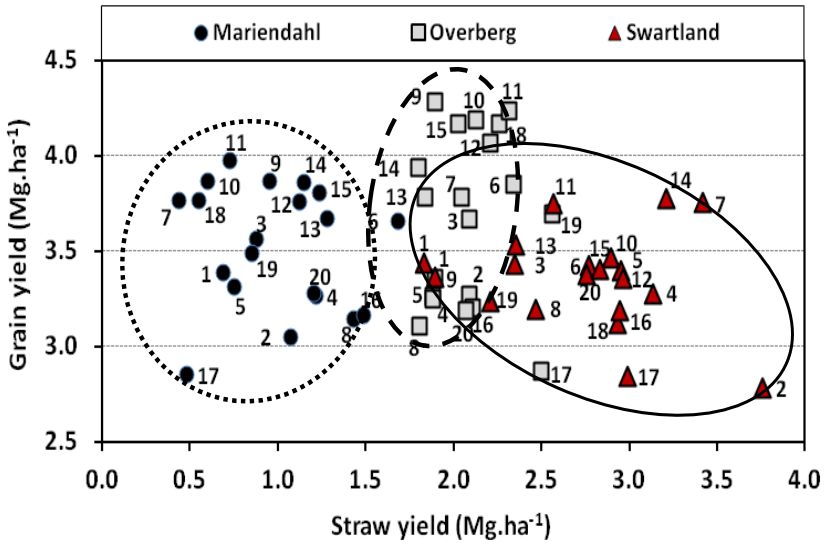


Figure 9-1: Mean values of grain and straw yields expressed in Mg.ha-1 for all of the triticale samples in the trials per cultivar and site of origin. Numbers 1-20 next to the marker symbol on each site correspond to the code label for the particular cultivar. Dotted and continuous lines enclose the general trend followed by the cultivars at each specific site

9.1.2 Feedstock quality variability

Starch grain composition, grain protein was found highly steady. Starch grain varied only between 59.1 and 65 g.100 g^{-1} DRM whilst grain protein was 9.8 g.100 g^{-1} DRM between cultivars and sites, respectively.

Straw quality was assessed on carbohydrates (cellulose and hemicellulose), extractive compounds, lignin and ash as major components found in straw composition. Quality regarding processibility during pretreatment and rated in terms of sugars released from the combined process pretreatment-enzymatic hydrolysis was also assessed. Carbohydrate content in straw was found fairly stable and no significant variations among cultivars and between sites was found. Variations in lignin was observed only for Swartland with significant lower lignin content of 13.5 g.100 g^{-1} DRM on average with respect to Mariendahl and Overberg. Ash content ranged 1 - 6.3 g.100 g^{-1} DRM among cultivars and was found highly site-related (p < 0.001). Mariendahl site showed consistently the lowest ash content in straw with 1.5 g.100 g^{-1} DRM on average, a reduction nearly 70% against

Overberg and Swartland straws (Figure 9-2). Reduction in ash content in straw showed to improve processibility: Mariendahl straws with reduced-ash content were found significant higher ($P < 0.05$) in xylose yield from pretreatment with average value of 14.8 g.100 g^{-1} DRM (xylose recovery about 62% of theoretical xylose in straw), nearly 10 and 25% more xylose recovered than Overberg and Swartland sites, respectively. Better processibility of Mariendahl straws was also found in higher EH-glucose outputs with 22.1 g.100 g^{-1} DRM with comparable glucose recovery of 73% to Swartland but close to 28% more recovery from enzymatic hydrolysis on average than Overberg. Ash content and sugar release from the combined sugars after pretreatment –enzymatic hydrolysis was found negatively associated as shown in Figure 9-2. Mariendahl-originated straw yielded 40.8 g.100 g^{-1} DRM (65.5% of total sugars recovered from theoretical in straw) against ~34.6 g.100 g^{-1} DRM Overberg and Swartland straws (with recoveries of 53.8 and 57.3% on average, respectively).

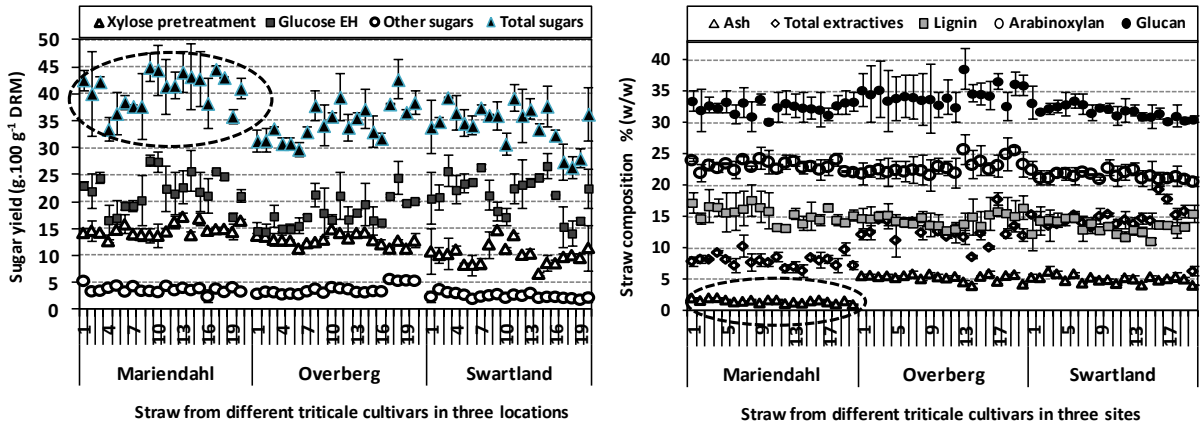


Figure 9-2: Left: monomeric sugars yield (gram of sugar per 100 grams of dry raw material) after dilute-acid pretreatment followed by enzymatic hydrolysis for straw from 20 triticale cultivars; enclosed area shows sugar yield from Mariendahl-originated straw. Right: straw composition found in triticale cultivars. Discontinuous lines enclose the trend in ash content for Mariendahl straws.

9.1.3 Cultivars with improved sugars output

Cultivars with preferred traits in grain and straw quality and straw processibility were sought and selected in this study to realize improved ethanol yield. Feedstocks with consistent and desirable quality are required for a viable cellulosic ethanol industry; thus identified traits regarding ash content and superior processibility of straw find applicability in breeding programs and categorizing preferred sites for triticale cultivation.

Selection of cultivars with the highest yields of total sugars from straw (pretreatment-enzymatic hydrolysis) was possible and highly site-related to Mariendahl site. Ten top cultivars with the highest

yields of sugars that displayed similar performance in Mariendahl site (p>0.05) although significant higher (p<0.05) against their counterparts originated in Overberg and Swartland were selected (Table 9-1).

Table 9-1: Averaged yields of released sugars (pretreatment - enzymatic hydrolysis) for the cultivars with the highest yields of sugars and averaged ethanol yield per hectare.

Parameter (yield)	Highest- yielding sugar cultivars (**Mariendahl originated**)									
	1	**3**	**9**	**10**	**11**	**14**	**17**	**18**	**13**	**15**
Xylose[1] (g.100^{-1}g DRM)	14.6±0.32[a]								17.1±0.28[b]	
EH-glucose (g.100^{-1}g DRM)	25.3±1.77[a]									
Total sugars[2] (g.100^{-1}g DRM)	43.2±1.66[a]									
Ethanol yield(L.Mg^{-1})[3]	265±7.76[a]									

[1]released from pretreatment.
[2]from pretreatment-enzymatic hydrolysis.
[3]estimate ethanol yield from xylose + EH-glucose at theoretical conversion (0.44 g ethanol. g^{-1} sugars consumed.
The values in each row correspond to the average value from ANOVA analysis among cultivars within Mariendahl site. Values having similar superscript letters do not differ between each other at a significance level of 0.05.

Selected cultivars with the highest yields of sugar were consistent in released sugars from pretreatment, enzymatic hydrolysis, total sugars and corresponding estimate ethanol yield per Mg of straw, except for cultivars 13 and 15 which showed to be statistically higher (p 0035 and 0.0102) in xylose yield (Table 9-2)

9.1.4 Cultivars with improved areal ethanol output

This study aimed at selecting cultivars with favourable traits of high areal ethanol output. Preferred cultivars could be further used on pretreatment optimization to potentially realize maximum areal 2G ethanol yield with little or no compromise with grain yield or 1G ethanol output potential. Thus applicability of the outcomes would certainly benefit farmer's revenues. Cultivars with significant superior 2G ethanol yield were compared to total ethanol yield estimate (Figure 9-3) to measure potential compromise with 1G ethanol output among the cultivars. Final screening stage concluded with the selection of the top 10 performer straws that showed superior areal ethanol yield estimate in the pair-wise analysis comparison (Table 9-2).

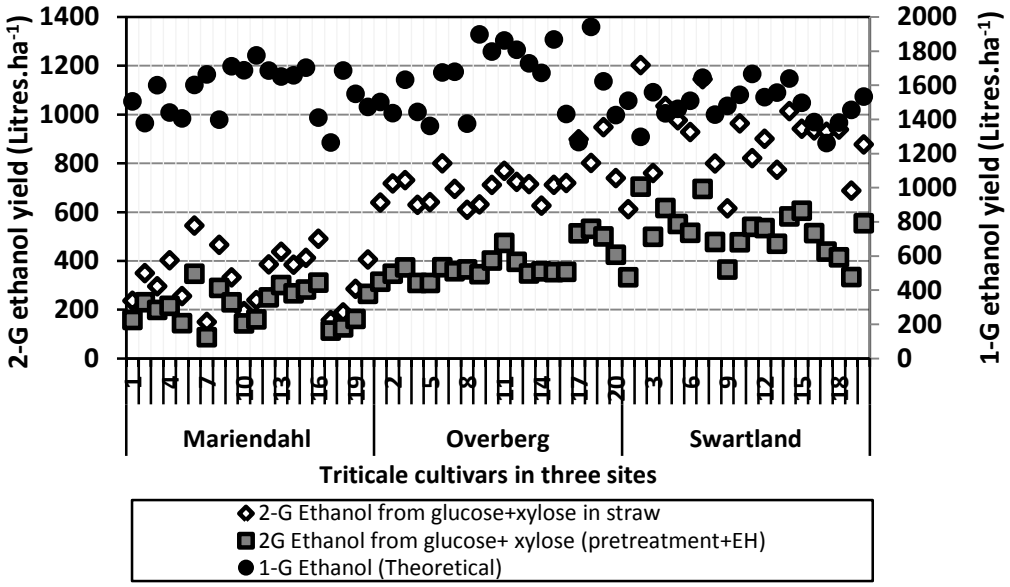


Figure 9-3: Mean values of estimated ethanol yield from starch grain (1G theoretical), estimated ethanol yields from glucose and xylose potential after pretreatment-enzymatic hydrolysis of straw (2G potential) and 2G ethanol yield potential in straw at theoretical conversions for triticale cultivars. Cultivars appear in numerical order 1-20 per site.

Selection of the cultivars with the highest yields of 2G ethanol was performed to realize low compromise with grain yield (or 1G ethanol yield potential). Cultivars 2, 4 and 5 (Table 9-2) displaying high compromise with grain were also included in the selection as a result of the high 2G ethanol productivity and promote studies on grain yield improvement. Additionally, pair-wise analysis showed similarities in areal 2G ethanol output between these three referred cultivars and the cultivars with the highest yields 7, 11, 12, 14, 15 and 20 that show low or no grain yield compromise (Table 9-2); thus observed high compromise of the formers may preferably be avoid by deciding on the latter cultivars from Swartland site and/or cultivar 18 Overberg-originated.

Table 9-2: Cultivars with the highest 2G ethanol yield per hectare (Swartland-originated) and attributes per site

Cultivar analysis			
Cultivar	**Ethanol ($L.ha^{-1}$)**	**Improved ethanol yield (%)[2]**	**Grain compromise**
2	704.5 ± 25.3^{a}	50~67	Very high
4	$617\pm63.8^{a,b,d,e,f,i}$	50~65	High
5	$551.5\pm45.3^{b,d,e,f,g,i}$	44~73	High
7	$695.1\pm11.1^{a,c,}$	48~87	No
11	$539\pm41.6^{d,e,f,g,h,i}$	12~70	No
12	$534.5\pm86.3^{e,f,g,i}$	26~53	Low
14	$582.4\pm20.6^{f,g,i}$	38~54	Low
15	$605.8\pm69.7^{a,g,i}$	41~53	Low
18[1]	532 ± 53.5^{h}	22~76	No
20	554.4 ± 149.0^{i}	23~52	Low
Site analysis			
		Attribute/Shortcoming	
Mariendahl	**Better straw processibility**/Lower stray yield		
Overberg	**Higher grain yield at consistent straw yield**		
Swartland	**Higher straw yield**/High straw yield instability		

The values in each row having similar superscript letters do not differ between each other at a significance level of 0.05.
[1] Cultivar 18 Overberg-originated.
[2] Refers to the improved ethanol yield of the cultivars with the highest yields cultivar in Swartland, except for cultivar 18 which is Overberg-originated, with respect to the ethanol outputs in other sites, expressed in percentage.

9.1.5 Selection of cultivars with improved sugar output and processibility

Final screening selection of cultivars targeted straw from the top 5 cultivars with the highest 2G ethanol per hectare for further study. Straw from preferred cultivars would be required in larger quantity during subsequent pretreatment optimizations at bench- and pilot-plant scales; thus availability of straw from cultivar Overberg- and Swartland-originated was a technical limitation faced in this study. Selection of top cultivars was then based on improved processibility in straw as performed in the screening selection (Table 9-3) and linked to deciding on providing coverage to all the three sites by assessing the best performer straw per site. Preferred cultivars with improved processibility during pretreatment were identified in the screening selection stage: Mariendahl originated- Cultivars 9, 13 and 14 (Table 9-3) were finally selected for further study on pretreatment optimizations due to availability of straw in stock. Straw from cultivars 19 and 7 originated in Overberg and Swartland were also selected (Table 9-3).

Table 9-3: Preferred cultivars with improved processibility during pretreatment-enzymatic hydrolysis.

Origin	Mariendahl			Overberg	Swartland
Cultivar	**9**	**13**	**14**	**19**	**7**
Total sugars yield (g.100 g^{-1} DRM)	45.0	44.1	43.3	36.7	35.5
P-site variability *	0.0387	0.0079	0.0051	0.0003	<0.0001

*refer to the variability in estimate ethanol from pretreatment-enzymatic hydrolysis at theoretical sugars conversion (0.44 g ethanol. g^{-1} sugars consumed) from ANOVA.

Straw from the top 5 cultivars underwent pretreatment optimization at bench-scale to refine the selection to only three preferred straws based on superior processibility during pretreatment. At this stage, processibility considered not only improved sugar output from the combined process (pretreatment - enzymatic hydrolysis) but also lessen pretreatment requirements to reach maximum yield of total fermentable sugars (TFS).

Pretreatment optimization of preferred straws at bench-scale showed differentiation between sugar productivity and pretreatment requirements to reach maximal total sugars yields (Table 9-4). Straws from Mariendahl site gave higher total sugars yields around ~10 -13% when comparing the highest outputs between sites (Table 9-4). Straw from cultivar 9 showed comparable high sugar output at lower severity factor and close to 25% lower acid concentration compared to straw from cultivars 13, the highest value between straws nearly 51 g.100^{-1}g DRM (Table 9-4).

Table 9-4: Predictive pretreatment conditions for maximum TFS yield (TFSY) from pretreatment and enzymatic hydrolysis. Desiderability function given in values between 0 and 1.

Cultivar	Origin	Optimum conditions[a]					Sugar yield (g/100 DRM)			Desira-bility
		Entry	Temp. (°C)	Acid conc. (% w/w)	Time (min)	SF	X_Y [b]	G_Y	TFS_Y [c]	
9	M	(*01T43*)	**181.7**	**0.39**	15.1	**3.59**	(9.0)	**36.7**	**50.6**	0.89
13	M	(*27thITYN39*)	186.7	0.53	17.0	3.78	(10.1)	**38.1**	**51.6**	0.97
14	M	(*98T376*)	185.4	0.48	18.0	3.77	(11.0)	33.8	48.8	0.97
19	O	(*00T207*)	190.0	0.53	**13.0**	3.76	(6.0)	30.2	44.9	0.87
7	S	(*BACCHUS*)	189.0	0.60	18.0	3.88	(11.5)	33.4	46.7	0.98

[a]Predictive pretreatment conditions for maximization of TFS_Y. SF stands for severity factor [b]Pretreatment variable not optimized. Values in brackets represent the yields of monomeric xylose at the optimum pretreatment conditions that maximized TFS yield.
[c]Optimum yield. Values in bold highlight lowest pretreatment conditions and maximum total sugar yield between straws.
M:Mariendahl O:Overberg S:Swartland.

Conversely, the poorest total sugar output (≤ 46.7 g.100^{-1}g DRM) were observed for straw from cultivars 19 and 7 (Overberg and Swartland originated, respectively), and particular cultivar 7 with the highest pretreatment requirements (Table 9-4). Straws from cultivars 9, 13 and 14 with the

highest yields of sugars were selected for pretreatment optimization at pilot-plant scale to realize the maxima combined sugars from the combined process.

9.1.6 Preferred cultivars with maximized combined sugars yield

Selected cultivars from the preceding screening selection study were subjected to pretreatment optimization at pilot-plant scale to realize the maxima combined sugar yields (CSY) from pretreatment – enzymatic hydrolysis. Uncatalyzed and SO_2-catalyzed steam explosion (SE) pretreatments were evaluated. Optimization of combined sugar at both types of pretreatment was constrained to low fermentation inhibitors production during optimization. Figure 9-4 summarises the maximum yields of combined sugars and inhibitors after pretreatment optimisations of the uncatalyzed and SO2-catalysed pretreatments of the straw samples obtained in this study.

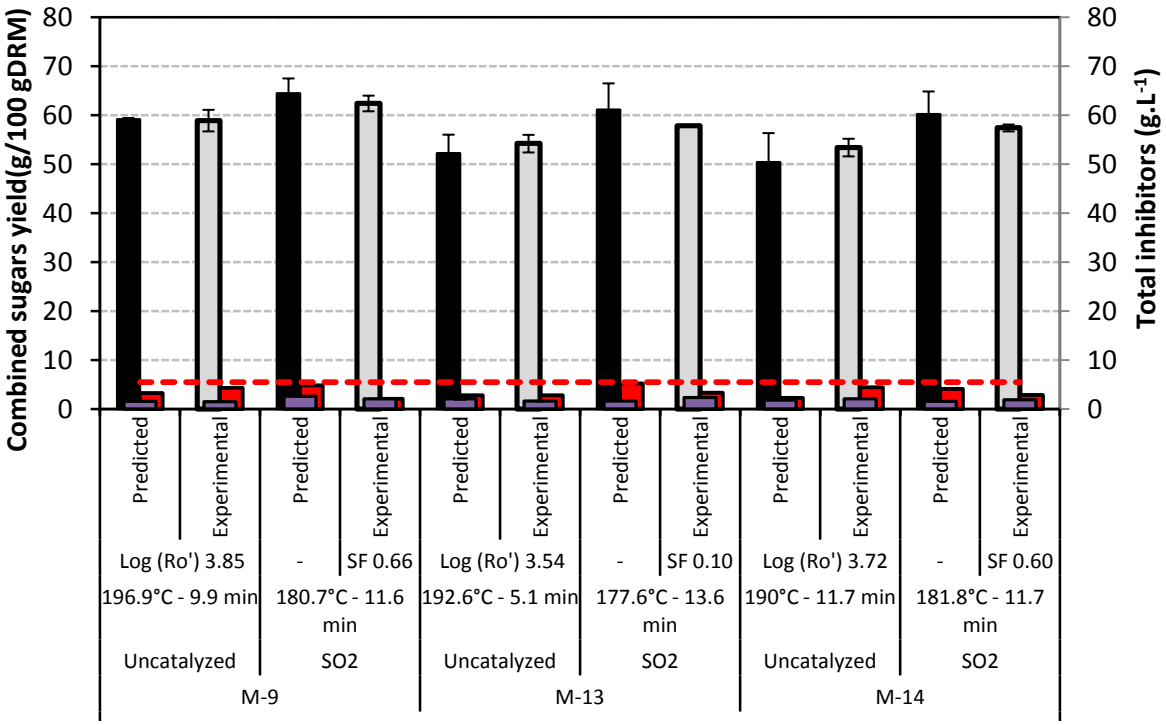


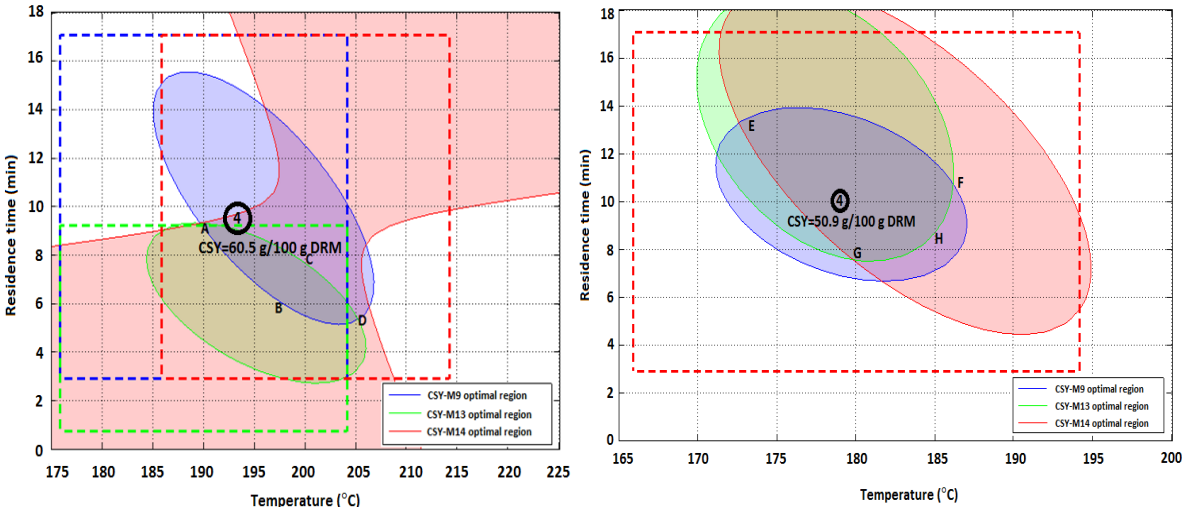
Figure 9-4: Optimal steam explosion pretreatment conditions (uncatalyzed and SO_2-catalyzed) and yield of combined sugars for straw from preferred cultivars 9, 13 and 14 (Mariendahl-originated) constrained to low inhibitors (HMF, furfural, formic acid and acetic acid). Black and grey bars represent the predicted and experimental optimal yields of combined sugars (pretreatment-enzymatic hydrolysis) by each type of pretreatment from optimization and model validation, respectively. Corresponding total concentration of inhibitors in pretreatment liquor (predicted and experimental) as the total of HMF, furfural, and acetic and formic acids is given in $g.L^{-1}$ (red bars). Red discontinuous line shows the minimum threshold of HMF, furfural, formic acid and acetic acid as total inhibitors concentration (5.5 $g.L^{-1}$) taken as reference at an acetic acid concentration of 2 $g.L^{-1}$. For maximum inhibition concentration, acetic acid corresponds to 6 $g.L^{-1}$ and threshold would correspond to 9.9 $g.L^{-1}$. Residual acetyl groups in exploded material are also given in $g.L^{-1}$ (blue bars). Pretreatment severity for uncatalyzed and SO_2-SE pretreatments is given according to Overend and *et. al.* $\mathbf{Log}\,(R_0') = \mathbf{Log}\left(t \cdot \exp\left(\frac{T-100}{14.75}\right)\right)$ and SF= Log (Ro') - pH. Pretreatment severity for predicted SO_2 is not given as combined severity factor is based on experimental pH.

Pretreatment optimization at pilot-scale resulted in cultivar differentiation regarding sugars yield and pretreatment requirements between straws of preferred cultivars. Even though both types of pretreatment resulted effective in realizing high CSY after optimization, the nature of the sugar hydrolysate released from pretreatment (mostly hemicellulose-derived sugars) varied accordingly to the type of impregnation: More oligomeric sugars from uncatalyzed- and more monomeric sugars from SO_2-SE pretreatment. Compared to uncatalyzed, SO_2 impregnation enhanced the CSY for all of the feedstocks by 8-16% and led to near theoretical recovery of total sugars from straws 9 and 13 under the constraints of inhibitors production. Cultivar 9 (Mariendahl-originated) showed better processibility by uncatalyzed and SO_2-catalyzed SE. Higher maximal CSY of 59.1 and 64.4 g/100 g DRM (91 and 98.8% total sugars recovered) was respectively predicted for cultivar 9 by uncatalyzed and SO_2-SE against predicted maximal yields of 52.2 and 50.4 g/100 g DRM (sugars recovery of 88.6 and 82%) by uncatalyzed-SE and comparable yield around 61 g/100 g DRM by SO_2-SE (close to theoretical sugars recovery), respectively from cultivars 13 and 14 (Figure 9-4). Pretreatment requirements to reach maximal yields were also found to differ between straws. Cultivar 13 displayed the lower requirements in severity by uncatalyzed-SE (Log (Ro') 3.54) although with lower sugar productivity at optimum conditions compared to cultivar 9 with the highest required severity (3.85) between straws (Figure 9-4).

Inhibitors production was directly associated to pretreatment severity. Experimental concentration of furfural for all the straws was below 1.2 $g.L^{-1}$ for uncatalyzed-SE nearly 50% lower than SO_2-SE (Figure 9-4). Thus optimization led to little furfural-toxicity and under the threshold of inhibition of 2 $g.L^{-1}$. HMF reached levels of toxicity nearly 30% beyond the threshold of inhibition (0.5 $g.L^{-1}$) only by uncatalyzed-SE of M9 and not higher than 0.22 $g.L^{-1}$ for straws M13 and M14 by SO_2-SE (Figure 9-4). SO_2-SE showed less HMF-toxicity for all of the straws at $\leq$ 0.26 $g.L^{-1}$. Formic and acetic acids at experimental optimal conditions showed together not to reach levels beyond 1 and 2-6 $g.L^{-1}$, respectively (Figure 9-4).

9.1.7 Preferred cultivars with feedstock flexibility for industrial application

The predicted models (p<0.05) developed for the maximization of CSY under constrained inhibitors production were modeled throughout the input range of experimentation to result in at least 95% of the maximum CSY for all of the straws and types of pretreatment. Areas of pretreatment in common between straw from preferred cultivar were possible to be identified for each pretreatment mode by overlap of individual results of the straws (Figure 9-5).



Uncatalyzed pretreatment **SO_2-catalyzed pretreatment**

Figure 9-5:Contour plots representing the overlapping of steam explosion pretreatment (SE) temperature and time by enclosed areas A, B, C and D for uncatalyzed-SE (left) and E, F, G and H for SO_2-SE (right) that result in not lesser than 95% of the maximum yield of combined sugars (CSY) for all of the feedstock. Encircled number 4 represents the pretreatment conditions used on cultivar 4 (Mariendahl-originated) to experimentally validate results. CSY is expressed in grams per 100 grams of dry straw. The continuous and dotted coloured lines represent the area of conditions that maximize CSY and the input range of the independent variables into the experimental design, respectively.

Conditions in common for maximizing CSY from the straws were sought to provide results with feasible industrial application in feedstock flexibility at potential shortage of homogeneity in seasons. Uncatalyzed pretreatment conditions with temperatures between 190 and 205°C together with residence that result in Log (*Ro'*) between 3.35-3.79 and catalyzed conditions (SO_2 at 3% w/w) with temperatures between 173 and 187°C combined with residence times that will give a severity factor between 3.30-3.41 were found to maximize CSY for straw from preferred cultivars under constrained inhibitors production with no lessen than 95% of maximal individual sugar outputs. The range of pretreatment conditions found was experimentally tested with cultivar 4 from Mariendahl site (which showed the lowest processibility and poor agronomic performance at the screening selection stage).

CSY from uncatalyzed and SO_2-SE at common severities found for the straws was 60.5 and 50.9 g/100 g DRM for straw from cultivar 4 (Figure 9-5), which represented combined sugars recoveries nearly 98 and 82% respectively. Inhibitors concentration was in all cases much lower than the values found for the studied feedstocks with exception of residual acetyl groups in WIS that reached 2.5 $g.L^{-1}$.

Straw from triticale cultivars seems to be a better feedstock in terms of sugars productivity and pretreatment requirements after acid-catalyzed pretreatment optimization compared to sugarcane bagasse under similar analysis. At lower demands in temperature (~172°C ≥ Temp ≤ ~187°C) compared to (~184°C ≥ Temp ≤ ~200°C) and residence time (~7 min ≥ time ≤ ~13 min) against (~6 min ≥ time ≤ ~16 min), triticale straws are predicted to sustain yield of combined sugars no lower than 57 g/100 g DRM and reach fairly comparable maximum outputs in similar analysis with bagasse from sugarcane varieties as observed in Figure 9-6.

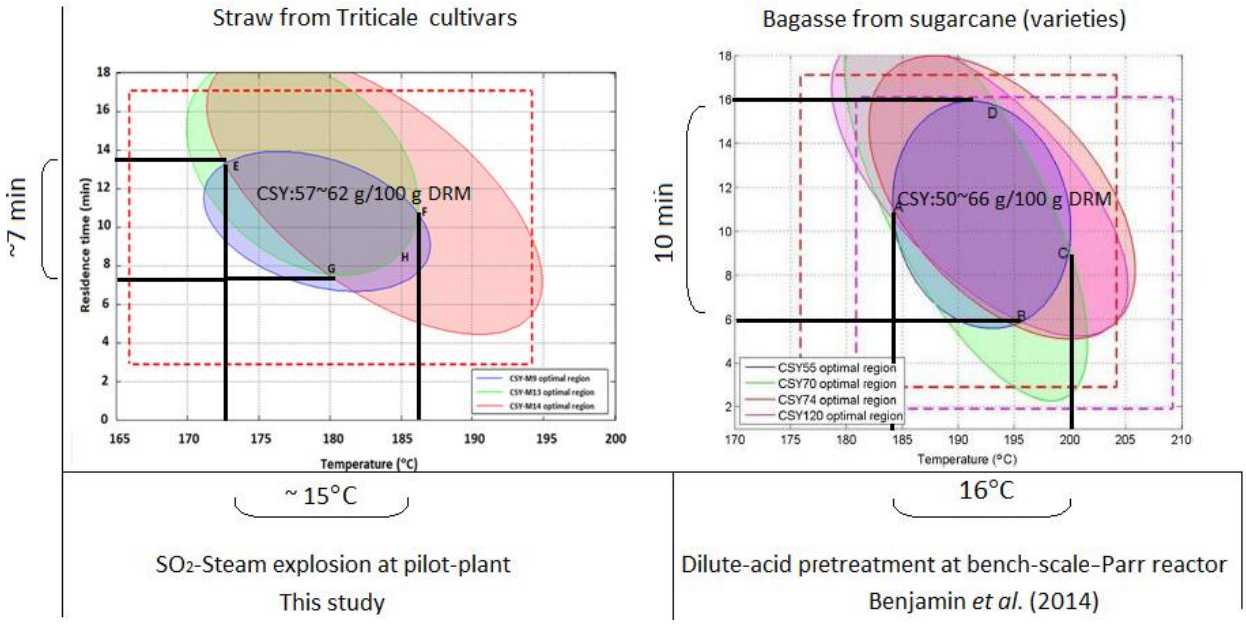


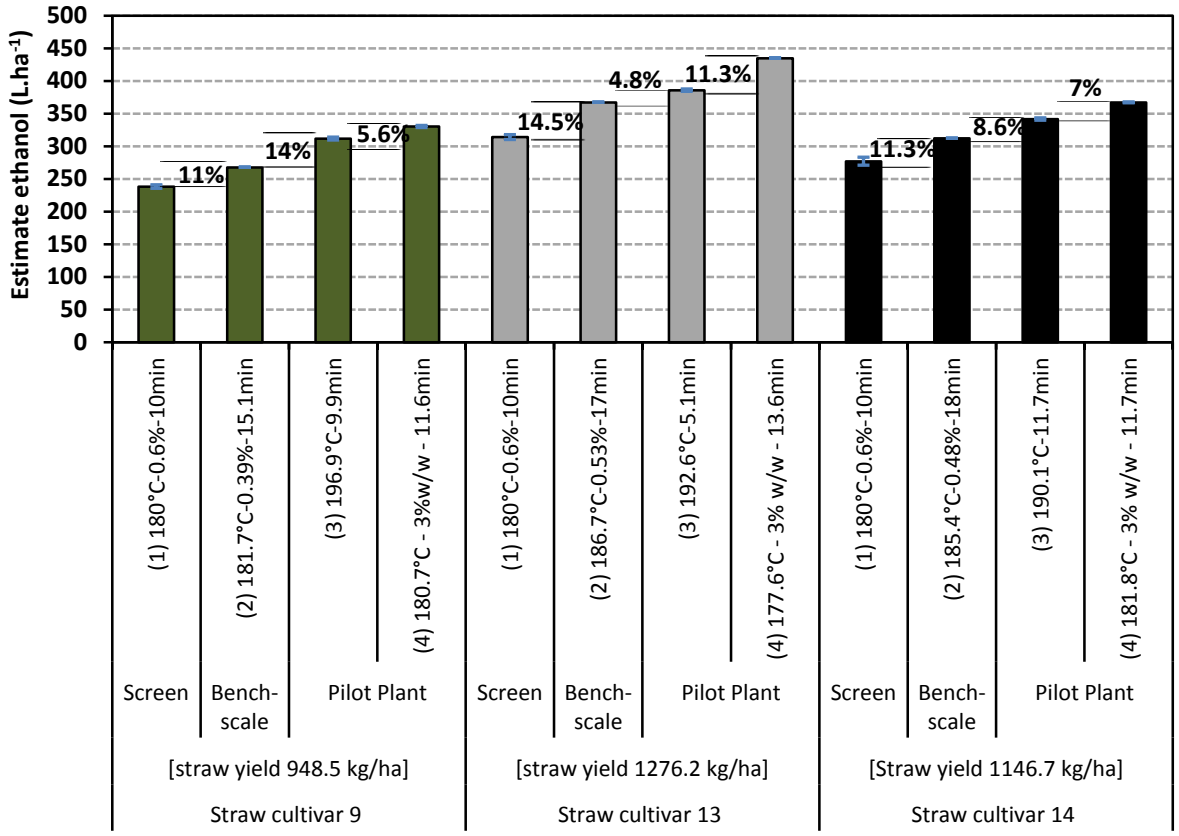
Figure 9-6: Comparative performance in combined sugars yield (CSY) for cultivars after pretreatment optimization between triticale straw (this study) and bagasse from sugarcane (Benjamin et al. (2014). Biotechnol. Biofuels, 2014, 7, 60).

9.1.8 Cultivars with improved ethanol output per hectare

The impact of cultivar selection and subsequent process optimization on ethanol yield from triticale cultivars was finally assessed. Straw from three preferred cultivars were subjected to consecutive pretreatment optimization: Dilute-acid pretreatment at bench-scale with total monomeric sugars yield (from the combined process pretreatment – enzymatic hydrolysis) as response. Subsequently, uncatalyzed and SO_2-catalyzed steam explosion pilot-scale with combined sugars yield (monomeric and oligomeric) from the combined process was optimized under constrained inhibitors production.

Figure 9-7 presents the estimate ethanol yield per hectare based on theoretical conversions (0.44 g ethanol/g of sugars consumed) of the experimental combined sugars yields achieved after

pretreatment-enzymatic hydrolysis at each stage of the study. Agronomic data of straw yield for each studied cultivar was used to refer estimate ethanol productivity per hectare of triticale cultivated.



(1) Pretreatment (bench-scale) at fixed dilute-acid conditions (Screening of cultivars stage). % acid in w/w.
(2) Pretreatment at optimal dilute-acid conditions at bench-scale. % acid in w/w.
(3) Predicted optimum conditions for uncatalyzed steam explosion at pilot-scale.
(4) Predicted optimum conditions for SO_2-catalyzed (3% w/w) steam explosion at pilot-scale.

Figure 9-7: Estimate ethanol yield per hectare based on experimental total sugars yields at theoretical conversions (0.44 g ethanol/g sugar consumed) and agronomic data of straw yield at each stage of the study for the preferred straw cultivars (Mariendahl-site). Screen: Screening selection; bench-scale: Dilute-acid pretreatment optimization at bench-scale; Pilot-plant: uncatalyzed and SO_2-catalyzed steam explosion optimization at pilot-plant.

First stage of pretreatment optimization (bench-scale) resulted in around 11-14.5% improved total fermentable sugars (Monomeric sugars) for the preferred straws; thus improved lignocellulosic ethanol yield estimate per hectare in the same magnitude was realized (Figure 9-7). Although pretreatment configuration and type of catalyst varied between bench- and pilot-scales, subsequent uncatalyzed pretreatment optimization at pilot-plant led to realize additional improvements in combined sugars outputs between 4.8 and 8.6% and correspondingly areal 2G ethanol yield estimate (Figure 9-7). The inclusion of SO_2 as catalyst during steam explosion resulted in about 5.6-11.3% additional improvements in combined sugars productivity among straws and therefore higher 2G

ethanol yield estimate per hectare up to 435 L.ha^{-1} for straw from cultivar 13 (Figure 9-7). The overall improvement in lignocellulosic ethanol yield estimate per hectare of triticale cultivated realized in the study was ~28% resulting from similar overall improvement in combined sugars yield from the combined process (pretreatment- enzymatic hydrolysis). Straw from cultivars 9 and 13 displayed similar improvement (~28%), although improvement in 25% of ethanol yield estimated was realized for straw 14 (Figure 9-7).

9.1.9 Impact of pretreatment optimization on the integrated saccharification-fermentation process

The strategies of solid loading (20 and 13% w/w) and type of substrate (pressed-slurry, unwashed WIS, pressed-washed material, and pressed-Intensive washed slurry) were evaluated experimentally by SSF. Solid loading of 20% w/w gave the lowest ethanol productivities in a preliminary SSF test. Experimental ethanol yields not higher than 171 L.ton^{-1} of straw were achieved at 13% solid loading for uncatalyzed-SE, with exception of slurry material conditioned by press-filtration and intensive washing from SO_2-SE of straw 14. Experimental volumetric productivities from preferred straws did not reach benchmark of 4% (v/v), high solid loadings and type of substrate (slurry or WIS) and conditioning (pressing and intensive washing) seemed to highly favour influence ethanol productivity. Thus fermentability of the pretreated material resulting from optimal SE conditions still remains a challenge to overcome high toxicity, mainly in acetic acid or acetyl groups in WIS if not conditioning in performed on slurries. This bottleneck to reach benchmark volumetric productivities of 4% v/v showed to be not inherent to triticale straw due to still comparable SSF performance with wheat straw at optimal pretreatment conditions.

Although not substantial differences in experimental SSF performance between cultivars were observed, main differentiation relays on theoretical yield that can potentially be achieved when considering the experimental glucose and xylose yields from pretreatment - enzymatic hydrolysis attained in the previous stage of the study. The effect of cultivar selection and pretreatment optimization on theoretical and experimental ethanol yields realized at the final stage of the study is represented in Figure 9-8.

Straw performance across the stages of the study was compared between cultivars to observe the highest 2G ethanol outputs estimate per hectare. Agronomic traits of grain and straw and overall performance were predominantly influential for improved total (1G + 2G) ethanol output estimate in the screening selection stage. Swartland-site as preferred location for maximum total ethanol

estimate per hectare and cultivar 7 as the performer with the highest yield of 2G ethanol (with no compromise with 1G ethanol) were the top in the selection. (Figure 9-8). Superior processibility for maximized total fermentable sugars (Table 9-4) was displayed by straw from cultivar 13 at the second screening selection (Figure 9-8). Regardless of the higher combined sugar yield achieved by straw from cultivar 9 after pretreatment optimization at pilot-plant, superior ethanol output per estimate per hectare would be maximized by straw from cultivar 13 (Figure 9-8) that displayed higher straw yield (1.20 vs 0.95 ton.ha^{-1}) in favour to straw 13. Pilot-plant optimization enabled nearly 15% of improvement in CSY in this straw and differences in straw yield compared to straws 9 and 14 resulted in 2G ethanol yield estimate per hectare around 430 L.ha^{-1} (Figure 9-8). Better fermentability of straw 14 achieved at 13% solid loading (press-filtration and intensive washing) led to realize experimental ethanol yield estimate per hectare above 200 L.ha^{-1} (Figure 9-8).

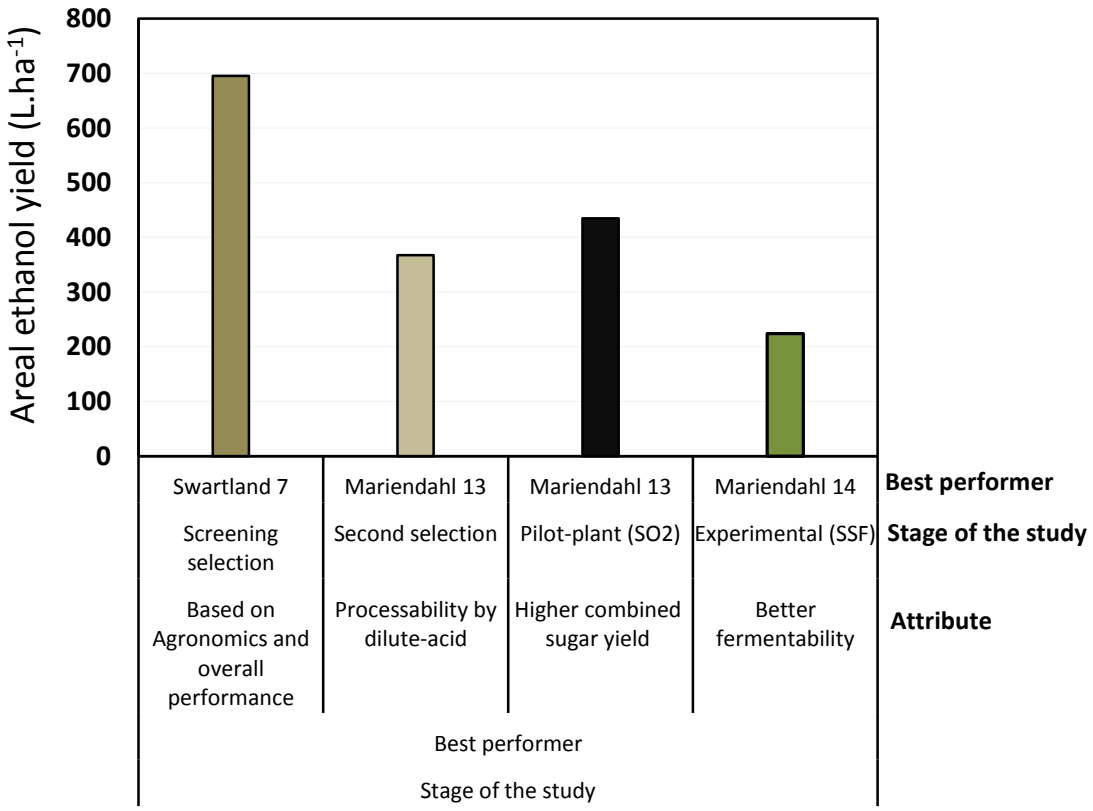


Figure 9-8: Estimate ethanol yield per hectare based on experimental total sugars yields at theoretical conversions (0.44 g ethanol/g sugar consumed) and agronomic data of straw yield at each stage of the study for the preferred straw cultivars (Mariendahl-site). Screen: Screening selection; bench-scale; Dilute-acid pretreatment optimization at bench-scale; Pilot-plant: uncatalyzed and SO_2-catalyzed steam explosion optimization at pilot-plant.

9.2 Conclusions

This dissertation addressed the gaps of cultivar and site specific variabilities in feedstock quality and processibility of straw from triticale and assessed the impact of cultivar selection on improved ethanol yield per hectare. Original contribution to knowledge was the establishment of a systematic approach based on selection of cultivars with superior traits in straw and preferred locations for improved processibility and sugars productivity **(Chapters 4** and **5), and** further development of predictive models with statistical significance for industrial application that maximize sugars yields through pretreatment optimization for maximum sugars at low toxicity levels yield (**Chapters 6** and **7**). Thus improved estimate ethanol yield per hectare of triticale cultivated is realized. Finally, experimental evaluation of the results from pretreatment optimization in an integrated configuration (**Chapters 8**) was investigated to determine the maximum experimental ethanol yield realizable and the fermentability of the treated material as concluding stage of the approach. Thus the conclusive implications of the empirical findings of this dissertation will follow the above direction showing how these conclusions address the proposed objectives and converge into the general aim of the study. Finally, delimitations imposed in the study and opportunities for future work are discussed in the final section of this chapter.

9.2.1 Cultivar Selection and Impact on Sugar Yield

Agronomic parameters of grain and straw outputs were found to be highly cultivar- and site-related variable for triticale; even though straw yield variation was more site-related and did not correlate with grain yield that showed more consistency and no grain quality (starch content) variation. These observations show both 1G and 2G ethanol outputs potential per area of land cultivated with triticale highly genotypic and environmentally influenced in South Africa, as consequence of the high impact of agronomic conditions. Even though other studies in triticale have similarly shown cultivar and agroclimatological influences on grain and straw output, the findings in the present work suggest that triticale cultivars in South Africa display high instability to sustain 1G ethanol but yet larger instability in straw output and consequently areal 2G ethanol yield. All three studied sites display particularities that necessarily should be considered to reduce negative impacts of agronomic variations on ethanol productivity per hectare: Mariendahl-specific agroclimatological conditions are favourable to cause improved processibility in straw although at the lowest productivities in sugars per hectare. Consequently, Mariendahl straws offer potential reduction in processing costs and better sugar productivity per ton of straw.

Maximization of total ethanol (1G + 2G) in Mariendahl-originated triticale certainly engages a compromise on both agronomic parameters, although at larger extent on straw yield caused by high on-side cultivar instability. Overberg and Swartland mega-regions in South Africa are recommended for superior total ethanol output at expense of lower processibility in straw compared to Mariendahl straw. Triticale cultivars are more adapted to Overberg-specific conditions in sustaining straw yield at a relatively high but yet variable grain yield. Better straw production at inferior grain yield from poorer-adapted cultivars in straw output is characteristic in Swartland site. High variability in agronomics of grain and straw yields has large influence on traits in feedstock quality and processibility and amplifies variation in maximum ethanol potentially resourced per hectare. This dissertation demonstrated that regardless of the high impact of agronomic conditions, selection of cultivar for improved total ethanol output per hectare is possible for South African triticale cultivars due to no association between grain and straw yields observed in this study.

Straw quality in triticale shows high consistency in carbohydrate potential together with high variability in ash content. Limited information is currently available on straw quality in triticale and its closer genetic-relative wheat straw although the latter has been found cultivar- and site-related variable. These findings inherently show South African cultivars more highly consistent in theoretical 2G ethanol potential per ton of straw if only carbohydrate potential (excluding straw processibility) is considered as criteria; thus local triticale cultivars are physiologically robust in sugars potential. Ash content in straw is highly site-related variable and more cultivar-stable.

Characteristic climatological conditions and sandy-loamy soil in Mariendahl contribute to the low mineral uptake in triticale cultivars; thus accumulation of minerals in lignocellulosic biomass is significantly reduced at this site. Hence, the observed in Overberg and Swartland sites evidences negative influence of the specific agroclimatological conditions on mineral build-up in triticale. Studies on straw quality of wheat cultivars have showed detrimental in processibility during pretreatment of straw with high ash content. This dissertation found that ash-reduced straw in triticale cultivars has significant high impact on processibility during pretreatment. Cultivars with more amenability for sugars release in straw above 41.6 g.100 g^{-1} DRM with reduced ash of 1.5% are possible to be identified by screening selection. As further discussed in this chapter, better processibility not only infers high sugar productivity but also reduction in pretreatment requirements for additional economical benefits in the overall bioethanol process.

In view of these findings, improved 2G ethanol productivity estimate (based on theoretical sugar conversion 0.44 g ethanol. g^{-1} sugar consumed) from ~87 up to ~704 L.ha^{-1} (comparing the poorest and the best performer straws) were realized in triticale by cultivar/site selection with low or no compromise with grain yield, findings that substantiate the proposed objective 1 and contribute in the accomplishment of the overall aim of this dissertation.

9.2.2 Selection of Straw with Improved Processibility

Enhancement of sugar output from triticale was achieved in this study as essential step for improved ethanol yield per hectare. Local triticale cultivars display in general consistent lignin and hemicellulose contents, major structural features directly related to recalcitrance in biomass. Thus selection of cultivars with reduced recalcitrance due to other features such as ash content is accomplished through differentiation in response to pretreatment and processibility.

The second selection of top cultivars, based on results of the first selection coupled to straw availability constraint, led to the final top 3 straws that displayed higher total sugars productivity after pretreatment optimization at bench-scale. Straws 9, 13 and 14 (Mariendahl-site) showed superior pretreatment response in sugars yield (48.8 - 51.6 g.100 g^{-1} DRM) additional to lower pretreatment requirements (commonly temperature and acid concentration) compared to cultivars 19 (Overberg-site) and 7 (Swartland). Improvements in total sugars yield after optimization could be realized at an extent between 8.6 and 14.5% more estimate 2G ethanol output (up to 367 L.ha^{-1}) from theoretical conversion of experimental sugars achieved only at this stage of the study. Thus attributes picked up in feedstock after accomplishment of the cultivar selection resulted in maximum improved ethanol yield estimate per hectare of ~15%.

A close association between ash in straw and better processibility in sugars output at reduced pretreatment requirements was again confirmed for Mariendahl site cultivars. These observations may suggest that, as found in other herbaceous such as switchgrass, low ash content in triticale straw similarly contributes to decrease the buffer capacity of the feedstock during pretreatment with positive consequences in pretreatment efficiency (higher sugars) and better catalyst efficiency (reduced acid concentration). Favorable agroclimatological conditions in Mariendahl over cultivar variability are predominately to be displayed on triticale and result in better processibility; thus triticale cultivars with improved processibility in straw is realized by selecting Mariendahl as preferred site.

These findings confirm the positive impact of pretreatment optimization on improved combined sugar yield and cultivar/site differentiation according to sugar productivity and pretreatment requirements by the straws. Thus objective 2 of this dissertation is reached.

9.2.3 Preferred cultivars with maximized combined sugars yield

Cultivars 9, 13 and 14 (Mariendahl-site) that displayed better processibility at second selection stage were finally preferred for further pretreatment optimization at pilot-scale. Straw from cultivar 9 showed superior sugar productivity after optimization of uncatalyzed and SO_2-catalyzed SE with 91 and ~99% of total sugars recovery, respectively. These high sugars recoveries realized at constrained inhibitors production may suggest that feedstock traits exhibited by cultivar 9 regarding significant higher carbohydrate potential (38.8 ±0.63g.100 g^{-1} DRM) and reduced ash (2.4±0.1%) favoured near 9% more CSY compared to straws 13 and 14. Thus straw from cultivar 9 shows sustained better processibility in terms of sugar yield across the sequential pretreatment optimizations although at higher pretreatment requirements than straw 13 at pilot-scale pretreatment.

Agronomics of straw yield showed high influence on sugar productivity from the combined process pretreatment – enzymatic hydrolysis; although straw 13 resulted in comparable CSY to straw 14 and much lower than straw 9 after pretreatment optimizations at pilot-scale, considerable much higher straw yield of about ~1276 kg.ha^{-1} (Figure 9-7). Summary of findings) in straw 13 resulted in near ~430 L.ha^{-1} improved ethanol yield estimate (the highest output from experimental sugar yield achieved at pilot-scale among top cultivars). These observations suggest that the selection of cultivars with maximized sugar output from straw embodies a technical compromise between sought ethanol productivity and potential revenue expected by the farmers. That is, improved sugar productivity in feedstock per ton of straw is highly desirable from process feasibility point of view (as straw 9) whilst more ethanol yield estimate per hectare of feedstock is highly desirable for improving farmers' revenues (as straw 13).

Sequential pretreatment optimizations positively maximized even more the CSY from preferred straws: ~15 more sugars from both 13 and 14 straws and ~20% more from straw 9, which provides foundation to the systematic approach used in this study. Although it seemed that SO_2-catalyzed steam explosion at pilot-scale was more effective in realizing higher CSY when compared to dilute-acid pretreatment at bench-scale, pretreatment response of the latter accounted for only total monomeric sugars against all monomeric and oligomeric sugars accounted for in the former. However, subsequent pretreatment optimizations at different technology sizes resulted in an effective strategy to pick up differences in straw processibility between triticale cultivars if

considered that similar trend for maximal total or combined sugars yield was consistent for the straws at both pretreatment scales. The results in this study demonstrated that even though pretreatment conditions that maximize CSY necessarily engage a compromise between conditions for maximum hemicellulose-derived sugar release and those for maximum digestibility, the constrain of inhibitors production into the pretreatment parameters for optimization still allow for high combined sugar productivity at acceptable levels of toxicity downstream (below 5.5 $g.L^{-1}$ of total inhibitors) for both uncatalyzed and SO_2-catalyzed SE. Thus feasibility for application of the results from optimized pretreatments is achieved.

The approach followed in this study showed to be effective way to substantially improve ethanol output is by considering hemicellulose-derived sugars into the approach, as followed in this dissertation. Increments between 29.6 and 46.3% in theoretical ethanol yield among straws was found by considering the glucose and xylose streams from the combined process compared to the ethanol from only glucose. Improved sugars from straw at reduced pretreatment requirements was experimentally accomplished and validated for triticale straw Mariendahl-originated. Uncatalyzed steam explosion severities (Log (*Ro'*)) that maximized CSY from straw of individual cultivars 9, 13 and 14 were between 3.54 and 3.85. Further analysis to find pretreatment conditions in common for at least 95% of maximal individual output show Log (*Ro'*) between 3.35-3.79 for pretreatment of cultivars under Mariendahl-site influences. Thus pretreatment requirements to maximize sugars in triticale straw from Mariendahl site seem to be lower than those for wheat straw with Log (*Ro'*) ~ 3.65 [1; 2]. Acid catalyzed pretreatment severities also show to be reduced for Mariendahl-originated straws with Log (*Ro'*) between 3.30 and 3.41 if compared with Log (*Ro'*) 3.36 and 3.64 for maximum conversion yield to ethanol and sugars recovery in pretreatment liquor from acid-catalyzed SE pretreated wheat straw, respectively [3].

9.2.4 Integrated SSF configuration and fermentability

Type of pretreatment (uncatalyzed or SO_2-SE) impacts on fermentability of the pretreated material and solid loading strategy applied if the configuration of choice is SSF. Volumetric ethanol productivities at industrial benchmark can be potentially achieved by further improvements in yeast robustness, improvements in pretreated material conditioning or alternative fermentation strategies rather than major adjustments in pretreatment; this aiming at alleviating the inhibiting effects of mainly acetic acid (or acetyl groups) in the pretreated material as a result of effective hemicellulose-solubilization during pretreatment.

9.3 Delimitations and recommendations

This research work sought, in first instance, to experimentally generate reliable data regarding agronomic yields, feedstock quality of grain and straw, as well as pretreatment responses such as sugars from a large number of cultivars and representative locations in South Africa. The Department of Genetics, Stellenbosch University, participated in the execution of the field trials, straw and grain harvest and collection as well as agronomic data of grain and straw yields which were under coordination of Dr. Willem Botes. The first stage of the study, comprising compositional analysis of grain and straw, and pretreatment of straw material at a single pretreatment condition was kindly performed under coordination of James Batt (Department of Process Engineering). Consequently, a large number of replicated samples would be processed in a definite period of time. Cultivar and site-related variabilities in triticale traits were then prioritized in the study and possible variation through different seasons was delimited to 2009 growing season for the agronomic data set gathering. Although seasonal variations in agronomic conditions and resulting triticale traits may be expected, as typical occur in wheat as genetic-relative to triticale, robustness in cultivar stability across sites was determinant in assessing the potential for bioethanol production of South African cultivars. However, important attributes in straw quality such as composition and processibility were able to be assessed in the preferred cultivars for growing season 2010 and 2011. Future work covering different growing seasons is then recommended to observe cultivar stability/instability across climate variations during seasons. Thus possible seasonal fluctuations in traits would have incidence on ethanol yield potential.

Optimization of dilute-acid pretreatment at bench-scale was evaluated in terms of total fermentable sugars (monomers) conversely to combined sugars (monomeric and oligomeric) at pilot-scale optimization. This delimitation was set in the study to facilitate differentiation in processibility and sugar productivity as part of the screening selection taking into account that dilute-acid pretreatment is more effective in monomeric sugars release, while substantially improving enzymatic digestibility. Thus the inclusion of sugar oligomers would not represent major variation in differentiation of pretreatment response by the cultivars with no extra steps of post-hydrolysis analysis.

9.4 Areas for future research

High cultivar and site-related variabilities in agronomic yields were found in triticale cultivars in South Africa. Expected sustainability in grain and straw yields commonly reported for triticale as indication of good adaptability [4] were not generalized in this study. Grain to straw ratio at high grain yield is essential to maximize areal 2G ethanol with yet no detrimental grain productivity. Although selection of cultivars with improved areal total ethanol yield was realized in the study, cultivars in general displayed high grain compromise to maximize 2G ethanol per hectare. Thus future work on identifying genotypical traits that confer triticale such cultivar instability together with cultivar × site interaction variabilities [5] is recommended to provide triticale not only with more predictability but also more robustness in total (1G + 2G) ethanol yield per hectare.

Triticale cultivars with superior traits for improved estimated total ethanol yield per hectare were possible to be identified. Preferred cultivars identified after screening selection (Chapter 4) estimate up to ~700 $L.ha^{-1}$ in 2G ethanol yield at theoretical sugar conversion whilst cultivars with improved processibility and maximized combined sugars yield from steam explosion pretreatment- enzymatic hydrolysis (**Chapter 7**) realized up to ~430 $L.ha^{-1}$. Shortage in straw availability for further assessment of preferred cultivars from the screening selection certainly limited the continuation of the general approach to achieve even higher improvements (>>700 $L.ha^{-1}$) in 2G ethanol yield estimate and consequently in total ethanol yield as grain yield did not compromise much in the selection. Future work on steam explosion pretreatment optimization of straw from cultivars 7, 14, 15 and 20 originated in Swartland as well as cultivar 18 from Overberg is highly advisable to realize more substantial improvements in total ethanol per hectare of triticale.

Steam explosion pretreatment conditions that maximize combined sugars yield are recommended in this study for cultivars with ash-reduced straw (Mariendahl-originated). However, future work on preferred cultivars with superior areal ethanol yield estimate, as found in Swartland, is suggested to be evaluated on those pretreatment conditions to realize applicability of these pretreatment regions over other sites.

Agronomics of straw yield showed high influence on sugar productivity from the combined process pretreatment – enzymatic hydrolysis; although straw 13 resulted in comparable CSY to straw 14 but much lower than straw 9 after pretreatment optimizations at pilot-scale, considerable much higher straw yield of about ~1276 $kg.ha^{-1}$ (Figure 9-7) resulted in near ~430 $L.ha^{-1}$ improved ethanol

yield estimate, the highest output from experimental sugar yield achieved at pilot-scale among top cultivars. These observations suggest that the selection of cultivars with maximized sugar output from straw embodies a compromise between sought ethanol productivity and potential revenue expected by the farmers. That is, improved sugar productivity in feedstock per ton of straw is highly desirable from process feasibility point of view whilst more ethanol yield estimate per hectare of feedstock is highly desirable for improving farmers' revenues.

Cultivar selection based on agronomic yields and overall performance of cultivars (grain and straw qualities and pretreatment performance) followed by selection based on improved sugar productivity and subsequent pretreatment optimization finally led to improved ethanol output estimate per ton of straw and per hectare of straw cultivated. Fermentability test of the pretreated material by different strategies of solid loading (13 and 20% w/w) and types of conditioning of the material were assayed by SSF. Low fermentability of the pressed-slurry at high solid loading at 20% and improved but still low at 13% suggest that periodic neutralization of the broth during fermentation should be attempted alleviate to the inhibitory effect of acetic acid and potential acetyl groups released in form of acetic acid into the broth.

Considering the substantial improvement observed in fermentability through extensive washing of the remaining solids after pretreatment, further work is needed on conditioning the material for the SSF to able to minimize water-consumption and yet reduce toxicity at acceptable levels for the yeast.

Finally, the need of yeast with stronger capabilities to handle higher concentrations of inhibiting compounds and better performance at higher pH values as imposed by slurries with better hemicellulose-sugar solubilization is essential to be considered for optimized sugars streams reach benchmark ethanol productivities.

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