

SUGARCANE CULTIVAR SELECTION FOR ETHANOL PRODUCTION USING DILUTE ACID PRETREATMENT, ENZYMATIC HYDROLYSIS AND FERMENTATION

by

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Declaration

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Abstract

The development of “energy cane” varieties of sugarcane for ethanol production is underway, targeting the use of both sugar juice (first generation ethanol) and bagasse (second generation ethanol). Nevertheless, identification of the preferred varieties represents the biggest challenge to the development of energy cane due to large number of samples produced during breeding. In the present study, dilute acid pretreatment, enzymatic hydrolysis and fermentation processes were used to evaluate the processability of bagasse (fibrous residue generated after juice sugar extraction) from different varieties of sugarcane to select preferred varieties with the properties of improving combined ethanol yield (ethanol from juice and bagasse) per hectare. The impact of variety selection on combined ethanol yield (ethanol from juice and bagasse) per hectare was also assessed.

In the first part of this study, 115 varieties of sugarcane originated from classical breeding and precision breeding (genetic engineering) were screened based on agronomic data and experimental data from biochemical processes (dilute acid pretreatment and enzymatic hydrolysis) applied to the bagasse fraction of each variety. The results showed wide variations in the chemical composition of bagasse between the varieties. Structural carbohydrates and lignin content ranged from 66.6 to 77.6% dry matter (DM) and 14.4 to 23.1% DM, respectively. The majority of precision breeding varieties showed higher arabinoxylan, lower lignin and lower ash content than most of classical breeding varieties. Combined sugar yield from the bagasse after pretreatment and enzymatic hydrolysis also varied significantly among the varieties. Up to 27.9 g/100g (dry bagasse) difference in combined sugar yield was observed. Combined sugar yield was inversely correlated with lignin as well as ash content, but it correlated positively with structural carbohydrates content. Total potential ethanol yields per hectare, calculated based on cane yield, soluble and non-soluble sugar content also differed significantly

among the varieties (8,602–18,244 L/ha). Potential ethanol from bagasse contributed approximately one third of the total potential ethanol yield. Interestingly, some of the varieties had combined properties of high potential ethanol yield per hectare and improved bagasse convertibility. Thus, six varieties (3 from each breeding technology) were selected as preferred varieties for further investigation.

To enhance sugar yield from bagasse, optimisation of pretreatment was conducted on the selected varieties. Industrial bagasse was included for comparison purposes. The pretreatment optimisation was based on maximising combined sugar yield from the combined pretreatment-hydrolysis process. A central composite design (CCD) was applied to investigate the effects of temperature, acid concentration and residence time on the responses and was later used to determine the maximum combined sugar yield. Pretreatment optimisation was conducted at gram scale (22.9 ml reactor) and at bench scale (1000 ml reactor). Significant differences in sugar yields (xylose, glucose, and combined sugar) between the varieties were observed. The combined sugar yields from the best performing varieties and industrial bagasse at optimal pretreatment-hydrolysis conditions differed by up to 34.1% and 33% at gram and bench scale, respectively. A high ratio of carbohydrates to lignin and low ash contents increased the release of sugar from the substrates. At mild pretreatment conditions, the differences in bioconversion efficiency between varieties were greater than at severe conditions. This observation suggests that under less severe conditions the conversion efficiency was largely determined by the properties of the biomass. Furthermore, it was demonstrated that the pretreatment conditions with temperature ranged from 184 to 200 °C and varying residence time to provide a severity factor between 3.51 and 3.96 was observed to be the area in common where 95% of maximum combined sugar yield could be obtained.

Simultaneous Saccharification and Fermentation (SSF) was performed on the unwashed pressed-slurry from bagasse pretreatment at conditions for maximum combined sugar yield at bench scale. Batch and fed-batch SSF feeding strategy at

different solid loadings and enzyme dosages were used aiming to reach an ethanol concentration of at least 40 g/L. The results revealed significant improvement in overall ethanol yield after SSF for the selected varieties (84.5–85.6%) compared to industrial bagasse (74.8%). The maximum ethanol concentration from the best performing varieties was 48.6–51.3 g/l and for poor performing varieties was 37.1–38.3 g/l. Ethanol concentration in the fermentation broth was inversely correlated with lignin content and the ratio of xylose to arabinose, but it showed positive correlation with glucose yield from pretreatment-enzymatic hydrolysis. The overall assessment of the varieties showed greater improvement in combined ethanol yields per hectare (71.1–90.7%) for the best performing varieties with respect to industrial sugarcane.

The performance in terms of ethanol yields of selected varieties from a number harvest years was evaluated. The results showed considerable variations in ethanol yields across harvests. The results showed that the best variety in terms combined ethanol yield was not maintained across harvests. The differences in ethanol yields were greater among the varieties than across the harvests. Prolonged severe drought significantly affected the ethanol yields of all varieties represented by lower and intermediate lignin content for cane yield compared to that which had highest lignin content. However, carbohydrates content in the bagasse and sugar yield/recovery between the harvest years did not change for the most of the varieties.

In summary, the present study provides evidence of the impact of cultivar selection and pretreatment optimisation in increasing conversion efficiency of bagasse. The results demonstrate that varieties with lower lignin and ash content, as well as highly substituted xylan resulted in higher sugar and ethanol yields. These results suggest that lower process requirements can be achieved without adversely affecting juice ethanol and cane yield per hectare. Nonetheless, an attempt to reduce lignin content in the bagasse, to reduce processing requirements for ethanol production, can also target the improvement of crop tolerance toward severe drought conditions.

Opsomming

Die ontwikkeling van “energie-riet” rasse vir etanol produksie is goed op dreef, waar beide die sap (eerste generasie etanol) en die bagasse (tweede generasie etanol) geteiken word. Die groot aantal monsters wat tydens teling geproduseer word, bied egter die grootste uitdaging vir die identifisering van nuwe rasse ten einde energie-riet te ontwikkel. In die huidige studie is verdunde suurvoorbehandeling, ensimatiese hidrolise en fermentasie-prosesse gebruik om die verwerkbaarheid van bagasse (veselagtige residu gegeneer na sap suiker ekstraksie) van verskillende suikerrietrasse te evalueer om nuwe variëteite te selekteer wat eienskappe van verbeterde gekombineerde etanolopbrengs (etanol van sap en bagasse) per hektaar toon. Die impak van variëteit-seleksie op gekombineerde etanol opbrengs (etanol van sap en bagasse) per hektaar is ook beoordeel.

In die eerste deel van hierdie studie het uit ‘n siftingsproses van 115 suikerriet rasse bestaan wat deur klassieke en presisie (geneties gemodifiseerde) teling gegeneer is. Die sifting was op agronomiese data gebaseer, asook op data van verdunde suur voorafbehandeling en ensimatiese hidrolise eksperimente wat op die bagasse fraksie van elke ras uitgevoer is. Die resultate het op groot variasie in die chemiese samestelling van die bagasse van verskillende rasse gedui. Die strukturele koolhidrate het tussen 66.6 en 77.6% droë massa (DM) gewissel, terwyl die lignien inhoud ‘n variasie van 14.4 en 23.1% DM getoon het. Verder het meeste van die presisie-teling variëteite ‘n hoër arabinoxilaan, maar ‘n laer lignien en as-inhoud as meeste van die klassieke teling rasse gehad. Die gekombineerde suikeropbrengs (GSO) van die bagasse na voorafbehandeling en ensimatiese hidrolise het ook beduidend tussen rasse gewissel, waar ‘n verskil van tot 27.9 g/100g (droë bagasse) waargeneem is. Daar was ‘n omgekeerde korrelasie tussen die gekombineerde suikeropbrengs en die lignien en as-inhoud gewees, maar die opbrengs het ‘n sterk positiewe korrelasie met die strukturele koolhidrate getoon. Die totale potensiële etanol opbrengs per hektaar wat vanaf die suikerriet se oplosbare en nie-

oplosbare suikereinhoud bereken is, het ook beduidend tussen rasse verskil (8,602–18,244 L/ha), waar die potensiële etanol opbrengs van die bagasse gedeelte ongeveer een derde van die totale potensiële etanol opbrengs beslaan het. Interessante bevindinge het op sommige rasse met gekombineerde eienskappe van hoë potensiële opbrengs per hektaar asook 'n hoë omskakelingsvermoë gedui. Derhalwe is ses variëteite (drie van elke telingstechnologie) as voorkeurvariëteite vir verdere studie gekies.

Om die etanol opbrengs vanaf die bagasse te verbeter was voorafbehandeling van die voorkeurvariëteite geoptimeer, en waar industriële bagasse vir vergelykingsdoeleindes ingesluit was. Vir die optimering was dit ten doel gestel om die gekombineerde suikeropbrengs van die gekombineerde voorafbehandeling-hidrolise proses te maksimeer. 'n Sentrale saamgestelde ontwerp (SSO) is gebruik om die effek van temperatuur, suurkonsentrasie en residensityd op die responsveranderlikes vas te stel wat uiteindelik gebruik is om die maksimum gekombineerde suikeropbrengs te bepaal. Die optimering van die voorafbehandeling is op gram-skaal in 'n 22.9 ml reaktor, asook op bank-skaal in 'n 1000 ml reaktor uitgevoer. Beduidende verskille in die suikeropbrengs (xilose, glukose en gekombineerde suiker) is tussen die voorkeurrasse waargeneem. Tussen die rasse wat die beste gevaar het, asook die industriële bagasse, het die gekombineerde suikeropbrengs by optimale voorafbehandeling-hidrolise toestande onderskeidelik met tot 34.1% en 33% op gram-skaal en bank-skaal gevarieer. 'n Hoë verhouding van koolhidrate tot lignien, asook 'n lae as-inhoud het tot 'n toename in die vrystelling van suiker uit die substraat gelei. By matige voorafbehandelingstoestande was die verskille in omskakelingseffektiwiteit tussen rasse groter as onder hewige toestande, wat daarop gedui het dat omskakelingseffektiwiteit grotendeels deur die eienskappe van die biomassa bepaal is. Verder is daar ook gedemonstreer dat die voorbehandelingsomstandighede met temperatuur tussen 184 en 200°C en verandering van die residensityd om 'n hewighedsfaktor van tussen 3.51 en 3.96 te verskaf, 'n gemeenskaplike area gelewer het waar 95% van maksimum gekombineer suiker opbrengs (GSO) verkry kon word.

Gelyktydige versuikering en fermentasie (GVF) is na voorafbehandeling op ongewaste, gepersde bagasse substraat by toestand vir die maksimum gekombineerde suikeropbrengs op bank-skaal uitgevoer. Bondel en voerbondel SSF voerstrategie by verskillende vaste ladinge en ensiemdoseringe is gebruik om 'n etanol konsentrasie van ten minste 40 g/L te bereik. Ná GVF was die algehele etanol opbrengs vir die voorkeurvariëteite (84.5–85.6%) beduidend beter relatief tot die industriële bagasse (74.8%). Die maksimum etanol opbrengs na SSF van die rasse met die beste prestasie was 48.6–51.3 g/L en 37.1–38.3 g/L vir rasse wat swak presteer het. Die etanol konsentrasie in die fermentasiesop was omgekeerd met lignien en die verhouding van xilose tot arabinose gekorreleer, maar was duidelik positief met die glukose opbrengs vanaf voorafbehandeling-hidrolise gekorreleer. 'n Algemene assessering het op 'n duidelike verbetering van die voorkeurvariëteite in terme van gekombineerde etanol opbrengs per hektaar gedui (71.1–90.7%), relatief tot die industriële suikerriet.

Die prestasie in terme van etanol opbrengs van geselekteerde variëteite is oor 'n reeks oesjare ge-evalueer. Die resultate het aansienlike variasies in etanol opbrengs oor oesjare getoon. Die resultate het gewys dat die beste variëteite in terme van gekombineerde etanol opbrengs nie volhou is oor oesjare nie. Die verskille in etanol opbrengste tussen variëteite was groter as die verskille oor oesjare. Verlengde ernstige droogte het die etanol opbrengs van alle variëteite met laer en intermediere lignien inhoud vir rietopbrengs aansienlik beïnvloed, in vergelyking met dié wat die hoogste lignien inhoud gehad het. Die koolhidraatinhoud in die bagasse en suiker opbrengs/lowering tussen die oesjare het vir die meeste variëteite egter nie gewissel nie.

Ter opsomming, die huidige studie verskaf bewyse van die impak van kultivarseleksie en voorbehandelings optimisering op die verhoging van die omskakelingsdoeltreffendheid van bagasse. Die resultate wys dat variëteite met laer lignien- en asinhoud, en hoogs-gesubstitueerde xilaan hoër suiker- en etanol opbrengs gelewer het. Hierdie resultate stel voor dat verminderde voorbehandelingsvereistes bereik kan word

sonder om die sap etanol en rietopbrengs per hektar te benadeel. Nieteenstaande, 'n poging om die lignien inhoud van die bagasse te verminder om die verwerkingsvereistes vir etanolproduksie te verminder, kan ook die verbetering van gewas-toleransie tov ernstige droogte-toestande teiken.

Dedication

This work is dedicated to my lovely daughter Jacqueline, who was born while I was away and stayed with the mother for the whole period of this study. It is also dedicated to my lovely wife Evaline, for moral, constant loving and time devotion to take care the family during my absence. It is also dedicated to our almighty God, for him everything is possible.

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List of Abbreviations

AIS	Acid insoluble lignin;
ANOVA	Analysis of variance
CCD	Central composite design;
CSY	Combined sugar yield;
DRM	Dry raw material
DSA	Dilute sulphuric acid
EH	Enzymatic hydrolysis,
FPU	Filter paper unit
HHV	High heat value
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
LAPs	Laboratory analytical procedures
NREL	National renewable energy laboratory
RM	Raw material
RSM	Response surface methodology
SASRI	South Africa sugarcane research institute;
SB	Sugarcane bagasse
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation;
WIS	Water insoluble solid
IU	International unit

Chapter 1

1. Introduction

1.1. Background

The excessive utilisation of fossil fuels, mostly in the transport sector, has accelerated the depletion of oil reserves in the world. Moreover, transport is one of the leading sectors responsible for the increasing carbon dioxide emissions into the atmosphere - the main cause of global warming due to the greenhouse effect [1]. Liquid biofuels, such as ethanol, produced by fermentation of monomeric sugars (mostly glucose) derived from sucrose (i.e. in sugarcane), starch (i.e. cereal grains from maize and wheat) or cellulose/hemicellulose (i.e. bagasse) are an interesting alternative in the short-term to displace a large part of the petrol usage in the transport sector whilst reducing greenhouse gasses (GHG) emissions [2]. It is well known that the feedstocks for first generation ethanol are generally expensive, compete with the food market [3] and of limited economic and environmental benefit [4,5]. On the other hand, lignocellulose biomasses such as grasses, wood or agricultural residues are a more sustainable feedstock as demonstrated by life cycle analysis [6–9]. However, because of the recalcitrant nature of lignocellulose its structure requires a more intricate technology so as to obtain its fermentable sugars through cleaving the polysaccharides (cellulose, hemicellulose) by enzymes. Different areas of research are being targeted to facilitate its development for industrial implementation. Some of these areas are discussed in the next subsections related to the aspects evaluated in this thesis.

1.1.1. Sugarcane and whole plant utilization

Sugarcane represents a preferred crop for bio-energy (ethanol) production in tropical and subtropical countries. It has high biomass yield per hectare and high sugar content that can be directly be fermented to ethanol [10]. The fibrous residue generated after juice extraction referred to as bagasse with high carbohydrates is currently burned inefficiently to generate steam and electricity to power mills. With the current technology in the development

of using bagasse for ethanol production, the solid residue (mostly lignin) left after fermentation of bagasse is sufficient to generate steam [11]. Therefore, the integrated approach of using juice and bagasse for ethanol represents a best scenario for energy and raw material allocation for sustainable development of biorefinery [12]. Integrated utilisation of the juice and bagasse will not only increase ethanol yield per unit land but will also increase revenue for the farmers. However, to obtain fermentable sugars from bagasse two processes of pretreatment and enzymatic hydrolysis are required. These two processes are still the limiting factors for the commercialisation of cellulosic ethanol [13,14].

1.1.2. Strategies to overcome lignocellulose recalcitrance

Engineering sugarcane for improving feedstock bioconversion is a prime strategy for reducing the processing costs. New varieties of sugarcane can be developed either by classical breeding or precision breeding (genetic engineering). The latter breeding technology has been shown to increase the sucrose content and bagasse with carbohydrates and low lignin content [15,16]. Jung et al. [17] has shown that it is possible to reduce lignin content in bagasse of transgenic sugarcane without adversely affecting the plant performance under controlled environment conditions. In this study [17] the *Caffeic acid O-methyltransferase (COMT)* activity on cell wall was down-regulated from 67 to 97%. The reduction of COMT gene, lowered lignin content by 3.9–13.7% compared to wild type (181.4 mg/g of raw material). The cellulose digestibility of untreated and dilute acid treated bagasse was improved up to 29% and up to 34%, respectively. The genetic engineering technology has also been reported in improving cell wall digestibility of other feedstock such as sorghum and maize [18–20]. Hence, selection of novel varieties with reduced recalcitrant without compromising agronomic properties will be a positive gain towards crop development that enables the use of the whole crop for ethanol production.

1.1.3. Challenges regarding selection of varieties with improved properties

Experiments assessing the quality of new varieties produce large numbers of samples. This means that development of methodology to identify the preferred varieties presents the biggest challenge. Furthermore, limited information is available on the chemical composition characterisation and the sugar yields after the pretreatment and enzymatic hydrolysis of the bagasse from large number of samples. This data is very important during screening of cultivars that are less recalcitrant to pretreatment-hydrolysis. In this way, we might be able to identify genetic traits that result in desirable physical-chemical properties that promote high fermentable sugar yields in a cost-effective manner, which could be engineered to develop new cultivars.

1.1.4. Shortcomings in 2G technology

1.1.4.1. Pretreatment

It has been proven that pretreatment is a crucial first step for efficient conversion of lignocellulose biomass to ethanol [13,21–23]. This step is responsible for unlocking the natural recalcitrance of lignocellulose. However, this process needs energy and chemicals to disrupt the building blocks of cell walls thereby improving enzymatic access to the polysaccharides (hemicellulose and cellulose). The pretreatment represents a limiting factor of the production process due to environmental and cost implications [1,13,14]. Sugarcane bagasse, like other lignocellulose materials must therefore be pretreated to enhance the rate and extent (yield) of enzymatic hydrolysis to fermentable sugars.

Several technologies for pretreatment of lignocellulose prior to enzymatic hydrolysis have been reported [13,21,22,24,25]. Among these, the dilute sulphuric acid pretreatment technique represents the most researched method for different types of feedstocks at different reactor setup [13,21,23,24]. The use of a well-established method for pretreatment of sugarcane bagasse will allow comparison of the existing data in the research database with the results obtained with new varieties in this study.

1.1.4.2. Optimum pretreatment conditions

For the dilute acid pretreatment technique, the biomass soaked in aqueous sulphuric acid is held at a particular temperature for specific periods of time, ranging from hours to seconds [26,27]. The almost complete hydrolysis of hemicellulose into the hydrolysate liquor is realised even at mild severity conditions, leaving the pretreated solid enriched in cellulose and lignin [27–29]. The pretreated solid can now be easily hydrolysed by enzymes into glucose during the enzymatic hydrolysis process. However, severe pretreatment conditions, in particular high temperatures (for example 200 °C), are required for higher cellulose conversion [30,31]. This requirement contributes to excess degradation of lignocellulosic sugars into chemicals compounds that are non-fermentable, thus reducing the amount of fermentable sugars [27]. The formed compounds are also inhibitive to the downstream process (enzymatic hydrolysis and fermentation) [32]. Severe conditions also mean lower pretreated solid recovery, and consequently, lower glucose yield is obtained. Furthermore, severe conditions can lead to high solubilisation and transformation of lignin into various phenolic compounds, which are inhibitive to enzymatic hydrolysis and fermentation [30]. The differences in the pretreatment requirements between the hemicellulose and cellulose necessitate the conditions to be optimised based on which sugar is in target (either for maximum hemicellulose-sugar recovery or the highest cellulose conversion) [33]. However, neither of these conditions can lead to maximum combined sugar yield (sum of all sugar released after pretreatment and enzymatic hydrolysis) [34]. To maximise combined sugar yields, other researchers have proposed a two-stage hydrolysis process [27,35]. The first step is conducted at low temperature to target hemicellulose-sugar recovery and the other is performed at high temperature for high cellulose conversion. However, an additional separation process is required before the second step, hence such suggestion is questionable with regard to economics and utilities (water and energy).

Another attractive approach is to find an optimum pretreatment condition that could maximise combined sugar yield by one stage hydrolysis. Lloyd and Wyman [34] have demonstrated that higher combined sugar yield from corn stover of up to 92.4% could be

obtained when the optimisation is based on combined sugar yield. By using their approach high fermentable sugars per feedstock could be obtained, thus eliminating the need for two stage pretreatment processes. However, in order to quantify the effects of pretreatment parameters (temperature, acid concentration and residence time) on the combined sugar yield, the experiments should be performed according to a statistical design such as central composite design (CCD), which permits simultaneous changes in the process variables. The pretreatment requirements of samples may differ from one variety to another depending on the chemical composition and structure properties [19]. Therefore, the effects of temperature, time and catalyst on the combined sugar yield from the varieties needs to be thoroughly investigated in order to establish the maximum effectiveness for each feedstock.

1.1.4.3. Ethanol production process configuration

After the pretreatment the pretreated solid is subjected to enzymatic hydrolysis to release glucose to be fermented to ethanol. Enzymatic hydrolysis and fermentation processes can be conducted either separately by the Separate Hydrolysis and Fermentation (SHF) process or together in the same vessel through the Simultaneous Saccharification and Fermentation (SSF) process. The main advantage of the SSF over the SHF configuration is that it can minimise the end-product inhibition of the enzymes through the continuous removal of the sugars [36]. This allows the use of higher solid loading for high ethanol concentration in the fermentation broth [37–39]. Ethanol concentration of at least 40 g/L is crucial for the economic viability of the distillation process [40]. Research shows that a solid loading higher than 15% WIS is required to obtain this benchmark concentration (40 g/L) [37,41]. However, solid loading greater than 15% (w/w) implies higher viscosity and mass transfer problems in the bioreactor. This can make stirring difficult, which results in low mass and heat transfer, lower sugar yield and thus, lower ethanol production [37,42]. In addition, high solid loading implies high inhibitors concentration, especially when using pressed (not washed) solids [11]. This problem can be alleviated/minimised when a fed-batch strategy is applied, employing gradual hydrolysis and the addition of the substrate [43,44]. Additionally, the mixing problem

can become less prominent by using feedstock of greater digestibility in addition to high carbohydrate content. Higher ethanol concentration can be obtained by low solid loading than that applied to the material with low digestibility. The variations of ethanol yields depending on cultivars have been reported in literature. Negative correlation between ethanol yield and lignin content has mostly been observed on corn stover samples [45] This kind of correlation has not been demonstrated on the sugarcane varieties.

1.1.5. Effect of harvest on ethanol yields

Various factors such as water, variety, diseases, soil, environmental conditions and agricultural practice can affect the agronomic properties and chemical structure of various crops such as sugarcane, sweet sorghum and maize [20,46,47]. However, water is the most important factor for the growth of sugarcane as a lack of water can reduce crop productivity up to 50% [48]. Hence, irrigation is commonly practiced in those areas with water deficiencies. Such necessity can be minimised by the development and selection of sugarcane varieties that are tolerant to drought. Furthermore, in recent years, South Africa has experienced unpredictable weather with low rainfall, which has affected sugarcane productivity [49]. Therefore the performance of selected varieties from different harvests needs to be evaluated.

1.2. General objective

Against that background, the overall goal of this study was therefore to contribute to the increasing knowledge of sugarcane crop development for bio-ethanol production in South Africa by developing a methodological approach to consider during selection of novel varieties of sugarcane, with improved potential for combined ethanol production from sugar juice and bagasse. Biochemical conversion processes which involve dilute acid pretreatment, enzymatic hydrolysis and fermentation were employed to determine the processability of bagasse from different varieties, and thus select preferred varieties on the basis of total ethanol yield (sugar juice plus bagasse) per hectare.

1.3. Thesis outline

The work described here is within the project “Engineering of sugarcane cultivars for lignocellulose hydrolysis and fermentation” at the Department of Process Engineering at Stellenbosch University, in collaboration with the South African Sugarcane Research Institute (SASRI), aimed at energycane development in South Africa. This study was the first attempt to collate research related to sugarcane development for combined ethanol production from juice and bagasse. The dissertation workflow is as follows. **Chapter 1** is the introduction consisting of background information, general objective and thesis outline. **Chapter 2** discusses the state of art of lignocellulose biomass, sugarcane and conversion processes of sugarcane bagasse (lignocellulose) to ethanol. **Chapter 3** describes how the objectives fit into the gaps identified during literature review. **Chapter 4** contains the results of varieties screening and selection. Pretreatment optimisation at gram scale of the selected variation is addressed in **Chapter 5**. **Chapter 6** provides the process integration (from pretreatment to fermentation) to demonstrate the importance of cultivar development and selection and pretreatment optimisation. Comparison of cultivars from the different harvests was presented in **Chapter 7**. **Chapter 8** provides the summary of main findings and **chapter 9** proveds the main conclusions and recommendations.

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Chapter 2

2. Literature Review

This chapter presents an overview of lignocellulose biomass, such as sugarcane bagasse, as feedstock for biochemical conversion to ethanol by means of pretreatment, enzymatic hydrolysis and fermentation. Firstly, it introduces lignocellulose material and its chemical composition and structure, which will impact on both the process requirements and yield. Secondly, the potential of sugarcane cultivar as a bioenergy crop in Southern Africa is evaluated. Lastly, the biochemical conversion processes (pretreatment, enzymatic hydrolysis and fermentation) of lignocellulose materials are reviewed.

2.1. Lignocellulose materials and their composition

2.1.1. Lignocellulose materials

Lignocellulose (fibrous) plant biomass is a complex biological material considered to be the most abundant of plant biomass available on the Earth, contributing to about 50% of world's biomass [1]. The sources of lignocellulose materials include: by-products and waste of forest and agriculture crops, municipal solid wastes, wood, fast growing trees and herbaceous biomass [2,3]. The energy content in biomass is directly associated with photosynthesis reaction whereby the energy from the sun enables the plant to utilise carbon dioxide and water to form carbohydrates (cellulose and hemicellulose) and lignin [4]. The formed carbohydrates and lignin are directly stored as building blocks of the cell wall, parallel with solar energy. Therefore, lignocellulose biomass can be transformed into various biofuels (solid, liquid and gas) [4]. The liquid fuel such as bio-ethanol, bio-butanol as well as gas fuel such as hydrogen can be used in the transportation sector [5], whereas biofuel in the form of solids can be used for power generation as well as for home use (for instance charcoal) [6].

2.1.2. Chemical composition of lignocellulose biomass

Lignocellulosic plant biomass is mainly composed of carbohydrates polymers (cellulose and hemicellulose) and lignin. It can also contain other minor components such as soluble sugars, extractives, minerals, ash and oil [2]. The actual chemical composition and structure of lignocellulose materials depends on several factors such variety, environmental conditions, geographic location, tissue, harvest period, agricultural practice, breeding technology, harvest season and maturity [7–9]. Table 2-1 shows the variations in chemical composition of different lignocellulose materials classified as hardwood, softwood, herbaceous plants and agricultural residues. In general, hardwoods and softwoods contain higher cellulose than that of herbaceous plants or agricultural residues. Softwoods are also characterised by higher lignin content compared to the content of hardwoods. Softwoods also have lower acetyl groups (part of hemicellulose) than other lignocelluloses.

Table 2-1: Chemical composition of different lignocellulose materials (% dry weight)

Substrate	Cellulose	Hemicellulose	Lignin	Reference
¹ Straws	32–47	20–30	5–24	[10]
¹ Sugarcane bagasse	35–45	23–35	16–24	[11–14]
² Grass	24–50	12–38	6–29	[15–18]
³ Pine and firs	41–50	11–33	19–30	[19–21]
⁴ Aspen and eucalyptus	39–53	19–36	17–24	[20–23]

¹Agricultural residues, ²herbaceous plants, ³softwood, ⁴hardwood

Cellulose, hemicellulose and lignin occur very closely and are linked to each other by covalent bonds, thereby making lignocellulose structure very recalcitrant to biological degradation and conversion (see Figure 2-1) [24]. Cellulose forms a skeleton surrounded by hemicellulose and both are protected by a lignin sheath [25]. The most important properties of these components (cellulose, hemicellulose and lignin) and their organisation in lignocellulose are briefly discussed in the next section.



Figure 2-1: Organization of lignocellulosic structure [24]

2.1.2.1. Cellulose

Cellulose is the largest homopolysaccharide occurring naturally as it composed of cellobiose units. The cellobiose units are linked in a linear fashion through β -(1-4) glycosidic bonds (see Figure 2-2) to form a cellulose molecule. The degree of polymerization can range from 500 to 15,000 glucose units depending on the type of the lignocellulose [25]. These cellulose molecules are joined through numerous hydrogen bonds (OH groups) generating the cellulose fibres. The hydroxyl group forms the functional group, with three hydroxyl groups in each glucose unit [26]. This causes a cellulose unit to have great numbers of the hydroxyl groups. The hydroxyl groups interact with one another within the same molecule or with other hydroxyl groups of neighbouring glucose units to form strong intra-molecular and inter-molecular hydrogen bonding, which agglomerate to create a cellulose unit (fibril structure) [27]. The hydrogen bonds also exist between hydroxyl groups of cellulose units and water molecules, which makes cellulose more hydrophobic and insoluble in water [28]. Cellulose chains are also stabilised by the presence of hydrogen bonding in the chain direction and thus form highly crystalline micro-fibril structures [25]. These micro-fibril cellulose structures are held together by strong hydrogen bonds. Cellulose structures are therefore robust and highly resistant to solvent and degrading agents such as enzymes.

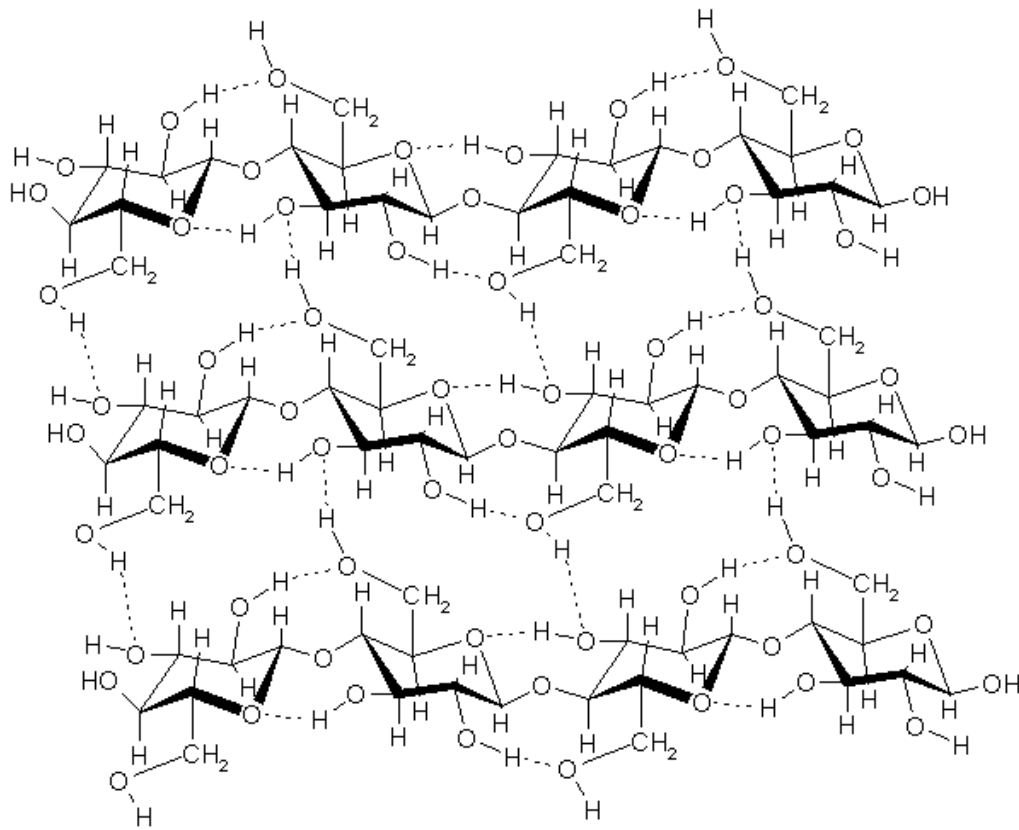


Figure 2-2: Simplified chemical structure of cellulose [25].

Although cellulose is in crystalline form, the micro-fibrils have some areas which are amorphous [29]. In these areas, the cellulose unit loses its linearity to acquire disorganised fashion (Figure 2-3). This area is considered to be the weakest part of cellulose and it can be easily attacked by catalysts such as enzymes and acids.

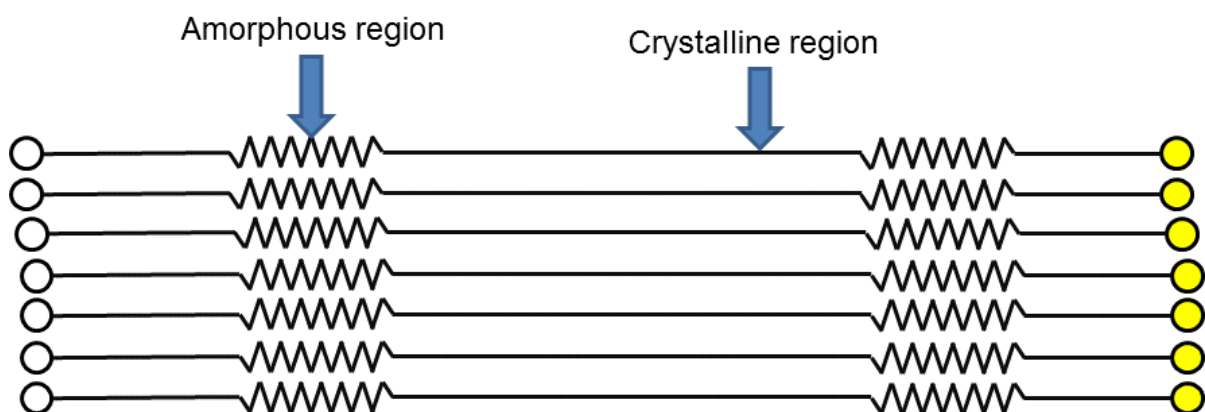


Figure 2-3: Schematic diagram illustrating a cross view of the cellulose units, showing crystalline and amorphous regions.

It is broadly accepted that highly crystalline cellulose is less accessible to cellulase hydrolysis than amorphous cellulose [30,31]; therefore crystallinity affects the efficiency of

enzyme contact with cellulose. Negative correlations between crystallinity index and hydrolysis rates have been observed in various feedstocks such as sugarcane bagasse [32] and sorghum bagasse [33,34]. A negative correlation between crystallinity and digestibility has been reported [35,36]. However, other researchers have proposed that the effect of reduced crystallinity on hydrolysis rates might be a consequence of simultaneous decreases in particle size [37,38] or an increase in surface area during pretreatment [35,36]. As a consequence it is difficult to analyse one factor separately.

2.1.2.2. Hemicellulose

Hemicellulose is the second most abundant polysaccharide in lignocellulose after cellulose. It is bound to cellulose and lignin in the plant cell wall. Unlike cellulose, hemicellulose polysaccharides are heterogeneous, highly branched and very amorphous with a maximum degree of polymerization of less than 200 units [7]. These characteristics make hemicellulose easily hydrolysed by acids to monomers of pentose (xylose and arabinose), hexoses (mannose, glucose and galactose) and small amounts of sugar acids (glucuronic and galacturonic acid) and organic acids (acetic acid) [20]. Hemicelluloses are classified according to the main sugar that composes the backbone [39]. For example, xylan is the predominant constituent of hemicelluloses in hardwoods and annual plants (agricultural residues and herbaceous plants), whereas glucomannan represents the largest fraction of hemicellulose in softwoods [40]. However, hardwood xylan differs to that isolated from annual plants xylan. Xylan isolated from hardwood mostly consists of glucuroxylan [41]. It is highly acetylated heteroxylan with 4-O-methylglucuronic acid groups linked through (1-2)-glycosidic bonds [41]. In contrast, xylans found in straws and grasses mostly consist of xylose units as a backbone linked through β -(1-4) bonds, heavily branched with arabinofuranose, xylopyranose, galactopyranose and trisaccharides units as side groups [42]. Generally, the xylans of straws and grasses are mostly dominated by polysaccharides of xylose and arabinose simply called arabinoxylan. Sugarcane bagasse hemicellulose is typically similar to that found in grasses [43]. It is characterised by a xylan backbone with substitution of arabinose, glucose and acetyl

groups and traces of mannose and galactose [43]. It may also contain traces of mannose and galactose [44]. Figure 2-4 shows the structure of sugarcane bagasse hemicellulose.

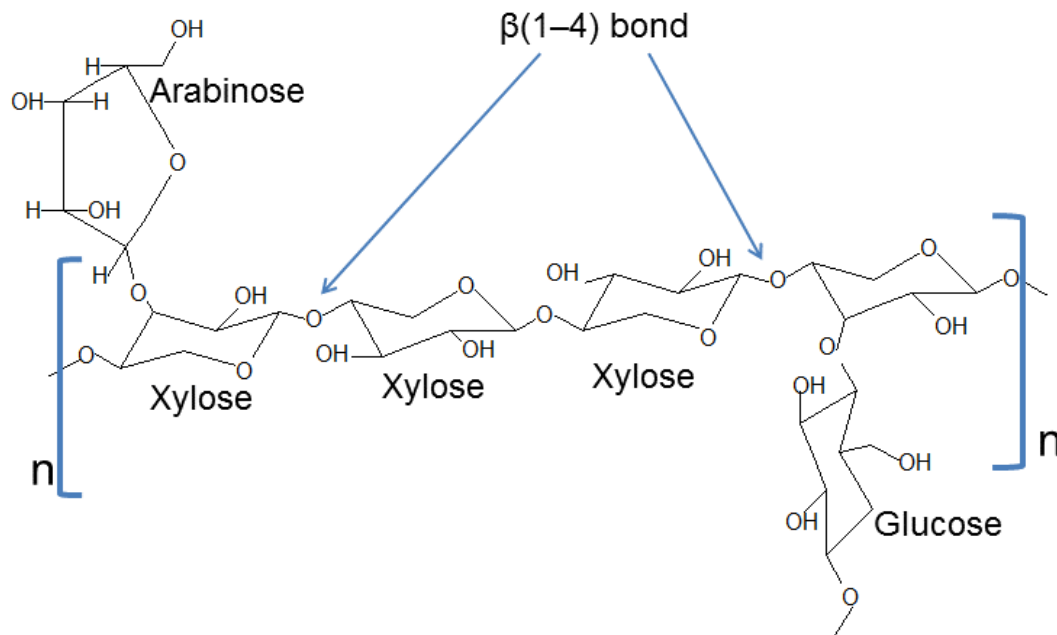


Figure 2-4: Simplified hemicellulose structure [44].

Both hemicellulose and lignin coat cellulose microfibrils in the plant cell walls, forming a physical barrier to hydrolytic enzymes, and removal of hemicellulose by dilute acid pretreatment has been reported to increase the enzymatic hydrolysis of cellulose [45]. The removal of hemicellulose has also been reported to increase surface area and pore area, which further improves cellulose accessibility [46]. It has also been reported higher enzymatic hydrolysis yield is obtained on biomass containing highly substituted xylan [47]. On the other hand, the presence of acetyl group adds steric hindrance to the cellulase activity due to the interference with enzyme recognition, thereby slowing the hydrolysis rates [48]. Hence, its removal during pretreatment has been shown to reduce the steric hindrance of enzymes and greatly enhanced cellulose digestibility of the pretreated solids [48–51].

2.1.2.3. Lignin

Lignin is a highly branched polyphenolic compound found in the plant cell wall, bounded within the spaces of cellulose and hemicellulose. It is formed by three radicals of coniferyl

alcohol, p-coumaryl alcohol and sinapyl alcohol as depicted in Figure 2-5 [52]. These units are joined together by various carbon-carbon and carbon-ether bonds, which makes the degree of polymerization vary between 450 and 500 units [53]. Some of these bonds are difficult to break, thereby limiting lignin microbial biodegradation [54]. However, most of lignin constituents are delignified by alkaline, except for the guaiacyl lignin, which tends to restrict fibre swelling [24]. Furthermore, lignin forms covalent bonds with hemicellulose thereby providing rigidity to the cell wall [39]. The lignin-polysaccharides linkages together with lignin structure complexity make it difficult for the complete isolation of lignin. As such, a complete understanding of lignin structure is still unknown [55].

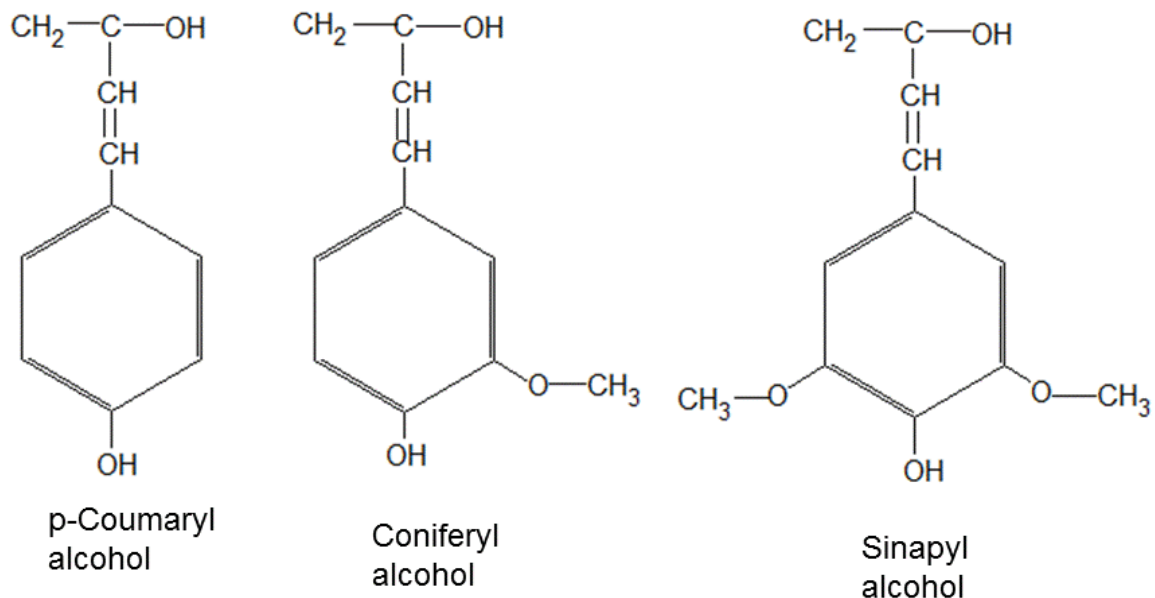


Figure 2-5: The chemical blocks of lignin [52]

It is generally accepted that lignin content largely contributes to the recalcitrant nature of lignocellulose. Lignin can limit cellulose digestibility in various ways such as preventing swelling and steric hindrance to cellulolytic enzymes. Limiting fibre swelling can prevent increase in accessible surface area [56] thereby preventing effective binding of enzymes into the biomass [57]. As such, cellulose digestibility is improved with increasing lignin removal [32,58]. Various models correlate the rate of hydrolysis to the extent of delignification. These correlations generally hold true up to the point where 50% of the original lignin has been removed [50,59]. However, some correlations have been extended to 90% delignification [48].

It is therefore clear that lignin is one of the important parameters limiting the accessibility of the lignocellulose biomass.

2.1.2.4. Summarized: Effect structures features on lignocellulose bioconversion

In general, the structural features of the lignocellulose biomass affecting the enzymatic hydrolysis can be categorised into physical and chemical features, as summarised in Table 2-2 [60]. Physical structural features include cellulose crystallinity, degree of cellulose polymerization, pore volume, accessible surface area, and particle size. Chemical structural features include the contents of lignin, hemicellulose, and acetyl groups. Although these structural features are divided into two groups, interactions exist among them. For example, lignin removal changes the percentage of cellulose and hemicellulose, pore volume, and accessible surface area.

Table 2-2: Summary of relationship between structural features and digestibility [61].

Structure features		Relationship with digestibility
Physical	Surface area	Positive
	Crystallinity	Negative/No correlation
	Degree of polymerization	Negative/No correlation
	Pore volume	Positive
	Particle size	Negative/No correlation
Chemical	Lignin	Negative
	Hemicellulose	Negative
	Acetyl group	Negative

Based on the impact of chemical and structural properties described above on processing requirements, research efforts are directed towards the development and/or selection of feedstocks with desirable properties, making lignocellulose more amenable to biological conversion through pretreatment-hydrolysis-fermentation.

2.2. Sugarcane as an energy crop

Selection of bioenergy crop depends on many factors, some of which are economical and others are related to feedstock properties. The economic factors such as land use constraints [62] and the impact of the energy crop on the edible crops [63] can be addressed by public policy. On the other hand, the factors related to feedstock quality are addressed through plant development (classical and genetic engineering), as discussed in section 2.3.3.

2.2.1. Sugarcane

Sugarcane (*Saccharum spp hybrids*) represents one of the major crops planted in tropical and subtropical countries. Its main distinguishing features include high biomass yield, high sucrose content [64], high efficiency in assimilating solar energy [65], high water use efficiency in terms of litres per kilogram of aerial biomass comparable to other categories of crops such as sugar beets [65] and higher energy balance than other types of feedstock as shown in Table 2-3 [66]. The estimated annual production is about 1.68 billion wet tons of sugarcane worldwide [65,67]. Brazil is the largest producer, contributing 33% of the world's production [65].

Table 2-3: Energy balance of ethanol production from different feedstocks [66].

Feedstock	Energy output/input ratio
Sugarcane	8.2–10.2
Sugar beet	1.6–2.3
Wheat straw	0.7–2.4
Maize	0–1.6
Wood	0.4–2.2

Sugarcane is characterised by segmented stem (stalk) and leaf blades. The leaf blades form approximately 24% of the whole plant and the remaining percentage is the stalk [68,69]. During the harvesting of sugarcane, leaf blades (leaves, tops and trash) are left in the field. These harvest residues (leaves, tops and trash) are burned in open air, thus generating

particulate emissions, which are reported to cause respiratory diseases [70]. Whereas the sugarcane stalks are transported to the mills, and crushed to extract juice for sugar or ethanol production [71]. For every 1 ton of stalk that is processed, approximately 260 kg of fibres (bagasse) are generated [68,69]. Bagasse is then inefficiently burned in the sugar mill to generate steam and electricity to power the factory. With the improvement in efficiency of the turbines, significant amount of bagasse will be available for other applications, such as ethanol production.

The harvest residues and bagasse can be converted efficiently to fuel ethanol in the “whole plant” conversion approach for an increase in ethanol output per unit land. This approach will not only reduce air pollution but also will add revenue for the farmers. However, bagasse and harvest residues differ in physical nature and process requirements [68,69]. These differences in process requirements suggest that separation of the two fractions is important during processing for optimal ethanol production. The present study is focused on the bagasse fraction because it can be easily integrated into the current processing plant [23].

2.2.2. South Africa: Sugarcane production trends

Sugarcane is recognized by the South African government as a bio-ethanol crop. However, it has been faced a major decline in sugarcane production since 1999 (Figure 6) [73]. This decline in productivity is a consequence of decrease in area under the cane assigned for sugarcane production (Figure 2-6). The decrease of area under the cane is attributed to the decrease of profit margin due to the increased rise in cost of production, unpredictable weather conditions experienced in recent years and land reform issues [74].

Moreover, sugarcane is the official bioenergy crop in Southern Africa [75]. The history of using this crop for bioethanol production in Southern Africa can be dated back in 1980, with the most successful plants developed in Malawi, Zimbabwe and Kenya [76]. The plants from these countries were able to produce up to 88 million litres of ethanol annually from sugarcane molasses [76]. However, most of these plants have closed production due to constraints such

as financial crisis, lack of raw material due to severe drought and land issues [76]. However, there are currently many opportunities to expand sugarcane-ethanol in Southern Africa. For example, the recent analysis study conducted by Watson [77] using the global information system (GIS), has confirmed that there are 6 million hectares of new land suitable for sugarcane plantation in Southern Africa without negatively affecting biodiversity, ecology or food production. This land can potentially produce up to 60 billion litres of ethanol. The estimation is based on 10000 litres of ethanol per hectare per year when juice and bagasse are used for ethanol production [78].

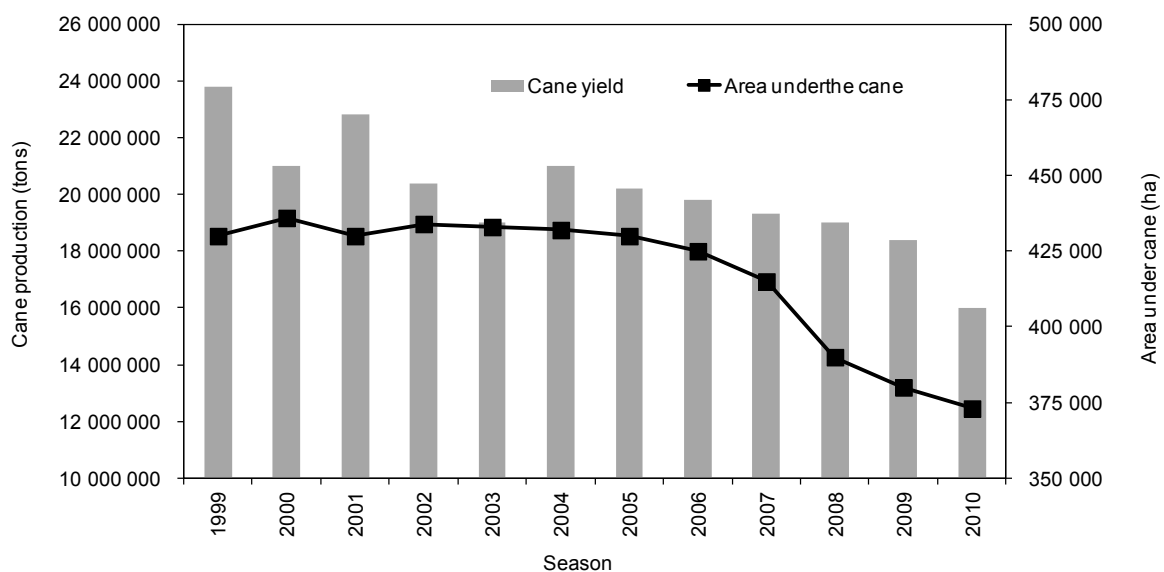


Figure 2-6: South African sugarcane production and the area under cane since 1999 (redrawn from Singels et al. [79])

2.2.3. Sugarcane cultivars development and selection

The prime target of crop development for lignocellulose conversion to ethanol has been the modification of the cell wall to reduce lignin content, thus increasing enzymatic hydrolysis. The evaluation of transgenic forage sorghum with reduced lignin content showed that the sugar conversion efficiency and ethanol yield were increased as the lignin content was reduced [80]. In this study, double brown midrib (bmr) was used to down-regulate the gene codifying for cinnamylalcohol dehydrogenase and O-methyl-transferase that are enzymes apparently specific for 5-hydroxyconiferyl aldehyde, a lignin precursor, which resulted in a

complete removal of active enzymes in tissues. Loss of enzyme activity resulted in an increase in soluble phenolic and lowered p-coumaric and ferulic acid as well as lignin content into the cell wall, of between 13% and 15%, which in turn increased the glucose yield up to 34% after enzymatic hydrolysis of dilute acid pretreated solid. The ethanol yield was also increased up to 43% compared with the wild type. Similarly, the evaluation of other type transgenic lines such as switchgrass [81], wheat [82] and maize [83], in which the COMT (*Caffeic acid 3-O-methyltransferase*) gene was down-regulated, leading to a reduction in lignin content and increase in fermentable sugar yield after pretreatment and enzymatic saccharification. A recent study, Jung et al. [84] has shown that it is possible to reduce lignin content in the cell wall of transgenic sugarcane without adversely affecting the plant performance under controlled environment conditions. In this transgenic plant the COMT gene in the cell wall was down-regulated to almost no expression. The reduction of the COMT gene expression lowered lignin content by 3.9–13.7% compared to wild type (181.4 mg/g dry raw material). The cellulose digestibility of untreated bagasse was improved up to 29%. After the dilute acid pretreatment, the glucose yield was also increased up to 34%.

Moreover, previous studies on other annual crops including maize and wheat have also proven that the breeding programs are able to improve the lignocellulose digestibility without adversely affecting for the yield and agronomic traits [9,85]. These findings are also relevant during breeding and selection of sugarcane for biorefinery.

In the present study, two approaches of breeding, namely classical and precision breeding (genetic engineering) will be used to produce new varieties of sugarcane. The classical breeding will use a traditional technology of cross breeding to produce new varieties targeting to increase biomass yield per hectare. The precision breeding will target to produce varieties of increased sucrose content by down-regulating expression of an endogenous enzyme UDP glucose dehydrogenase (Figure 2-7) as described elsewhere [86].

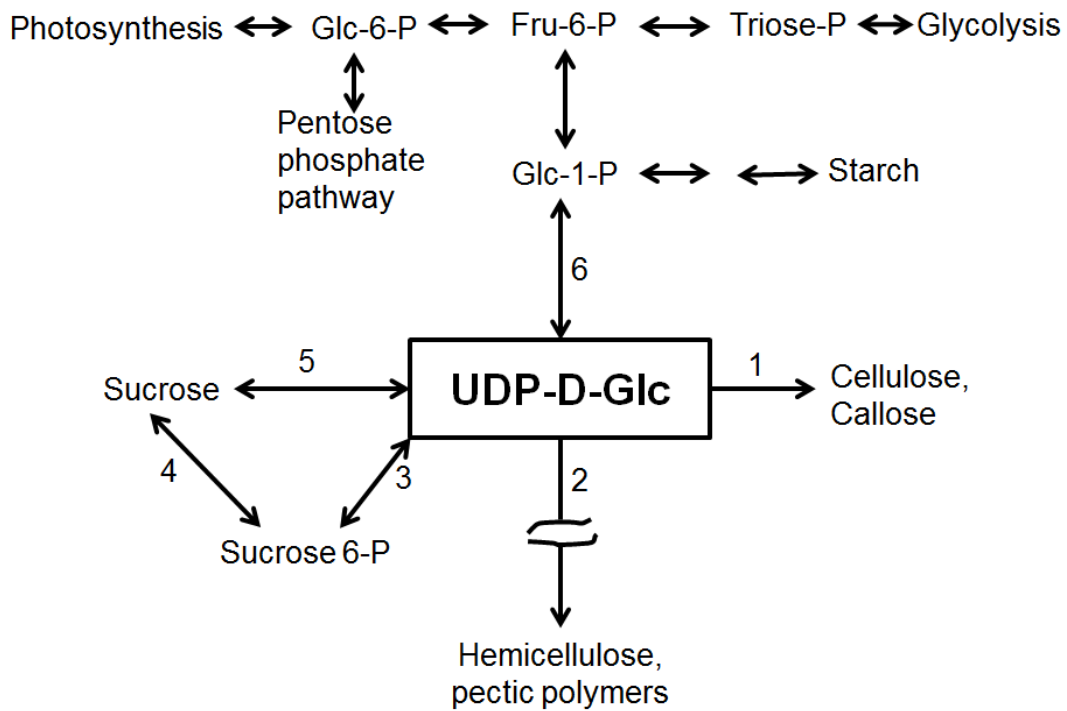


Figure 2-7: Central position UDP-glucose in plant carbohydrate metabolism. 1- Cellulose/callose synthesis, 2-UDP-Glucose dehydrogenase, 3-Sucrose phosphate synthesis, 4-Sucrose phosphatase, 5-Sucrose synthase, 6-Glucose pyrophosphorylase (adopted from Bekker [86]).

2.3. Sugarcane bagasse conversion to ethanol

Converting sugarcane bagasse to ethanol involves three major steps [23]. The first is the pretreatment, which is responsible for the disruption of the structure matrix to increase accessibility by the enzymes. The second step involves the conversion of cellulose and hemicellulose into monomeric sugars through an enzymatic hydrolysis process. The last step is the fermentation of monomeric sugars to ethanol using yeast. After fermentation the ethanol produced can be purified to meet fuel standards. The residual structural carbohydrates and lignin after enzymatic hydrolysis and fermentation are used as boiler fuel for electricity or steam production.

2.3.1. Pretreatment

Pretreatment refers to the process that alters the natural recalcitrance of lignocellulose material, to make material more readily susceptible to enzymatic hydrolysis [87]. During this

process hemicellulose, lignin or both are removed and/or modified, depending on the pretreatment technology applied, thereby exposing cellulose for enzymatic hydrolysis. The removal of either hemicellulose or lignin can drastically increase the cellulose digestibility [60]. Figure 2-8 illustrates how the pretreatment alters the structure of lignocellulose material [24]. Various pretreatment techniques can be employed in the pretreatment of sugarcane bagasse [24,60,61,88]. Nevertheless, pretreatment techniques should meet the following criteria to be economically feasible [61]: (1) to improve the combined sugar yield (refers to the sum of all sugars released after pretreatment and enzymatic hydrolysis) of pretreated samples compared to untreated material; (2) to avoid sugar degradation; (3) to avoid excessive formation of by-products, which are inhibitory to the downstream processes of enzymatic hydrolysis and fermentation; (4) to minimise energy requirement and (5) to reduce cellulose crystallinity. Dilute sulphuric acid represents one of the few pretreatment methods that satisfy most of these requirements [61], and therefore was selected as the pretreatment method to be investigated in this study. Application of the dilute acid pretreatment will also allow comparison of the performance of the bagasse from new sugarcane cultivars to an extensive literature database on lignocellulose pretreatment with this method. The purpose of this section is to review the dilute acid pretreatment method. Other important leading pretreatment techniques such as steam explosion and liquid hot water/auto-hydrolysis and are also briefly reviewed because they also target hemicellulose solubilisation, similar to dilute acid pretreatment.

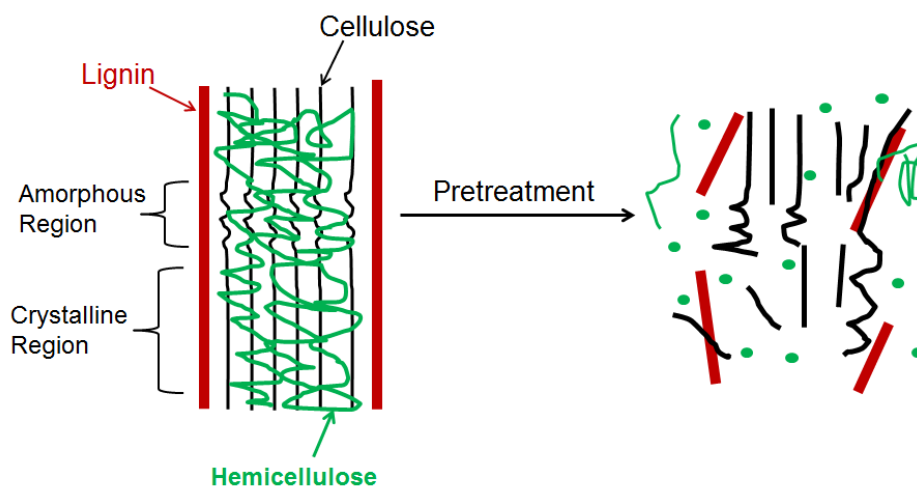


Figure 2-8: Impact of pretreatment on lignocellulose structure [24]

2.3.1.1. Dilute acid pretreatment

Dilute acid pretreatment represents the most widely researched technology on different types of feedstocks ranging from agricultural to woody residues, and is considered to be one of the methods with great potential for commercial application [24,60,61,88]. During this technique, biomass pre-soaked in acid solution is subjected to high temperatures from a few seconds to minutes. During the pretreatment most of hemicellulose is hydrolysed into the liquid fraction (hydrolysate or pretreatment liquor), thus leaving the solid material (pretreated material) enriched in cellulose and lignin [89]. The removal of hemicellulose makes the material porous and weakens the carbohydrate-lignin matrix structure, which increases the accessible surface area and therefore cellulose digestibility [60,61]. In addition, the application of high temperature can also assist in weakening the lignocellulose structure, which further increases digestibility of the substrate [90]. The acids that have been investigated are sulphuric, hydrochloric, nitric and phosphoric, formic hydrochloric, acetic organic acids, and phosphoric acid [91]. Of these, sulphuric acid is the most common as it is cheaper and more effective than others [91].

Dilute acid pretreatment of lignocellulose material such as bagasse is commonly conducted in batch processes. Through this process the xylan (main component of sugarcane bagasse hemicellulose) is hydrolysed into oligomers and xylose but, depending on the severity of pretreatment, might also undergo some level of degradation to by-products (downstream inhibitors) [92]. Most of the cellulose remains in the pretreated solids, but is more amenable to enzymatic hydrolysis for effective release of glucose, although a minor amount of glucose degradation may also occur. During pretreatment, lignin is partially solubilised and degraded, leading to inhibitory phenolics in the pretreatment liquor.

The optimisation of pretreatment conditions can be aimed at maximising either xylose recovery or cellulose digestibility [92–94]. However, the conditions for the maximum digestibility require severe conditions, which often result in a large amount of xylose degradation. Conversely, maximising xylose recovery often requires a much lower severity of pretreatment than that required to achieve acceptable cellulose digestibility. This means that

the single batch process cannot be employed to maximise both xylose and glucose from pretreatment hydrolysis, due to differences in pretreatment process requirements [20]. Other researchers have proposed a two-step process to minimise sugar degradation [20,95]. The first step is performed at low temperature/severity to maximise xylose recovery. Then the whole pretreated material, also referred to as slurry, is separated into solid and liquid streams. The second step is conducted on the solids at a higher temperature/severity to obtain a solid substrate with high digestibility. However, this kind of process adds additional costs for the separation of streams and the two stages of pretreatment, also resulting in higher energy demand. Thus, a compromised yield between xylose and glucose is proposed under a single batch process, while minimising sugar degradation. Such a single step pretreatment is aimed at maximising the combined sugar yield, the sum of xylose and glucose released during pretreatment and subsequent enzymatic hydrolysis. This approach, as applied in this study, assumes that both C5 and C6 sugars can be fermented efficiently, which is possible with commercial yeasts available today [96,97].

Maximising xylose recovery

Maximum xylose recovery depends on temperature, reaction time and acid concentration. Aguilar et al. [98] showed how maximum xylose production from sugarcane bagasse can be obtained by variation of pretreatment conditions. In this study, the temperature was varied from 100 to 128 °C and residence time between 0 and 300 minutes. Acid loadings were 2%, 4% and 6% (w/w), whereas solid loading was kept constant at 10% (w/v). Time series of xylose yield at different temperature and acid loading is depicted in Figure 2-9. Higher xylose yields were obtained at a temperature of 122 °C. The maximum xylose concentration in the pretreatment liquor was 21.6 g/L, equivalent to 92% of the xylose in the raw material, observed after 20 minutes of hydrolysis at 122 °C using 2% H₂SO₄. At a higher temperature (128 °C), the rates of xylose formation and degradation were faster compared with a low temperature (100 °C).

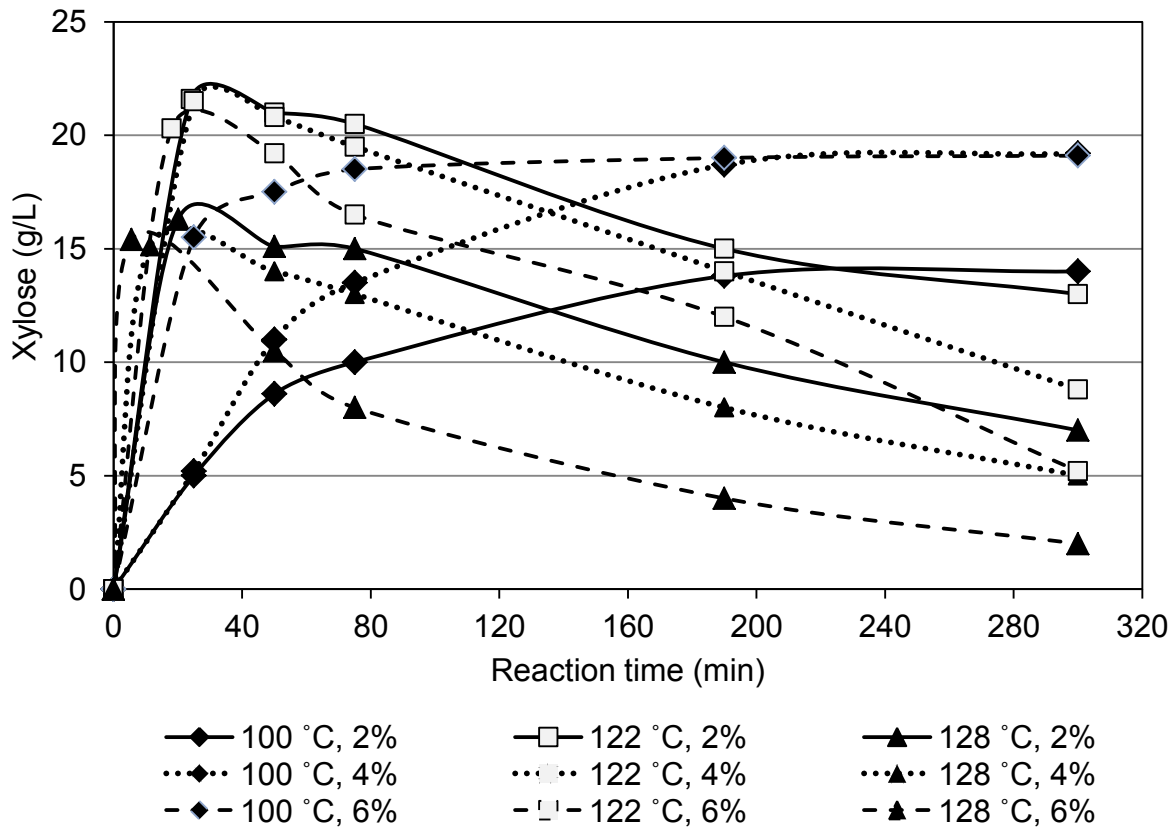
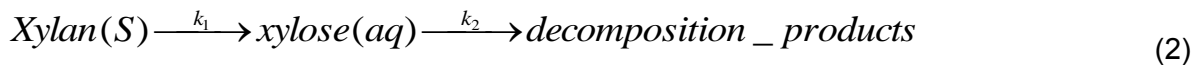


Figure 2-9: Experimental xylose production at different temperature, acid concentration and reaction time (redrawn based on the experimental data obtained from Aguilar et al. [98]).

Different models have been postulated to explain the kinetics of the acid-catalysed hydrolysis of sugarcane bagasse [98–100]. Among these, the model developed by Lavarack et al. [100], covered a wide range of pretreatment conditions of temperature (80–240 °C), acid concentration (0.25–8.00%), and reaction time (10–4000 min). In this model, xylan is first hydrolysed to xylo-oligosaccharides (equation 1). Xylo-oligosaccharides can further be degraded to xylose in presence of acid catalyst [24]. Furthermore, xylose can be further hydrolysed to furfural and other degradation products. The rates constants (k_1 and k_2) shown in equation 2 are temperature dependent as they follow the Arrhenius equation [100]. However, at high temperatures, the rate of xylose degradation to furfural (k_2) is always higher than the rate of xylose formation (k_1). This means that to enhance xylose recovery and to avoid xylose degradation, low temperature is required. However, low temperatures imply low cellulose digestibility as discussed in the next section. Similarly higher acid concentration (for example 8%) and longer residence time (for example 60 min) are required during

pretreatment. Due to these shortcomings, the intermediate temperature (150–200°C) and low acid concentration (below 1%) and shorter residence time (1–20 min) were selected in study, with the purpose of maximising the combined sugar yield from both pretreatment and subsequent enzymatic hydrolysis.



Maximising cellulose digestibility

Previous studies have shown that more severe pretreatment conditions are required for maximum cellulose digestibility, compared to those for maximum xylose recovery [93,101]. According to Castro et al. [102] the pretreatment of rapeseed straw at 144°C, 2% (w/v) sulphuric acid for 6 min was the optimal for the highest xylose recovery (92.3% of theoretical), but this condition did not give the highest cellulose digestibility. The maximum cellulose digestibility of 100% was obtained at 200°C, 0.4% (w/v) H₂SO₄ for 27 min. High temperatures have been reported to improve cellulose digestibility of grass (Figure 2-10). This is because high temperature can cause agglomeration of lignin [103], consequently increases cellulose digestibility. Nonetheless, high temperature can also cause lignin recondensation and reduce cellulose digestibility thereof [104]. Furthermore, although higher pretreatment severity has shown to improve cellulose digestibility, it can also lead to lower glucose recovery from the pretreated material due to lower solid recovery. Therefore, optimisation process should be based on the combined glucose yield/recovery from pretreatment and subsequent hydrolysis, rather than just the digestibility of cellulose in the residual solids after pretreatment.

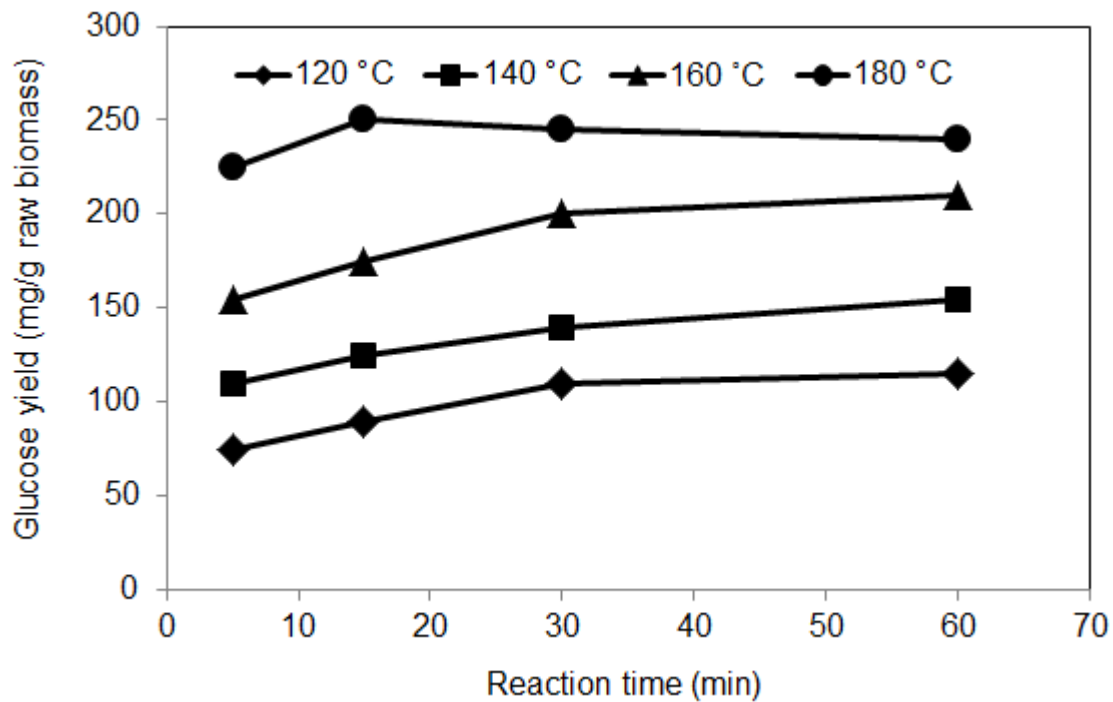


Figure 2-10: Glucose yield after enzymatic hydrolysis of coastal Bermuda grass as a function of reaction time at different temperature and acid concentration was kept constant at 0.3% w/w (redrawn based on the experimental data obtained from Redding et al. [90]).

Maximising combined sugar yield

Dilute acid pretreatment optimisation according to combined sugar yield has been observed as the promising alternative strategy for maximising fermentable sugar production from lignocellulose. Lloyd and Wyman [105] evaluated the effect of dilute sulphuric acid pretreatment on sugar yields from corn stover, and showed that the best condition for maximum xylose yield did not lead to maximum combined sugar yield (Figure 2-11). Similarly, conditions providing the maximum glucose yield from enzymatic hydrolysis did not coincide with conditions giving the highest combined sugar yield, due to xylose degradation and lower solid recovery (Figure 2-11). The condition that provided the maximum sugar yield was therefore a compromise between that for maximum xylose recovery and that for maximum cellulose digestibility. The pretreatment optimisation method aimed at combined sugar yield was selected on this study. The goal is to maximise pentoses and hexoses recovery from pretreatment-hydrolysis, while minimizing inhibitors formation. This will allow the use of the

whole pretreated material (hexoses and pentoses) by improved microorganisms (with both capacities of co-fermentation and coping with the toxicity of the slurry). However, in order to study the effect of temperature, acid concentration and time on the combined sugar yield, the experiments should be performed according to central composite design (CCD). The CCD allows for a better optimisation with reduced number of experiments and it allows determining interactions between the variables evaluated in the responses studied.

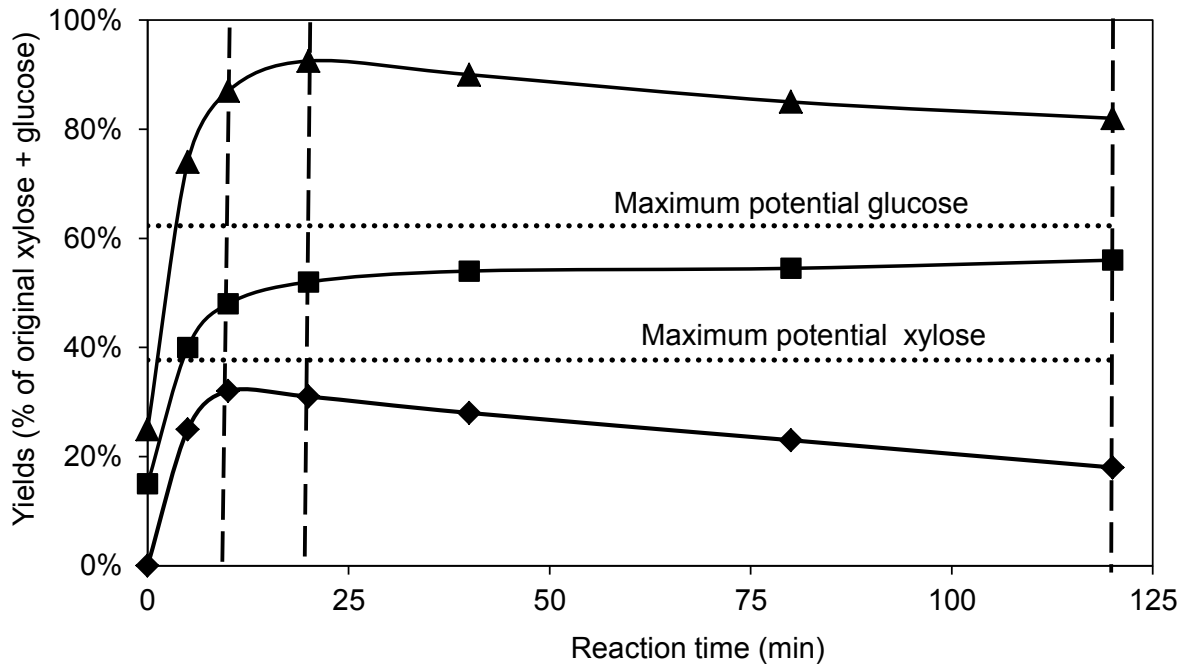


Figure 2-11: Sugar yields of corn stover as the function of reaction time for dilute acid pretreatment performed at 160 °C and 0.49% H₂SO₄ and enzymatic hydrolysis run for 72h with enzyme loading of 60 FPU/g of original glucan before pretreatment (adopted from Lloyd and Wyman [105]. Symbols key: (♦) xylose yield after pretreatment; (■) glucose yield after enzymatic hydrolysis; and (▲) combined sugar yield, the sum of all glucose and xylose obtained after pretreatment and enzymatic hydrolysis. Dashed line shows the maximum yields of xylose, glucose and combined sugar.

Regardless of the numerous studies that have reported on the optimisation of combined sugar yield from feedstocks, to the best of our knowledge, there is not such a study on bagasse from different varieties. The pretreatment requirements differ from one variety to the other depending on the physical-chemical properties [47].

2.3.1.2. Liquid hot water/Auto-hydrolysis

Liquid hot water pretreatment is similar to dilute acid pretreatment as both of them aim to hydrolyse hemicellulose to enhance the cellulose digestibility. However, no catalyst is used in liquid hot water pretreatment. In this process high pressure is applied to maintain water in a liquid state at elevated temperature (for example 120–220 °C), so as to penetrate into the lignocellulose structure [106–108]. The infiltration of hot water into the fibres causes significant solubilisation of xylan, cleavage of acetyl group to acetic acid (depending on feedstock) and partial hydrolysis of lignin [109]. The organic acids generated act as a catalyst to accelerate the “auto-hydrolysis” reactions during such pretreatment [29]. This method has also been employed on different lignocellulose materials such corn [109], sugarcane bagasse [110,111], wheat straw [101], oil palm fronds [112], Aspen chips [113] and *Eucalyptus grandis* [114] and has shown to solubilize up to 100% of the hemicellulose in these lignocelluloses. The main advantages of liquid hot water pretreatment is that there is no need of addition of chemicals, consequently, no need of corrosive resistance equipment for hydrolysis reaction. Furthermore, the pretreated material generated by liquid hot water pretreatment requires less chemicals for pH adjustment and removal of inhibitors, compared to other pretreatment techniques such as dilute acid. However, the main disadvantages of the liquid hot water are high utilities demand (water and energy) and is not developed up to the commercial level [60]. Another important drawback is low level of solid loading applied during pretreatment (1% to 8%) [115], which result in low concentration of sugars (0.6–5.8 g/L) from hemicellulose [24], consequently, evaporation step is required to concentrate the hydrolysate before fermentation. Furthermore, most of sugars hydrolysed during pretreatment remain in oligomeric form, therefore, cannot directly be fermented. One step of post hydrolysis is required prior to fermentation, to convert oligomeric sugars into fermentable monomeric sugars, which could possibly increase the production cost.

2.3.1.3. Steam explosion

Steam explosion is one of the most cost effective pretreatment methods, well documented and it has been demonstrated to be effective on several lignocellulose materials ranging from forest [116–118] to agricultural residues [119–122]. This method has been applied at pilot-scale and commercial scale [123,124]. Steam explosion is similar to dilute acid pretreatment due to the fact that both of these techniques use acid catalyst to enhance sugars conversion efficiency through the hydrolysis of hemicellulose. However, steam explosion employs direct saturated steam injection at a temperature between 160–260 °C corresponding to a pressure of 6.2–46.9 bar to the biomass for a specific period of time. During this period biomass undergoes auto-hydrolysis through release of acetyl groups and other organic acids within the biomass to solubilize the hemicellulose. After the holding time is completed the material is decompressed through a sudden pressure release, thereby discharging the material from the steam reactor into a holding vessel for rapid cooling [60,104]. The sudden drop in pressure results in evaporation of the water that is in the lignocellulose structure, causing an “explosion” of the fibres, which causes physical, structural and thermal disruption on the biomass and opens structure of the biomass thereof [125]. This results in significant solubilisation of hemicellulose, lignin depolymerization and reduction of crystallinity of cellulose, hence, making the material more digestible [24]. The most important parameters to control under the steam explosion are temperature and residence time [121,122]. High temperature promotes fast hydrolysis of the hemicellulose, which might result in sugar degradation [120,126]. Particle size and moisture content of the biomass has also been reported to effect the sugar conversion [127]. The impregnation of material with water and/or acidic catalysts prior to pretreatment has shown to improve the subsequent enzymatic hydrolysis step. Working on sugarcane bagasse and switchgrass, Ewanick and Bura [128], observed a slight difference on sugar yield between soaked (80% moisture content) and unsoaked (12%) lignocellulose during unanalysed steam explosion. Impregnation with SO₂ significantly improved the sugar recovery.

Steam explosion is also effective for pretreatment of lignocellulose with large particle size, compared to dilute acid pretreatment, thus requiring less energy during feedstock preparation [129]. Ballesteros et al. [130], investigated the effect of three particle sizes (2–5, 5–8 and 8–12 mm) on unanalysed steam explosion of softwood, and observed that at mild pretreatment conditions the large particle size (8–12 mm) exhibited less hemicellulose solubilisation but at severe conditions almost complete extraction of hemicellulose was obtained. The enzymatic hydrolysis results showed that the large particle had higher glucose conversion compared to small particle sizes. However, with herbaceous agricultural waste, the enzymatic hydrolysis results were different, small particle sizes performed better (almost 100% of potential glucose in the pretreated material) compared to large particle sizes (85%) [131]. The drawbacks of this process are similar to dilute acid pretreatment since both of them can result in sugar degradation when severe conditions are employed.

2.3.1.4. By-products and detoxification

By-products

One of the drawbacks of pretreatment is the formation of compounds that can inhibit enzymatic hydrolysis and fermentation. Figure 2-12 shows the possible compounds that can be measured in the hydrolysate liquor after dilute acid pretreatment of sugarcane bagasse. Examples of these inhibitors include aliphatic acids (acetic acid, formic acid and levulinic acid), furan derivatives (furfural and 5-hydroxymethylfurfural) and phenolic compounds. Furfural and 5-hydroxymethylfurfural (HMF) are formed through chemical decomposition of pentoses (such as xylose and arabinose) and hexoses (such as glucose) sugars, respectively [92]. Furfural and 5-hydroxymethylfurfural can further breakdown to formic acid as well as levulinic acid [20]. Furfural and HMF can cause lag phase in yeast growth because they are consumed first before sugars are converted to ethanol [132,133]. Phenolic compounds are produced through the depolymerization of lignin, whereas acetic acid is formed through the cleavage of the acetyl group present as a side chain in the hemicellulose (xylan) structure [90]. Acetic acid decreases intercellular pH, which in turn affects yeast metabolism [132]. On the other hand, various

phenolic compounds have been reported to deactivate cellulase and β -glucosidase activities, hence, reducing the enzymatic hydrolysis yields [107].

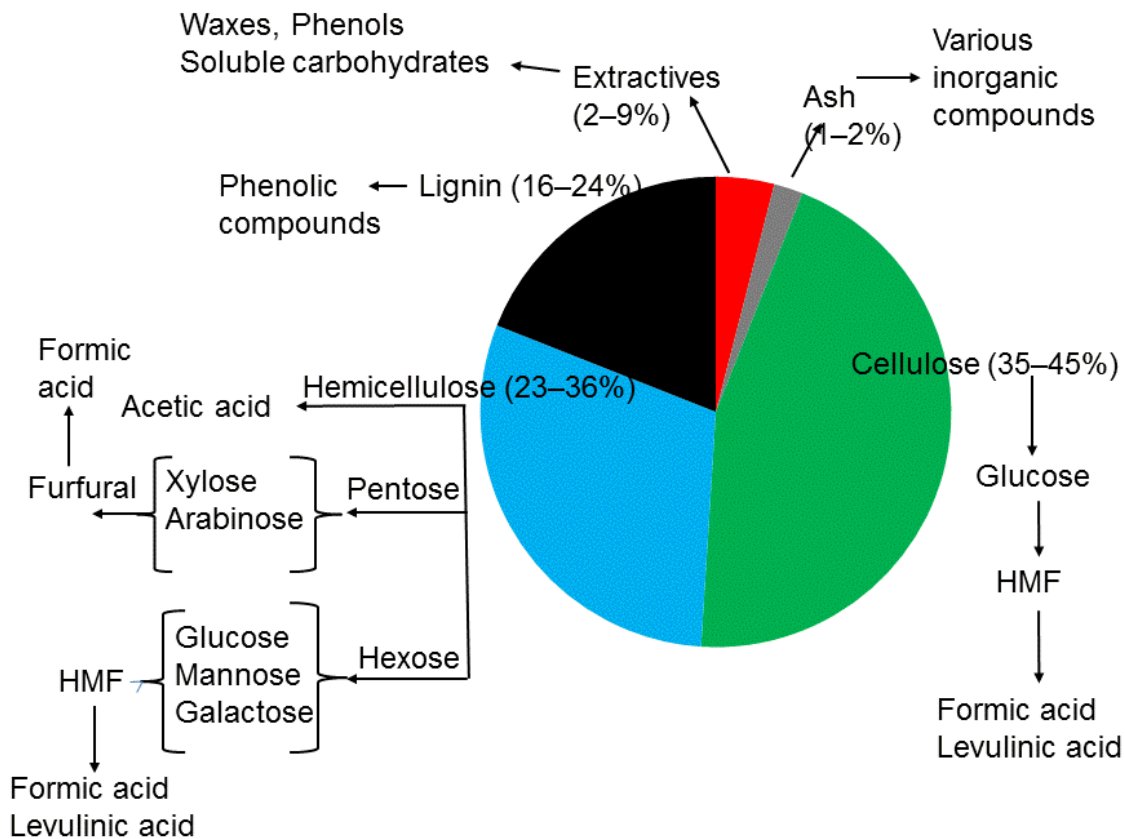


Figure 2-12: Chemical composition of sugarcane bagasse and the possible hydrolysis products after dilute acid pretreatment (adopted from Taherzadeh and Karimi, [20]).

Detoxification

Detoxification process is commonly performed to reduce the inhibitors concentration in the hydrolysates generated by lignocellulose pretreatment. This process can be performed in several ways such as evaporation [135], overliming with calcium hydroxide [135] and use of enzymes with phenoloxidase or laccase [136], metabolic processes by the yeast [135] and extensive washing [137]. Evaporation can significantly remove volatile compounds such as HMF, furfural and acetic acid, but it may also lead to the increase in concentrations of non-volatile compounds [135]. Overliming lowers the concentration of various by-products, but also results in some sugar loss, whereas phenoloxidase and laccase enzymes remove phenolic compounds [136]. Metabolic processes by the yeast represent a method of biological

detoxification through the use of hardened strains (strains that have been pre-conditioned in the pretreated liquor) to tolerate the inhibitors. This is the best approach for detoxification, provided that the yeast can be sufficiently “hardened.” On the other hand, intensive washing (1 to 3 times with clean water) can effectively remove all of the inhibitors from pretreated solids. The present study will use pressing as an alternative method to washing, but this does not eliminate the need for detoxification. The hydrolysate from pressing/washing is still high in inhibitors, which should either be detoxed, or fermented by a resistant yeast.

2.3.2. Enzymatic hydrolysis

After the pretreatment the pretreated solid is subjected to enzymatic hydrolysis, which converts structural carbohydrates into fermentable monomeric sugars. Previously, this hydrolysis was achieved using concentrated sulphuric acid [20]. However, the hydrolysate remained very toxic to fermentative organisms and glucose yields were less than 60% due to kinetics constraints [138]. Other disadvantages of concentrated acid include: the corrosion of equipment, which mandates the material of construction to be highly resistant to corrosion; high energy requirement during acid recovery, increasing the process cost; similarly, large amount of gypsum are produced during neutralization of the acid stream, thus, creating environmental concern [20]. For these reasons, commercial potential interest of this process is very small. Conversely, enzymatic hydrolysis is more specific thus higher glucose and ethanol yields are obtained.

Cellulases are enzymes that can hydrolyse/cleave β -1-4-glucosidic bonds to release glucose. These enzymes are produced by several microorganisms such as anaerobic hyperthermophilic bacteria, filamentous fungi, aerobic actinomycetes and anaerobic fungi [123]. The cellulases are divided into three categories depending on the specific activity in hydrolysis of the cellulose. The first category is endoglucanase (1,4- β -D-glucan-4-glucanohydrolases) and it is responsible for reducing degree of polymerization by randomly attacking the amorphous regions [123]. The second category is exoglucanase (1,4- β -D-glucan cellobiohydrolases, which specialize in hydrolysing the ends of glucan molecules to release

cellobiose units. The last category is the β -glucosidase (β -glucoside glucohydrolase). This kind of enzyme is responsible for hydrolysing cellobiose unit into glucose units [123]. All these enzymes belong to the glycosil-hydrolases family [139]. Although cellulases are broadly classified into these previous categories, there are new activities such as oxidative enzymes that improve cellulose conversion. These mono-oxigenases (initially considered glycosil-hydrolases and designated as GH61) are believed to promote the efficiency of cellulase by acting on the surfaces of the insoluble substrate, where they introduce chain breaks in the polysaccharide chains without the need of first “extracting” these chains from their crystalline matrix [140].

Enzymatic hydrolysis is affected by various factors related to operating conditions and cellulase inhibition, while others are specific to feedstock quality. The operating conditions such as temperature, time, residence time, pH, solid loading and enzyme dosage can be optimised for maximum yield [139]. Cellulase inhibition factor is related to the slow-down of cellulase activity during enzymatic hydrolysis due to the irreversible adsorption of cellulase onto the cellulose/lignin [141,142]. As a consequence, high enzymes dosage is required for acceptable cellulose hydrolysis. On the other hand, the feedstock properties elucidated in section 2.2.3 can be addressed through plant breeding. Figure 2-13 depicts the cellulose convertibility of untreated bagasse from different sugarcane clones with different lignin content [11]. The highest cellulose conversion for the clone with the lowest lignin content (31%) was doubles to that the highest lignin content (13.5%) [11]. However, in this study [11], the effect of pretreatment on enzymatic hydrolysis was not evaluated.

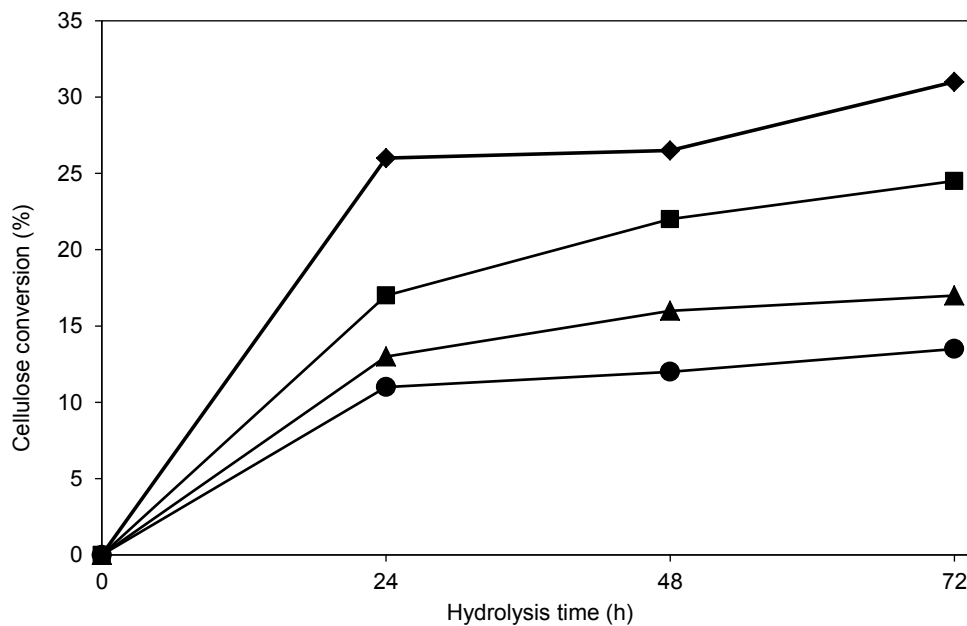


Figure 2-13: Conversion of cellulose to glucose obtained by directly enzymatic saccharification of untreated of sugarcane bagasse samples with different lignin content at cellulase dosage of 20 FPU plus 40 IU of β -glucosidase per gram of bagasse (adopted from Masarin et al. [11]). Symbol key: (\blacklozenge), clone 89; (\blacksquare), clone 146; (\blacktriangle), clone 166); and (\bullet), mill bagasse. The lignin content for clone 89, 146, 166 and mill bagasse are 16.8, 18.6, 19.6 and 24.0% dry weight, respectively

Enzymatic hydrolysis yield can also be affected by degree of substituted hemicellulose. It has been reported that higher cellulose conversion can be obtained on biomass with xylan of higher degree of substitution [47].

Enzymatic hydrolysis has been tested on a wide range of enzyme dosages, varying from 2.5 to 60 Filter Paper Unit (FPU) per gram WIS [139]. Nevertheless, a dosage between 10 and 20 FPU/g WIS is preferred, since it gives high glucose yield after 48 to 72 hours of hydrolysis. In this study the amounts of enzyme was limited to 1.5 to 20 FPU/g total solids. This enabled the present study to identify the most digestible varieties. Through this way it is possible to reduce enzyme requirements and reduce process cost thereof.

2.3.3. Simultaneous saccharification and fermentation (SSF)

Fermentation is the last step of bio-conversion of sugarcane bagasse to ethanol. In this process monomeric sugars are converted to ethanol by using fermentative microorganisms such as yeast. *Saccharomyces cerevisiae* was considered since it is the yeast used globally for ethanol production.

Enzymatic hydrolysis and fermentation can be performed by either Separate Hydrolysis and Fermentation (SHF) or Simultaneous Saccharification and Fermentation (SSF) processes. SHF is performed in two steps, one for enzymatic hydrolysis to generate sugar syrup that is subsequently fermented, whereas SSF allows these two processes to happen simultaneously in a single step (reaction vessel). Product inhibition is the single most important factor for using SSF over SHF. During SSF the glucose generated by enzymatic hydrolysis is immediately removed, thus, no end-product inhibition of the cellulases is observed [143–145]. This allows the use of higher substrate loading for high ethanol concentration in the fermentation broth. However, SSF operates at temperature which is suboptimal to cellulases, which might require higher enzyme dosages. The optimum temperature for enzymatic hydrolysis is at 45-50 °C. Conversely, SHF process allows these two processes (enzymatic hydrolysis and fermentation) to be optimised separately since they operate in different vessels. Based on the above advantages of SSF over SHF, SSF process was selected to be investigated in this study.

Over the decades, several research groups have focused on SSF to improve the parameters such as substrate loading, enzyme loading, temperature and pH. Most of these works are compiled in a review of SSF process by Olofsson et al. [137]. Only solid loading, enzyme dosage and substrate related factors are reviewed.

Traditionally, SSF is conducted in a batch setup. However, several limitations arise when the substrate loading is higher than 15% (w/w) [146]. Among these limitations, it is a stirring problem as a result of high viscosity, which results into low mass and heat transfer. As a consequence, low monomeric sugar release and low ethanol yield can be observed at high solids loading [143]. This problem can be reduced by employing fed-batch SSF, employing the gradual feeding of the substrate and hydrolysis [147,148]. This feeding strategy, maintains

low concentrations of inhibitors throughout the hydrolysis-fermentation, by allowing time of yeast-mediated detoxification, causing less inhibitory effect [137]. Ethanol concentration in fermentation broth of 4% (v/v) is considered as a benchmark for an economically viable distillation [149]. Figure 2-14, illustrates that a solid loading higher than 15 %WIS is required to obtain this concentration (4% v/v). This shows that fed-batch SSF is necessary for high ethanol yield for the economy of SSF process.

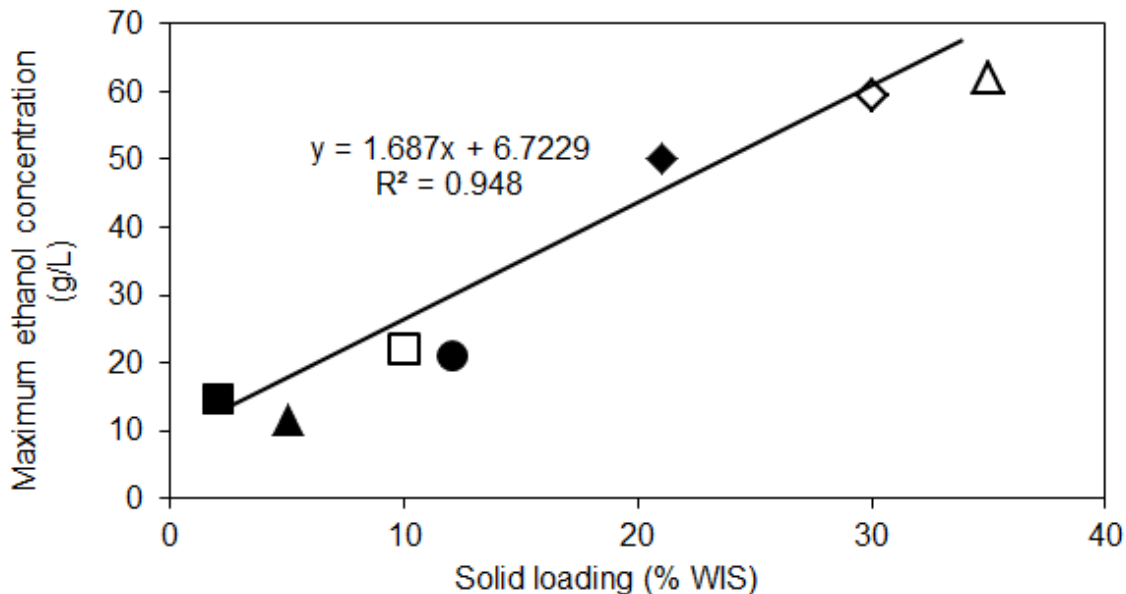


Figure 2-14: The influence of solid loading on ethanol concentration of different pretreated materials. Symbol key: (■) sugarcane bagasse [150]; (□) sugarcane leaves [69]; (◆) corncob [93]; (◇) corn stover [143]; (▲) wheat straw [119]; (△) wheat straw [144], and (●) sweet sorghum bagasse [151].

Previous studies have generally demonstrated that high enzyme dosages are required for high ethanol yield. Figure 2-15, depicts the experimental data ethanol concentration of steam exploded wheat straw and corn stover at different enzyme loadings [119,143]. The results showed positive correlation between ethanol concentration and enzyme dosage. This shows that enzyme dosage is a crucial factor for economic development of SSF process. However, it is still difficult to estimate a maximum enzyme dosage, since increasing enzyme dosages will increase ethanol yield, but at the expense of increasing operational costs. Economic estimates have shown that a reduction of 50% in enzyme dosage is beneficial if the

ethanol yield does not decrease up to 6% and the residence time is not increased by more than 30% [152].

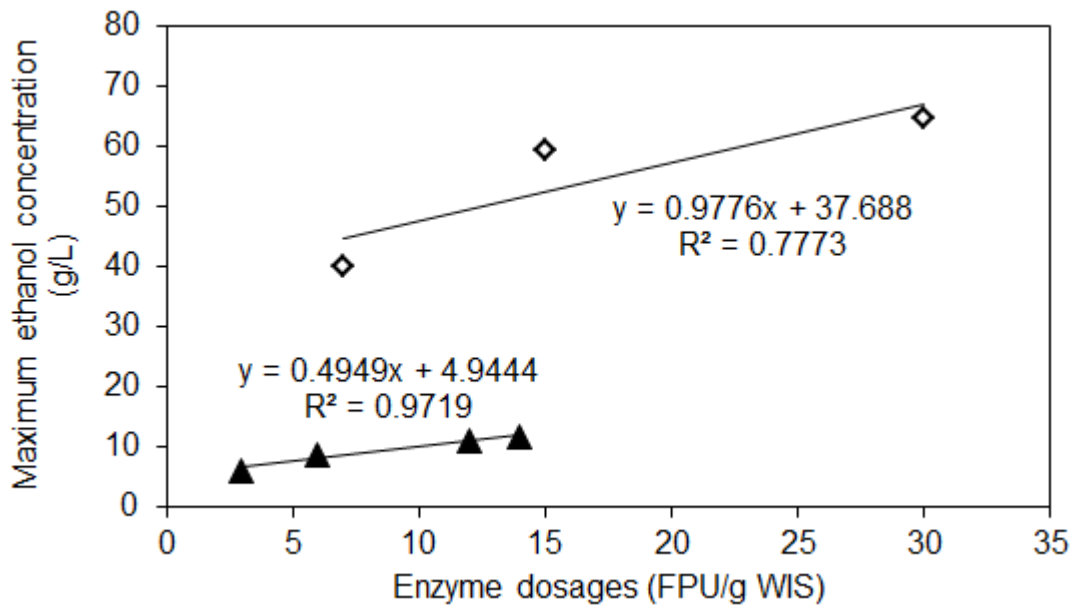


Figure 2-15: The influence of enzyme dosage on ethanol concentration of different pretreated materials. Symbol key: (◇) corn stover [143] and (▲) wheat straw [119].

The enzyme dosage can be reduced through cultivar development and selection of the most digestible substrates. Dien et al. [80] observed inverse correction between ethanol yield and lignin content of forage sorghum (Figure 2-16). In another study, Isci et al. [153] found that ethanol yield after the SSF of corn stover varied from 44.9 to 73% of the theoretical between genotype. Authors also observed strong inverse correlation between ethanol yield and lignin content. These findings show that ethanol yield could be improved when cultivars with low lignin content are selected. However, to the best of researcher's knowledge, the impact of sugarcane cultivar selection on ethanol yield has not been evaluated. Therefore, the current study evaluated also the impact of variety selection on ethanol yield during a fed-batch SSF process of pressed pretreated material.

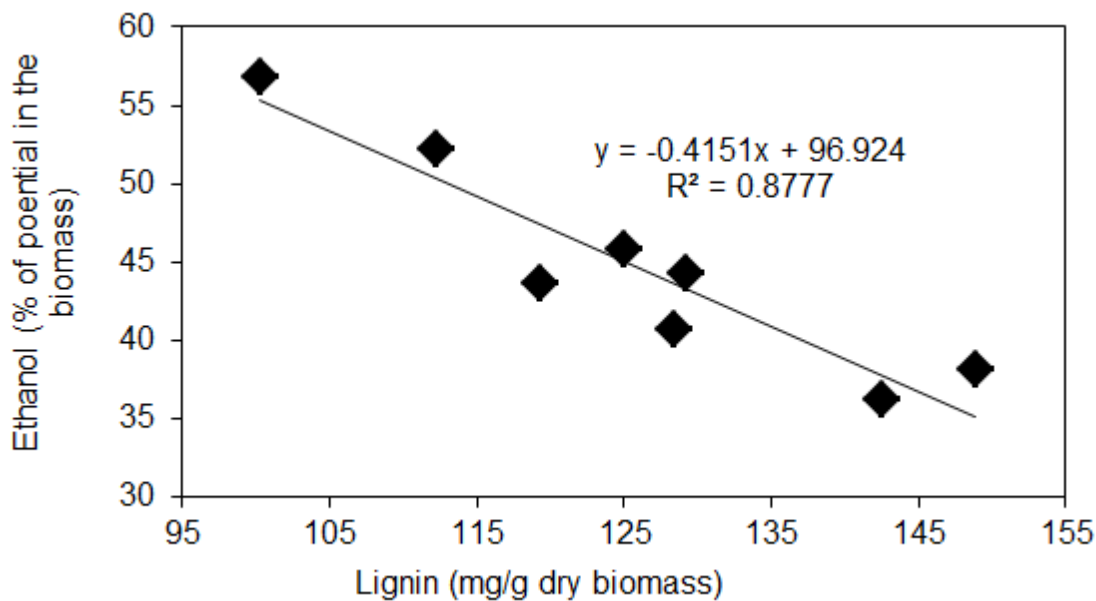


Figure 2-16: The influence of lignin content on ethanol yield of forage sorghum after dilute acid pretreatment [80].

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Chapter 3

3. Objectives

Sugarcane is one of the preferred crops for bio-ethanol production due to its high biomass yield per hectare and high fermentable sugar content in the juice [1]. Presently commercial ethanol production (1G) from sugarcane utilizes only the sugars present in the juice obtained after crushing the stalk [2]. The stalk residue after sugar extraction, referred to as bagasse, is rich in carbohydrates that could be also fermented to ethanol. However, the bagasse is currently combusted to supply electricity and steam to the sugar mill [3]. The advancements in energy efficiency of the combustion systems would generate additional bagasse for 2G ethanol [4].

To obtain fermentable sugars from lignocellulose material such as bagasse the material needs to be subjected to a pretreatment step prior to enzymatic hydrolysis [5]. In spite of the progress achieved on 2G technologies for cellulosic ethanol production, there are still shortcomings that are restraining its industrial application. Existing research to overcome these limitations is broadly focused on the development of efficient and sustainable pretreatments, reduction of enzyme dosage and/or use of more effective enzymes, and efficient fermentation of sugars, including hexoses and pentoses [6]. The ultimate goal is to achieve an ethanol concentration of at least 4% (v/v) in order to make the distillation stage economically viable [7].

One of the strategies suggested to promote 2G establishment is by integrating first and second technologies in the same plant [8]. Production cost can also be reduced by optimisation of the different steps of conversion in an integrated manner, given that each step will influence in the next [9]. In this context, simultaneous saccharification and cofermentation (SSCF) is one of the preferred configurations as it incorporates the hydrolytic enzymes and fermentative microorganism able to conferment hexoses (glucose) and pentoses (xylose) in the same vessel [9]. The direct use of the pretreated material, also referred to as slurry, as substrate of SSF is also preferred since it simplifies the processing of the pretreated material

avoiding filtration and washing steps. At the same time, the use of the whole slurry would easily meet the required concentration of potential fermentable sugars so that the target of 4% (v/v) ethanol can be reached. Nevertheless, the slurry contains not only the fermentable sugars but also inhibitory compounds whose concentrations rely on type and severity of the pretreatment process and characteristics of the lignocellulose itself. The toxicity of the slurry together with the mixing and mass transfer problems associated to high solids content are some other shortcomings in 2G ethanol production [9].

In such an integrated approach the impact of feedstock properties on the lignocellulose processing requirements and final ethanol yield, including chemical and structural characteristics, type of feedstock (hardwood, softwood or herbaceous lignocellulose), genetic variety, tissue, harvest, location, etc., should also be taken into consideration. The selection of varieties that provide both high sugar/biomass yields and lignocellulose (bagasse) coupled with bagasse that is less recalcitrant to biological conversion may enable the use of milder pretreatment conditions, reduced enzyme dosages and pretreated materials that are less toxic to the fermentative microorganism. The selection of such favourable feedstock properties must be subjected to high biomass/sugar productivity per hectare and high carbohydrate content in the lignocellulose.

The improvement in the performance of sugarcane varieties in terms of biomass yield, sugar content, pest resistance and drought tolerance has been achieved through classical breeding. Although this technology has produced traits that are beneficial for the biofuel sector, they have not been focused on the processability of bagasse. On the other hand, genetic engineering approach can produce varieties with high sucrose content and bagasse of improved properties. It is also faster than classical breeding and more specific, but has the disadvantage of creating less robust plants, possibly with lower biomass yield.

Although there are several studies about feedstock impact on lignocellulose conversion to ethanol, in terms of glucose and/or ethanol yield, the study of agronomic properties in combination with the bagasse processability has been overlooked. Moreover, studies encompassing the impact on ethanol yield of both sugarcane variety selection and process optimisation have not been reported. Taking into account the above considerations, the

general aim of the present dissertation is to evaluate the impact of sugarcane varieties, originated by classical and precision breeding, on the final ethanol yield per hectare considering the ethanol yield from juice and bagasse. The specific objectives are as follows:

3.1. Objective 1

To establish a database compiling agronomic properties, chemical composition and “processability” of the bagasse fraction from sugarcane varieties generated from classical and precision breeding compared to industrial sugarcane, in order to select varieties that are technically superior. Such selection will prioritize sugar juice yield per hectare and biomass yield per hectare over the amenability of bagasse to pretreatment-hydrolysis-fermentation; the “processability” of bagasse will only be maximised after selection of cultivars based on (i) superior sugar juice yield per hectare, and (ii) lignocellulose yield per hectare. This information will be used to establish correlations between the chemical composition (cellulose, hemicellulose, lignin, and ash) and structure (i.e. degree of substitution of hemicelluloses) of the bagasse samples with agronomic properties and processability.

In order to accomplish this objective, bagasse samples from 115 sugarcane varieties were characterised in terms of chemical composition. The recalcitrance of these samples was evaluated based on their response to dilute acid pretreatment at small scale (tubular reactors, gram scale) at typical pretreatment conditions and enzymatic hydrolysis with a conventional enzyme cocktail at standard enzyme dosage. Next, the bagasse of 34 preferred varieties were pretreated with a wider range of conditions (low, mild and severe conditions). The pretreated solids generated in this second screening were enzymatically hydrolysed with the same enzyme cocktail, but with a low and standard dosage, to identify bagasse samples with reduced processing requirements.

In both screening steps the ultimate selection considered availability of material and the agronomic information (cane yield, juice sugar and bagasse content) which was used to calculate the potential ethanol yield per hectare. The study performed to address this objective is detailed in **Chapter 4**.

3.2. Objective 2

To evaluate the impact of further optimisation of parameters of pretreatment on combined sugar of the selected varieties. The process requirements of the preferred varieties will be compared with those of industrial bagasse.

Several studies have addressed the optimisation of dilute acid pretreatment of bagasse in terms of recovery of hemicelluloses-derived sugars [10] or glucose [11]. However, relatively little research on optimizing combined sugar yield while reducing the inhibitors originated during pretreatment has been reported [12]. Moreover, most of the studies described thus far have been limited to industrial bagasse. The present study contributes to the existing knowledge by studying the aforementioned aspects on bagasse of preferred sugarcane varieties originated by classical (Chapter 5 and Chapter 6) and precision (Chapter 5) breeding.

The effects of pretreatment parameters (temperature, acid concentration and reaction time) of dilute acid on pretreatment-hydrolysis responses of bagasse of the selected sugarcane varieties were investigated by central composite design (CCD). These studies were performed at (i) gram scale (tubular reactor, **Chapter 5**), and (ii) bench scale (1 litre reactor, **Chapter 6**) at acid constant concentration loading. The use of CCD allows for a better process optimisation with reduced number of experiments while determining interactions between the independent variables evaluated for a particular response. Through this approach, quadratic models will be developed for the bagasse of selected varieties using the experimental data to predict the responses of interest within the studied range of the independent variables. These models will be validated and used to determine the optimum pretreatment conditions providing maximum value of combined sugar yield and minimise inhibitors formation. These pretreatment conditions, combined sugar yield and inhibitors formation will be compared with those of industrial bagasse. Based on the primary screening that considered reduced recalcitrance of samples (Objective 1), it could be hypothesized that the bagasse from selected varieties would require milder pretreatment conditions, and thus result in reduced inhibitors formation, to achieve maximum combined sugar yield.

3.3. Objective 3

To establish a common range of pretreatment conditions that can be applied to the sugarcane varieties to achieve near-to-the-maximum combined sugar yield.

The strategy to accomplish this objective was to use the mathematical models created in the pretreatment optimisation studies (Objective 2) to draw 2D contour plots representing the different conditions that could provide the maximum combined sugar yield for the preferred varieties (**Chapter 6**). In this way, it will be possible to establish the area in common where near-to-the-maximum combined sugar yield among the bagasse from preferred varieties and industrial cane. This set of conditions would be suitable for use in future screening of bagasse from different sugarcane varieties for combined sugar yield after pretreatment-hydrolysis.

3.4. Objective 4

To develop a simultaneous saccharification and fermentation (SSF) process adapted to the feedstock, optimum pretreatment conditions, enzymes combination and fermentative microorganism in order to reach the benchmark ethanol concentration of 40 g/L.

The pressed slurry generated from optimum pretreatment conditions will be used as substrate of SSF at different solid loadings and enzyme dosages. The fermentative microorganism selected for SSF process is an industrial strain of *Saccharomyces cerevisiae* (MH1000). Although this yeast is fairly robust, the use of pressed-slurry would minimise media toxicity and therefore allow for higher solid loadings. Moreover, this strain does not have the ability to ferment the xylose that is solubilized on the pretreatment liquor. Other strategies considered to enhance yeast's robustness are the inclusion of a pre-adaptation step prior to inoculation of SSF and a fed-batch feeding regime. This fed-batch approach will reduce the mixing and mass transfer problems associated with high solids loadings.

Batch and fed-batch SSF will be applied to both the preferred varieties selected and industrial bagasse, to determine the impact of variety selection and optimisation of pretreatment, enzyme cocktail and fermentation process on ethanol yield per hectare (**Chapter 6**).

3.5. Objective 5

To study the stability of the selected varieties from classical and precision technology by comparison of combined ethanol yield across different harvest

To accomplish this objective, the performance of the selected varieties across multiple harvests was evaluated in terms of combined ethanol yields (from juice and bagasse) considering agronomic properties, chemical composition and processability of bagasse. Assessing the variability between harvests is imperative for the ultimate selection of the varieties of superior characteristics for combined ethanol production.

3.6. Methodological consideration

Due to the large number of varieties in the database available for the present project, the majority of bagasse pretreatment experiments were conducted at small scale (gram scale) after rigorous representative sampling. As a sixth objective, the effect of scaling up from tubular reactors (gram scale) to Parr reactor (bench scale) on the responses (combined sugar yield and ethanol yield) was evaluated.

Dilute acid and various other promising pretreatment processes have been studied at bench-scale in order to generate models and understand the mechanism behind them [13], [14]. However, these studies have been limited to feedstocks such as poplar, corn stover and switchgrass [15]. Moreover, limited information is available about the effect of scale up of these technologies [16]. In this thesis, the effect of scale up from tubular reactors (Chapters 4 and 5) to Parr reactor (Chapter 6) was evaluated. These findings are described in Chapter 8.

Through this approach, a novel methodology for screening varieties of sugarcane was developed to be considered by agricultural and biofuels sectors in order to identify novel varieties with properties that improve combined ethanol yield.

3.7. References

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Chapter 4

4. Sugarcane varieties screening

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Title: “Evaluation of bagasse from different varieties of sugarcane by dilute acid pretreatment and enzymatic hydrolysis”

Authors: Yuda Benjamin, Hongbin Cheng and Johann Görgens

Objective of dissertation and summary of findings in present chapter

This chapter addresses **objective 1**. Varieties were evaluated for agronomic data (cane yields, juice sugar and bagasse content), and chemical composition and processability of the bagasse. The processability of the bagasse was tested at different pretreatment condition and different enzyme dosages. The agronomic data and combined sugar yield from the bagasse obtained after pretreatment and enzymatic hydrolysis were used to calculate the potential ethanol yield. Through this approach, sugarcane bagasse varieties with bagasse less recalcitrant (reduce process requirements) that at the same time have high biomass and juice yield were identified as preferred varieties.

The results showed considerable variations in chemical compositions (example structural carbohydrates, 59.3-69%; and lignin content, 14.3-23.1%) and combined sugar yield after pretreatment and enzymatic (27.3-55.2 g/100g dry raw material) among varieties: The CSY positively correlated with structural carbohydrates, but negatively correlated with lignin, ash content. The majority of precision breeding varieties showed improved bagasse properties (high arabinoxylan, low ash and lignin content), and hence higher CSY than many of classical breeding varieties. Some of the varieties had combined characteristics of high cane productivity and combined sugar yield after pretreatment-hydrolysis of the bagasse.

Candidate declaration

With regard to chapter 4 page numbers 64–104 of this dissertation, the nature and scope of my contribution were as follows.

Name of contribution	Extent of contribution (%)
Planning of experiments	60
Executing experiments	100
Interpretation of results	80
Writing the chapter	100

The following co-authors have contributed to chapter 4 page numbers 64–104 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Hongbin Cheng	hcheng@sun.ac.za	<ul style="list-style-type: none"> • Experimental planning • Reviewing the chapters 	10 40
2. Johann Görgens	jgorgens@sun.ac.za	<ul style="list-style-type: none"> • Experimental planning • Interpretation of results to correlate with literature • Reviewing the chapter 	30 20 60

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 64–104 in the dissertation,
2. no other authors contributed to chapter 4 page numbers 64–104 in the dissertation besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that any necessary arrangements have been made to use the material in to chapter 4 page numbers 64–104 of this dissertation.

Signature	Institutional affiliation	Date

Abstract

Lignocellulosic ethanol is a promising alternative to gasoline that can be produced by fermentation of sugars present in lignocellulosic biomass. Improved properties of energy crops and reduction of lignocellulose recalcitrance to biological conversion have the potential to reduce production costs. This study evaluated bagasse from 115 varieties of sugarcane for fermentable sugar yield. The purpose was to select the preferred varieties with fibre of high processability without compromising juice ethanol and cane yield. Dilute acid pretreatment was employed to improve the sugars yield from the bagasse. The results showed wide variations in structural carbohydrates (as monosaccharide) content (66.6–77.6% dry matter (DM)) and lignin content (14.4–23.1% DM) between varieties. Combined sugar yield obtained after pretreatment and enzymatic hydrolysis also varied significantly (27.3–55.2 g/100g DM). Further, it was demonstrated that some of the varieties had combined characteristics of high cane productivity and combined sugar yield after pretreatment-hydrolysis of the bagasse. These results suggest the incorporation of selection of varieties, given its contribution for developing a cost-efficient pretreatment and saccharification process.

Key words: sugarcane bagasse, classical breeding, precision breeding, pretreatment, enzymatic hydrolysis.

4.1. Introduction

Sugarcane is one of the preferred crops for ethanol production due to high biomass yields and high fermentable sugar content (Somerville et al., 2010). However, the fibrous residue (bagasse) generated after sucrose extraction for ethanol production may also be used to increase the ethanol yield per ton of harvested cane. The positive utilization of this abundant residue will bring a breakthrough to a complete utilization of the whole crop for ethanol production.

Like other agricultural residues, Sugarcane bagasse (SB) is mainly composed of cellulose, hemicellulose and lignin (Cardona et al., 2010). Cellulose is a homopolymer composed of

glucose molecules with the major part bounded by hydrogen bonds and forms a crystalline microfibril structure (Klemm et al., 2005). Hemicellulose is an amorphous polymer, mainly composed of xylose and arabinose monomers (Lavarack et al., 2002). Hemicellulose is linked to cellulose and lignin by covalent bonds and fewer hydrogen bonds. Lignin acts like a glue and bind cellulose and hemicellulose, which in turn makes structure more moisture resistant and recalcitrant to biological degradation. Due to this matrix structure, it is difficult for the enzymes to access cellulose if the material is in a native form (Cardona et al., 2010; Sun and Cheng, 2002). Employing a pretreatment process is an efficient way of reducing natural recalcitrance of the lignocellulose cell wall. In this process, the matrix structure is altered to increase the accessibility prior to enzymatic hydrolysis (Alvira et al., 2010; Wyman, 2007). Several pretreatment options have been actively researched (Alvira et al., 2010; Cardona et al., 2010; Wyman, 2007). Among these methods, dilute acid has been studied extensively since it satisfies most of the requirements of the pretreatment process (Sun and Cheng, 2002). The fundamental concept of the dilute acid pretreatment is based on the solubilisation of the hemicellulose, thereby increasing the cellulose accessibility by enzymes (Taherzadeh and Karimi, 2007).

Although pretreatment can significantly improve the cellulose accessibility it still remains a limiting factor in industrial application, due to the cost of processing. However, the cost of pretreatment could be reduced by breeding or selection of lignocellulose feedstocks that are more easily hydrolysable (possibly low lignin content) and with high structural carbohydrates content, without compromising other important agronomic characteristics such as high biomass yields and high sucrose/grain yields.

Previous studies have reported on reduction of the lignin content by breeding new varieties, as a way of diminishing the recalcitrance of the lignocellulose feedstock (Dien et al., 2009; Li et al., 2011). A study on grain yield and stover quality for cellulose ethanol of test crosses of 223 × Mo17 inbred of maize (*Zea mays L.*) has proven that the corn breeding programs are able to improve the stover digestibility without adversely affecting for the grain yield and agronomic traits (Lewis et al., 2010). Working on 79 wheat straw samples, Lindedam et al. (2012) found that ethanol yield estimated from sugar released after the pretreatment and

enzymatic hydrolysis varied from 161 to 203 l per ton dry straw between cultivars. The sugar yield showed a strong negative correlation with lignin as well as ash contents, and was positively correlated with structural carbohydrates. These findings are also relevant during breeding and selection of sugarcane for biorefinery. Masarin et al. (2011) showed a strong negative correlation between enzymatic digestibility of sugarcane bagasse and lignin content, thus favouring sugarcane clones with reduced lignin content. However, what has not been known is the selection criteria used during screening to reach to eleven clones and also the correlation between sugar yields and other chemical components such as structural carbohydrates and ash.

For many years, sugarcane breeding program at South Africa Sugarcane Research Institute (SASRI) has been focusing on only how to increase the sucrose content per unit biomass (Bekker, 2007). With the recent knowledge of producing ethanol from lignocellulose materials, it is also equally important to increase both total fermentable sugars and fibre yields per hectare, to maximise energy production per land used. Following recent developments, various research initiatives have been developed to find a way of enhancing ethanol production from SB. One of these initiatives is the use classical and precision breeding (genetic engineering) technologies to produce sugarcane with preferred fibre characteristics, such as higher biomass yields per hectare and physico-chemical properties that are more amenable to hydrolysis, which will significantly reduce the lignocellulosic ethanol production costs.

In this study, bagasse of one hundred and fifteen varieties of sugarcane developed by classical and precision breeding technologies were evaluated in terms of fibre compositions and fermentable sugar yields from dilute acid pretreatment and enzymatic hydrolysis. The influence of chemical composition and variety type in pretreatment and enzymatic hydrolysis were also evaluated.

4.2. Materials and methods

4.2.1. Raw material and samples preparation

One hundred and fifteen samples of bagasse from different sugarcane varieties were provided by SASRI. The feed stocks were sampled from mature sugarcane (12 months old) in an experimental field located at Mount Edgecombe (29.7000° S and 31.0333° E), KwaZulu-Natal. Fifty six varieties had a South African origin and the rest (59) were imported from USA, Barbados, Australia, India and Reunion. The local and imported varieties are shown in Table 4-1. Numbers enclosed in parenthesis were used for varieties identification from the two breeding technologies, labels 1–100 represented classical breeding varieties and the remaining varieties (labelled 101–115) were from precision breeding, as reported previously (Bekker, 2007). The international and local genotypes were first planted in field trials in 2002 and 2006 respectively. This means that the bagasse evaluated in this study were from 3rd ratoon for the local crops and 7th ratoon for the international crops. The plants were rain fed and no fertilizer was applied.

Twenty to thirty of cane stalks (not less than 6 kg) from each variety were randomly cut from the experimental field. The stalks were shredded and then blended with water (1.5 kg of sample and 3 l of water) for 20 min. Thereafter, the finely crushed shredded canes from the blending jar were washed with water three times and each wash was collected and measured for residue sucrose and other soluble sugars. The remained fibre was pressed to reduce water content and finally it was dried at 40 °C for four days until dry. The average moisture content of the materials after drying was about 6%. Prior to its use, the milled SB was sieved to obtain a representative particle size suitable for the raw material composition analysis and for the pretreatment studies. The particles retained between 425 and 825 µm were packed in zipped plastic bags. The prepared samples were 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 was 12 months.

4.2.2. Dilute sulphuric acid pretreatment

Dilute sulphuric acid pretreatment was carried out in a small tubular reactor (18 cm long and 1.27 cm internal diameter), according to Yang and Wyman (2009). 1.5 g Dry Material (DM) was soaked in 30 ml of dilute sulphuric acid solution or water for 12 h. Soaked samples were concentrated through filtering to a solid loading of 30% (w/v). The obtained wet biomass was loaded into the reactor and compressed by a metal rod to ensure uniform heat and mass transfer. The reactor was first submerged into a heating-up fluidized sand bath set at 30 °C above the target temperature. The reactor was heated until the target temperature was reached (approximately within 120 s), after which it was transferred into the second fluidized sand bath set at the target reaction temperature. After the reaction time was completed, the reactor was quenched by submerging into cold water bath. After cooling, the whole slurry was mixed with 100 ml of distilled water and vacuum-filtered into a solid and a liquid fraction. The solid fraction was further washed in three washes (each wash with 100ml) to raise the pH up to 5 prior to enzymatic hydrolysis, and is subsequently referred to as Water Insoluble Solids (WIS). One part of filtrate was analysed for monomeric sugars content and the other part was used to determine the total sugars in the pretreated liquor as monomers and oligomers by post-hydrolysis as described elsewhere (Jacobsen and Wyman, 2002). All pretreatments were performed on duplicate and average results are shown.

The pretreatment study was done in two phases. In the first phase, all 115 SB varieties were pretreated at 180 °C, 0.5%, (w/w) acid for 15 min. The temperature and residence time were selected from Diedericks (2013). In the second phase, 34 varieties were selected and pretreated at four different pretreatment conditions based on the preliminary study performed on one variety: (150 °C, 0.96%, w/w acid for 15 min); (160 °C, 0.96%, w/w acid for 15 min); (190 °C, 0.07%, w/w acid for 15 min); (200 °C, no-acid for 10 min). The pretreatment severities used in this study were those reported by others (Canilha et al., 2011; Jacobsen and Wyman, 2002; Neureiter et al., 2002; Um and Bae, 2011). The condition used in the first phase (180 °C, 0.5%w/w acid for 15 min) was repeated to check the effect of storage time on the sugar yield.

4.2.3. Enzymatic hydrolysis

The WIS fraction was subjected to enzymatic hydrolysis to evaluate the effect of pretreatment on the enzyme accessibility for each of SB. These experiments were conducted in 24 ml glass tubes. The tubes were loaded with 200 mg (dry weight) of WIS and 10 ml of 0.05 M citrate buffer (pH 4.8) with the enzyme solution. Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial contamination. Two commercial enzymes preparations were used: 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 activities of undiluted enzymes (Spezyme and Novozym 188) were determined by applying analysis protocol described elsewhere (García-Aparicio et al., 2010). Cellulase loading of 32.31 mg protein/g WIS (corresponding to 15 FPU/g WIS) of Spezyme CP supplemented with β -glucosidase of 2.02 mg protein/g WIS (equivalent to 15 IU/g WIS) was applied in all the experiments. Tubes loaded with the mixtures were placed in water bath shaker maintained at 50 °C by shaking at 90 revolutions per minute. Liquid samples were withdrawn after 72 h and prepared as described below and analysed for sugars by High Performance Liquid Chromatography (HPLC) (method described below). All experiments were performed in duplicate.

In the second phase of pretreatment study, two enzyme loadings were used. The first loading was the same as one used in the first phase described above. Then enzyme loading was reduced by 10-fold, resulting in a loading of 3.231 mg protein/g WIS (corresponding to 1.5 FPU/g WIS) of Spezyme supplemented with β -glucosidase at 0.202 mg protein/g WIS (equivalent to 1.5 IU/g WIS).

4.2.4. Chemical composition determination and analysis

The NREL procedure described by Sluiter et al., (2008b, 2005) was used for the composition of untreated SB. In brief, 3 g of milled dried raw sample was consecutively

extracted with water and with 95% ethanol for 48 h in a Soxhlet apparatus. 300 mg of extractives free material was hydrolysed with 72% sulphuric acid (3 ml) in a heating water bath set at 30 °C for 60 min. The acid was diluted with 84 ml of de-ionised water to make the final concentration 4 (%w/w) H₂SO₄ and the mixture were autoclaved at 121 °C for 60 min. The resulting mixture was cooled and filtered through a porous crucible. The solid fraction was incubated at 105 °C to constant weight and then heated in the furnace set at 575 °C for four h. The weight difference before and after incineration was considered as acid insoluble lignin. The acid soluble lignin concentration in the liquid fraction was measured by UV-spectrophotometer at a wavelength of 240 nm and using the value of 15 l/g.cmas the absorptivity of soluble lignin for bagasse (Sluiter et al., 2008b). For determination of ash content in the raw material, 1.5 g of dry extractives free material was combusted at 575 °C for four h. The ash content in extracted sample was calculated as the weight of the solid left after combustion per weight before incineration (Sluiter et al., 2008a). The composition of raw material was performed in four replicates.

For the pretreated material, the same procedure was applied, except that no water or ethanol extraction was carried out because the pretreatment removed of most of extractives. The acid soluble lignin of the pretreated material was not measured because was very small compared to acid insoluble lignin.

The concentration of glucose, xylose and arabinose from the liquid fractions resulting from untreated and pretreated materials compositional analysis, pretreated liquor, post-hydrolysis and enzymatic hydrolysis were quantified by HPLC on an Aminex HPX-87H Column equipped with 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 5mM sulphuric acid and a flow rate of 0.6 ml/min. Sugar concentration was measured with a RI detector (Shodex, RI-101) operated at 45 °C. Under these conditions, the column does not resolve xylose, galactose and mannose. The presence of mannose and galactose for some of untreated samples were checked on an Xbridge™ Amide column (4.6 x 250 mm, 3.5 µm particle size) equipped with an Xbridge™ Amide pre-column (Waters) at 30 °C, eluted at a rate of 0.7 ml/min with 0.05% ammoniumhydroxide in water (A) and 0.05% ammoniumhydroxide in 90%

acetonitrile (B). Sugars were detected by a Varian 380-LC evaporative light-scattering detector. No mannose and galactose peaks were detected. Therefore, the xylose quantification obtained on an Aminex HPX-87H Column was accurate. The glucan and arabinoxylan contents was calculated as $(0.95 \times \text{cellobiose} + 0.9 \times \text{glucose})$ and $0.88 \times (\text{xylose} + \text{arabinose})$, respectively.

4.2.5. Statistical analysis

One-way-analysis of variance (ANOVA) was determined to evaluate whether there were statistical differences in the chemical composition of the raw materials and sugar yields after pretreatment and enzymatic hydrolysis, both between varieties or among pretreatment conditions. ANOVA was carried out using Design Expert program version 8.0.3. The hypothesis was accepted or rejected at 95% confidence interval. Likewise, the correlation coefficients between chemical composition and sugar yield were calculated using STATISTICA (software, version 10) (Weiss et al. 2010; Lindedam et al. 2012).

4.2.6. Higher heating value calculation

The higher heat values (HHV) as the function of chemical composition of SB was calculated using equation proposed by Jimenez and Gonzalez, (1991).

$$HHV = \left[1 - \left(\frac{A}{Ca + L + E} \right) \right] (0.17389 \times Ca + 0.26629 \times L + 0.32187 \times E) (MJ / kg) \quad (1)$$

Where A, Ca, L and E are weight percentage of ash, structural carbohydrates (cellulose and hemicellulose), lignin and extractives on dry biomass basis, respectively. The cellulose and hemicellulose contents were calculated as $(0.95 \times \text{cellobiose} + 0.90 \times \text{glucose})$ and $0.88 \times (\text{xylose} + \text{arabinose})$, respectively.

4.3. Results

4.3.1. Chemical composition and potential energy yields of cultivars

The chemical composition of the bagasse from 115 varieties of sugarcane is depicted in Figure 4-1 (data presented in Appendix A-1). The composition on a dry weight basis ranged as follows: 32.6–40.7%, glucan; 23.6–31.0%, arabinoxylan; 14.4–23.1%, lignin; 3.5–12.4%, extractives; and 0.6–3.4%, ash. Arabinoxylan comprised of xylose and arabinose, with xylose as the major portion (88–94%). Varieties 13, 30, 31, 78 and 101 exhibited higher glucan content compared to varieties 37, 55, 73, 84, 89, 90 and 111. Varieties 31, 61, 81, 101, 102, 106, 107, 108, 109, 112 and 114 (mostly varieties from precision breeding) were characterised as having higher arabinoxylan compared to varieties 5, 8, 9, 11, 13, 43, 48, 50, 67 and 83 (from classical breeding). Based on glucan and arabinoxylan contents, the potential recovery of monomeric sugar varied from 66.6–77.6 g per 100g of dry raw material, which make these materials promising for ethanol production. The variety 101 presented the highest total sugar (77.6%), whereas the lowest (66.6%) was for variety 89. It was worth to notice that the lignin contents of some varieties such as 5, 7, 64, 99, 101, 102, 106, 107 and 114 (about 8% of all varieties) was below 17%, making their structural carbohydrates to lignin ratio to range between 4.0 and 4.7. In addition, about 47% of varieties had a lignin content above 20%, which obviously reduced their structural carbohydrates to lignin ratio (2.7–3.2). The lignin content of the remaining percentage of varieties (45%) was in between 17% and 20%.

A pair-wise comparison of SB compositions between varieties indicated significant ($P < 0.05$) differences in the contents of glucan, arabinoxylan, lignin, ash, and extractives while other varieties showed similarities. When compared to varieties from classical breeding, Table 4-2 shows that varieties originated from precision breeding were typically characterised by higher arabinoxylan (12.2% more) and higher structural carbohydrates (5.2% more). Furthermore, varieties from precision breeding also presented lower lignin (11.4% less) as well as lower ash (50% less) than classical breeding varieties. However, no significant difference was observed on average glucan content between the two breeding technologies (see Table 4-2).

The chemical compositions of various fibre samples were combined with the fibre content of the harvested cane, to estimate the total content of structural carbohydrates per unit of cane. The sugars in fibre differed significantly among the varieties (77–119 kg per ton of wet cane) (confidential). The highest sugar content per unit cane was for the variety exhibited the highest amount of structural carbohydrates per unit fibre. The ratio of non-structural (soluble, fermentable) sugar in the juice to the total sugar content of the fibre was also compared per unit of cane. It varied from 1.1–2.1, suggesting that ethanol production from lignocellulosic material could increase the yield of ethanol by up to up to 92.3% per unit cane or hectare.

4.3.2. Phase 1: Pretreatment and enzymatic hydrolysis of 115 SB varieties

For screening purposes, all SB samples were subjected to dilute acid pretreatment at 180 °C, 0.5% for 15 min, followed by enzymatic hydrolysis of the solid fraction with a conventional enzyme dosage (15 FPU/g WIS). The yields of xylose (considering both monomers and oligomers) after pretreatment, glucose in enzymatic hydrolysis, and combined sugar (sum of all sugars measured after pretreatment and enzymatic hydrolysis) of all the varieties are depicted in Figure 4-2. Statistical analysis of the variations in sugar yields after pretreatment and hydrolysis is presented in Table 4-3. The total xylose yields ranged from 8.8 g/100g DRM (Dry Raw Material) for variety 43–20.4 g/100g DRM for variety 61. Significant difference in the xylose yield was observed between varieties (Figure 4-2). Nevertheless, the overall average xylose yield between bagasse from classical breeding and precision breeding did not differ significantly (Table 4-3). Arabinose and glucose was also present in the pretreated liquor, and contributed 12–24% to the total sugar detected in the pretreated liquor (Appendix A-2).

The effectiveness of the pretreatment method is also determined by its ability to expose the cellulose to enzymatic hydrolysis. The average glucose yield after enzymatic hydrolysis (EH) of the WIS is depicted in Figure 4-2. Significant differences in the digestibility were observed among the varieties, with those from precision breeding being the most digestible (Table 4-3). The highest average glucose yield after EH was 33.5 g/100g DRM for variety 101

and the lowest was 7.4 g/100g DRM for variety 11. It is worth to notice that the highest glucose yield was obtained with the variety with the lowest original lignin content (14.4%) compared to others. In general, glucose yield was affected by lignin content.

The efficiency of the pretreatment and enzymatic hydrolysis processes was measured by the ability to maximise the yields of pentose and hexose sugars, calculated as the combined yield of all sugars after pretreatment and enzymatic hydrolysis. The combined sugar yield (CSY) varied between 27.3 and 55.2 g/100g DRM, depending on the variety (Figure 4-2). Variety 101 exhibited the highest yield while the lowest yield was for variety 31. A combined sugar recovery of above 70% of potential sugars was obtained for varieties 4, 5, 8, 19, 34, 51, 55, 59, 63, 67, 71, 89, 92, 96, 101, 102, 103, 104, 105, 106, 107 and 109, while varieties 2, 11, 31, 32, 56, 76 and 77 released substantially lower sugars (45% of theoretical) than to others, after pretreatment and hydrolysis. Furthermore, the ANOVA results indicated significant difference on combined sugar yield by comparing the varieties to one another. For comparing the substrates on the basis of breeding technology, the varieties from precision breeding exhibited higher combined sugar yield (13.2%) than the yield obtained from classical breeding varieties (Table 4-3).

Apart from structural carbohydrate and lignin content in the raw material, and the CSY after pretreatment and enzymatic hydrolysis, agronomic parameters including sucrose yields per hectare, biomass yields per hectare and non-structural sugar content in the juice per unit biomass, were considered for the selection of varieties for further studies. The sucrose yields per hectare and biomass yield per hectare are not included in this report, but were applied in the selection of 34 varieties for the second phase of pretreatment studies: 1, 4, 5, 6, 8, 12, 13, 15, 16, 20, 28, 30, 34, 54, 55, 57, 58, 63, 70, 71, 74, 87, 88, 89, 94, 97, 101, 102, 103, 104, 105, 106, 109 and 114.

4.3.3. Phase 2: Pretreatment and enzymatic hydrolysis of 34 SB varieties

Dilute acid pretreatment with the selected varieties was performed at four different conditions, in comparison to the conditions applied during the first phase of screening (180 °C,

0.5%, for 15 min). The selected four pretreatment conditions represented a number of alternative optimisation targets for pretreatment, and were based on preliminary optimisation with one variety (Appendix A-3): (150 °C, 0.96%, for 15 min, lowest pretreatment severity), (160 °C, 0.96%, for 15 min, high xylose recovery), (190 °C, 0.07%, for 15 min, high combined sugar yield) and (200 °C, no acid, 10 min, high digestibility).

4.3.3.1. Effect of pretreatment on xylose yield

The varieties were grouped based on the similarities in xylose yield at a particular pretreatment condition (Figure 4-3, data presented in Appendix A-4). The xylose yield ranged from 5.9 g/100g RM for variety 104 (200 °C, no acid for 15 min) to 19.6 g/100g DRM for variety 114 (160 °C, 0.96% for 15 min). At (200 °C, no-acid for 10 min) the xylose yield was relatively low, in particular for the precision breeding varieties compared to the classical breeding varieties. At this condition (200 °C, no-acid for 10 min), 32.8% to 75.6% of the total xylose in the liquor (hydrolysate) from pretreatment itself was in the form of oligomers. The monomeric xylose yield in the pretreatment liquor was improved at moderate temperature and high acid loading. Xylose yield was substantially increased when the temperature was changed from 150 °C to 160 °C. However, no significant improvement on the yield when the temperature was further increased from 160 °C to 180 °C, while the acid loading was lowered to 0.5%. Pretreatment at a temperature higher than 190 °C reduced the xylose yield, even at a low acid loading (0.07%).

An interesting observation here is how varieties responded differently in respect to pretreatment conditions. For example, in more than two pretreatment conditions, varieties 5, 6, 12, 34, 105, 106 and 114 consistently released higher xylose compared to varieties 20, 74, 94, 88 and 89. Generally, the breeding technology did not seem to impact xylose yield. However, the xylose yield variability between varieties was significantly influenced by temperature and acid loading.

In terms of xylose recovery (%), calculated as the xylose yield divided by the potential xylose in raw material of a particular variety, the highest recovery was 73.7% for variety 8, found at 160 °C, 0.96%, 15 min and the lowest was 23% for variety 102, observed at 200 °C,

no-acid for 15 min. Xylose recovered was very low when no or low acid was used but was significantly improved when high acid loading was applied. In general, xylose recovery was improved by the increase of pretreatment severity up to certain extent, where the degradation of xylose became significant.

4.3.3.2. Effect of pretreatment on solids digestibility

The effect of different pretreatment conditions was evaluated in terms of glucose yield after enzymatic hydrolysis of the pretreated WIS. The pretreated solid was hydrolysed under two enzymes loadings, with either 15 FPU/g WIS or 1.5 FPU/g WIS. The purpose of reducing enzymes dosage to 10-fold was to identify the varieties that are more easily digestible or reactive, thus requiring lower dosages.

Higher enzyme loading (15 FPU/g WIS)

Figure 4-4 depicts the varieties arranged in groups based on similar glucose yield from EH, after pretreatment at a certain condition, for example 101, 103 and 114, at 150 °C, 0.96%, for 15 min (data presented in Appendix A-5). The highest EH glucose yield was 34.6 g/100g DRM for variety 101, obtained at 200°C, no-acid, for 10 min and the lowest yield was 15.2 g/100g DRM for variety 57, found at 150°C, 0.96%, for 15 min. For most of the varieties the yield was substantially improved when temperature was raised from 150 °C to 160 °C. Interestingly, varieties 102 and 114 showed higher glucose at 160 °C, 0.96% and 15 min than that observed at 190°C, 0.07% and 15 min, while for varieties such as 1, 4, 5 and 8 the opposite outcome. For varieties as 20, 57, 104 the EH glucose yield did not differ significantly between these two conditions. In addition, the EH glucose yield was enhanced at (200°C, no-acid, for 10 min) compared to 160°C, 0.96%, for 15 min for most of the varieties. It was interesting to see high glucose yield when no acid was used (200°C, for 10 min) compared to low xylose yield from pretreatment (Figure 4-3), in particular for the precision breeding varieties (101, 103, 104, 105, 109 and 114). Varieties 101, 102, 103, 106 and 114 (precision breeding), released higher glucose during EH after pretreatment at different conditions,

compared to the yields obtained for varieties 20, 28, 57 (classical breeding). In general, most of precision breeding varieties performed better during EH (overall average glucose yield 27.5 g/100g DRM) than classical breeding varieties (overall mean 23.3 g/100g DRM).

Lower enzyme loading (1.5 FPU/g WIS)

The glucose yields after enzymatic hydrolysis of pretreated WIS at 1.5 FPU/g WIS (low dosage) also varied significantly among the varieties (Figure 4-5, data presented in Appendix A-6). The highest yield was 18.6 g/100g RM for variety 103, at 160°C, 0.96%, 15 min and the lowest was 7.3 g/100g DRM for variety 20, at 180°C, 0.5%, 15 min. Varieties 101, 103, and 114 (precision breeding) consistently released higher glucose than other varieties, followed by varieties 5, 13, 63, 102 and 105. Interestingly, varieties such as 20, 28 and 57 (classical breeding) consistently released lower glucose at both enzyme dosages (1.5 and 15 FPU/g WIS) than others (Figure 4-4 and Figure 4-5).

4.3.3.3. Effect of pretreatment conditions on combined sugars yield

The combined sugar yields also varied significantly among the selected pretreatment conditions and the sugarcane varieties (Figure 4-6). The highest CSY (55.2 g/100g RM) was for variety 101 observed at 180°C, 0.5%, 15 min and the lowest (28.1 g/100g RM) was for variety 58, found at 150°C, 0.96%, 15 min (data are presented in Appendix A-7). The CSY was more strongly correlated to the glucose yield than the xylose yield. From Figure 4-6, it can be seen that applying a moderate pretreatment temperature of 160 °C, combined with high acid loading (0.96%), was sufficient to deliver high CSY for varieties 102 and 114, compared to more severe pretreatment at a temperature of 180 °C and acid loading of 0.5%. However, for varieties such as 20, 28 and 57, the CSY was increased with an increase in pretreatment severity. Furthermore, varieties 5, 30, 63, 101, 102, 103, 105, 106 and 114 consistently delivered higher CSY compared to varieties 20, 28, 57, 88, 89 and 104.

4.3.4. Correlation of bagasse compositional factor and pretreatment responses

One of the purposes of this study was to evaluate whether the chemical composition of SB could predict the effectiveness of pretreatment and enzymatic hydrolysis. Correlation coefficients were determined between the SB chemical composition (glucan, arabinoxylan, lignin, extractives, ash and total structural carbohydrates) and the total xylose yield, or the glucose yield or the combined sugar yield, using data obtained during the second phase of pretreatment (34 varieties). As shown in Table 4-4, the xylose yield was not strongly correlated with any of the SB chemical components analysed. Glucose and combined sugar yields showed significant positive correlations with the glucan and total structural carbohydrates contents, but was negatively correlated with the ash and lignin content. Glucose yield was also significantly positively correlated with the arabinoxylan content.

4.3.5. The effect of variety type on the pretreatment and enzymatic hydrolysis responses

Seven groups of varieties with similar raw material chemical composition were selected to assess the contribution of variety type to the observed variations in the sugar yields, as shown in Table 4-5. Significant ($P < 0.05$) differences in xylose and glucose yields were found between varieties in a particular group at the same pretreatment-hydrolysis condition. Variety 57 consistently showed higher xylose yield than 63, whereas for the remaining groups no clear trend in xylose yields was observed. On the contrary to xylose yield, variety 63 was more digestible than variety 57. Similarly, variety 34 consistently released significantly higher glucose than variety 20. Similar result was also observed when varieties 30 and 58 were compared. Nevertheless, no significant difference on the glucose yield was obtained when varieties 88 and 97 or 103 and 105 were compared in most of the pretreatment conditions. The results also indicate that the majority of precision breeding varieties had higher glucose yields from pretreatment-hydrolysis compared to most of classical breeding varieties.

4.3.6. Relationship between energy content and potential ethanol yield

HHV calculated on the basis of the chemical composition is also depicted in Figure 4-1. The HHV values ranged from 17.5 to 19.2 MJ/kg. No significant difference was observed in most of the varieties. The HHV of the bagasse (MJ/kg) was converted to GJ/hectare energy yield, based on the fibre content of the harvested cane, and the cane yield per hectare. This was compared to the total potential ethanol yield per hectare, based on both the soluble sugars in the cane juice and the expected ethanol yield, based on CSY from pretreatment-hydrolysis, which were combined with the cane yield and its fibre/juice content as shown in Figure 4-7. The ethanol yield showed was positively correlated with the gross energy yield per unit hectare. However, potential ethanol ($R^2=0.9895$) had stronger correlation than that calculated based on the sugar in the juice and CSY ($R^2=0.9384$). The strong correlation infers that the variety with higher sugarcane productivity per unit land delivered higher potential ethanol and maximum energy per unit land than those exhibited lower cane yield.

4.4. Discussion

4.4.1. Variability in the chemical composition of bagasse samples

Significant differences in chemical compositions of SB samples were observed between the varieties of sugarcane used in this study (Figure 4-1). These differences could be attributed to the type of variety and breeding technology. The chemical compositions of SB samples were in agreement with the composition variability of SB reported elsewhere (Aguilar et al., 2002; Rabelo et al., 2008; Brienzo et al., 2009; Carrasco et al., 2010; Masarin et al., 2011). However, some of our varieties were characterised by higher arabinoxylan content and others had lower lignin content compared to arabinoxylan and lignin contents of industrial SB. The higher arabinoxylan content in these varieties could be more advantageous to ethanol production, provided that high sugar recovery is achieved in pretreatment-hydrolysis, and that yeast conversion of xylose and arabinose is efficient. Moreover, the relatively low lignin content of some of these varieties could improve enzymatic digestibility compared to industrial SB.

Varieties 13 and 101 exhibited the highest glucan content (40.7%), whereas variety 111 presented the lowest glucan content (32.6%). High glucan content is beneficial to ethanol production, because glucose can be converted to ethanol at high yields. Generally, the glucan content was similar independently of breeding technologies (Table 4-2). The varieties from precision breeding technology showed higher arabinoxylan content, with lower lignin and ash contents, compared to those from classical breeding technology (Table 4-2). The increase in the pentose content on these transgenic sugarcane varieties has also been reported by Bekker (2007). In these transgenic sugarcane varieties the expression of the UDP-glucose dehydrogenase in the pathway responsible for biosynthesis of UDP-glucose to hemicellulose and pectin polymers was down-regulated, to divert the assimilated carbon to the other competing sinks like sucrose and cellulose. The reduction of carbon flux through UDP pathway was compensated by the increase in the *myo*-inositol oxygenation (MIOP) pathway, an alternative pathway of synthesis of cell wall precursor, which in turn, increased the hemicellulose and pectin content. The increase in hemicellulose and pectin contents in turn decreased the lignin, ash and extractives contents, which favoured a higher content in structural carbohydrates. Similar observations were also reported on transgenic sugarcane in which *Caffeic acid O-methyltransferase (COMT)* activity was down-regulated, leading to a 3.9–13.7% decrease in lignin content (Jung et al., 2012), improved the enzymatic saccharification of up to 34% after dilute acid pretreatment. Similar improvements in lignocellulose conversion to ethanol were reported for forage sorghum (Dien et al., 2009), switch grass (Fu et al., 2011), sorghum and maize (Vermerris et al., 2007) through crop development.

Evaluating the composition data of classical breeding technology showed that the varieties with low amounts of lignin did not exhibit increased amounts of structural carbohydrates. This means that the reduction of lignin content was compensated for by the increase in other components, including extractives and ash, rather than structural carbohydrates. Similar observations were reported during the evaluation of chemical composition of bagasse from different sugarcane clones (Masarin et al., 2011).

Summarizing the results in Figure 4-1 and Table 4-2, this study found that the use of precision breeding technology probably could reduce lignin and ash contents leading to less

severe pretreatment-hydrolysis requirements. Improving structural carbohydrate contents could possibly increase ethanol production per unit biomass.

4.4.2. Pretreatment and enzymatic hydrolysis responses

This study has demonstrated the use of dilute acid as an effective way of hemicellulose solubilisation, as has been reported previously (Aguilar et al., 2002; Canilha et al., 2011). Xylose was the predominant sugar in the hydrolysate liquor, with the yields ranging from 5.9 to 20.4 g/100g DRM (Figure 4-2 and Figure 4-3). The differences in xylose yields between the varieties were significant. However, there was no significant correlation between xylose sugar yield and chemical composition of the fibres (Table 4-4). In this context, Weiss et al. (2010) evaluated the influence of corn stover composition on the dilute acid pretreatment effectiveness at three pretreatment severities. The xylose yield (monomeric xylose and total xylose) in their work was not strongly correlated with the corn stover chemical composition. However, their monomeric xylose yield was strong negative correlated with acid neutralization capacity and the soil content of the lignocellulose samples, which was not observed in the present study. The xylose yield from pretreatment of the SB samples was therefore primarily dependent on the pretreatment conditions selected, together with physical-chemical properties of SB not reflected in the chemical composition. Generally, xylose yield was enhanced by the increase in acid loading at moderate temperature. Lower xylose yield, and in particular more monomeric xylose, was obtained in most of varieties with water-only as catalyst (autohydrolysis), compared to those conditions with dilute acid as catalyst (Figure 4-3). This is due to the fact that the autohydrolysis pretreatment always depends on acetic acid generated by the cleavage of the acetyl group to complete the hydrolysis reaction of oligomer to monomeric xylose (Jacobsen and Wyman, 2002). As such, most of the sugars found in the hydrolysate remain as oligomers.

The best xylose yield observed in the present study (20.4 g/100g DRM) compared well the maximum xylose yields reported previously, in particular by Neureiter et al. (2002), with 22.95 g/100 g DRM after pretreatment at the optimal condition (177 °C, 0.025 mol/L, 4% dry

matter, for 13 min). Xylose yields as low as 12.8 g/100 g DRM (57.6% of theoretical) have also been reported by Canilha et al. (2011) using sugarcane bagasse at the best hydrolysis condition (150 °C, 2.5%, for 30 min). According to these authors, neither post hydrolysis nor pre-soaking of the material in the acid solution improved the xylose yield, while low yields were attributed to xylose degradation to furfural and others minor compounds. In the present study, some of the varieties consistently exhibited low xylose yields (Figure 4-3). This shows that xylose recovery could also be a function of the physical-chemical properties of lignocellulose biomass, in addition to the pretreatment conditions and the type of pretreatment reactor employed.

Glucose was the second most abundant sugar in the pretreated liquor, with the concentrations ranging from 0.6 to 6.5 g/100g DRM (Appendix A-2), corresponding to 2% and 15% of the potential glucose in the raw material of varieties 86 and 5, respectively. Arabinose was the third most abundant sugar, with the concentrations ranging in between 0.6 and 3.1 g/100g DRM (Appendix A-2), equivalent to 40% and 94% of arabinose present in the raw material of varieties 49 and 109, respectively. The relative low glucose hydrolysis during pretreatment was primarily associated with the greater recalcitrance of cellulose toward dilute acid hydrolysis, compared to xylan (Canilha et al., 2011). Thus glucose present in pretreated liquor might be linked to amorphous cellulose components, as previously reported by Martin et al. (2007).

The glucose yields from enzymatic hydrolysis subsequent to pretreatment, varied significantly (Figure 4-2, Figure 4-4 and Figure 4-5) in part depending on the chemical composition of SB (Table 4-4). It is well known that lignin hinders cellulose reactivity (Chang and Holtzapple, 2000; Palonen et al., 2004), which was also observed in the present study. The glucose yields were substantially higher for the samples with low lignin content than for those with high lignin content. Generally, glucose yields were increased by the increase of pretreatment severity, although, the impact was greater for the varieties with low lignin content than those with elevated amount of lignin (Figure 4-1, Figure 4-4 and Figure 4-5). There was a clear trend that SB from precision breeding consistently released significantly higher glucose yields than bagasse originated from classical breeding (Table 4-3, Figure 4-4 and Figure 4-5),

reflecting relatively the decrease in lignin content for precision breeding varieties (Table 4-2). Likewise, ash was inversely correlated with glucose yield, apparently related to the buffering capacity of the inorganic compounds found in biomass, which can neutralize the acid catalyst, thus reducing pretreatment effectiveness (Springer and Harris, 1985). However, the glucose yield from the biomass cannot be determined by lignin and ash contents alone, but was also a function of structural carbohydrates content (Lindedam et al., 2012), together with other physical-chemical properties of the SB samples. The glucose yield was impacted positively with high amount of glucan as well as arabinoxylan in the SB samples. It is known that hemicellulose can impact negatively glucose yield when is not sufficient removed (Chang and Holtzapfel, 2000). The observed positive correlation demonstrated that arabinoxylan was effectively hydrolysed after dilute acid pretreatment, leaving cellulose more exposed for enzymatic hydrolysis. Therefore, this finding suggests that the fibre composition modifications (such as reduction in lignin and ash contents and increasing arabinoxylan content) could play a crucial role to enhance cellulose amenability, in turn, favouring the precision breeding varieties.

The effect of a wide range of chemical-physical properties of SB could also contribute to the observed variability in glucose yields (Table 4-5). The differences in glucose yields could not be fully explained by changes in the chemical composition alone, but should rather consider the full scope of properties associated with SB from precision vs. classical breeding. Similar observation has also been reported by Li et al. (2011). In this study, cellulose digestibility of sixteen straws after dilute acid pretreatment was evaluated. It was found that although the straws had similar chemical compositions, their glucose yields differed significantly. The straws coming from genetically modified were found to be more digestible than those originated from mutant breeding. Likewise, precision breeding technology produced more stable varieties than classical breeding technology. However, further study is needed to investigate the effects of precision breeding technology on SB fibre structure in respect to saccharification.

Without pretreatment, the combined sugar yield was significantly lower (10.5–20.2 g/100 g DRM) compared to those reached after pretreatment (27.3 to 55.2 g/100g DRM). This

shows the importance of the pretreatment step prior to enzymatic hydrolysis to maximise sugar yield. As it was observed in glucose yield, the combined sugar yield was negatively affected by high amount of lignin and ash content in the biomass (Table 4-4). On the contrary, high content of structural carbohydrates impacted the yield in a positive manner. Furthermore, the pretreatment at low severity condition (150°C, 0.96%, for 15 min) enhanced the xylose yield, but failed to achieve high glucose yields from enzymatic hydrolysis, thus lowering the combined sugar yield (Figure 4-3, Figure 4-4 and Figure 4-6). Therefore, mutual balance of pretreatment and enzymatic hydrolysis steps are very essential to maximise both xylose and glucose yields, and to minimise by-product formation (HMF and furfural), thus maximising the total sugar yield. The use of acid catalyst was necessary to improve pentose sugars recovery, consequently, increasing potential ethanol production yields.

4.4.3. Correlation between energy content and potential ethanol yield

The HHV of SB samples was estimated on the basis of chemical compositions. It has been shown that lignocellulose holocelluloses (cellulose and hemicellulose) have a HHV of about 18.6 MJ/kg, whereas of the HHV of lignin varies from 23.3–25.6 MJ/kg, depending on the chemical structure (Demirba, 2001). The estimated HHV of various SB samples, based on the HHV of its chemical components, did not vary significantly between the SB samples from varieties investigated (Figure 4-1 and Table 4-2). This is because the lignin content differences between the varieties were not large enough to cause noticeable differences. The potential ethanol production from bagasse contributed approximately one third of the total potential ethanol. This maximum potential ethanol yield per unit hectare showed strong positive correlation with the maximum energy from the bagasse (Figure 4-7), indicating that cane yield and the content of structural and non-structural sugars in the cane, remains as the most important optimisation parameters for sugarcane application for ethanol production. Therefore, the selection of preferred varieties should incorporate crop productivity per unit land, in combination with structural and non-structural sugar content, with the latter based on the measured CSYs after pretreatment and enzymatic hydrolysis.

4.4.4. Selection of cultivars with improved agronomic properties and sugar yields

Reducing lignin content is widely regarded as the best way to reduce natural recalcitrance of the lignocellulose material to biological conversions (Chang and Holtzapfle, 2000). The present study indicated that cane varieties with low lignin content in the bagasse (14-16%) had higher sucrose content in the cane, as well as increases in the glucose yield and combined sugar yield from pretreatment-hydrolysis. However, the agronomic productivity of these varieties was lower (65–98 wet ton/hectare) than the observed productivity (115–126 wet ton/hectare) for plants with intermediate lignin content (18-20%) (Confidential). Still, the lignin content in SB observed in many samples in the present study was significantly lower than the content found in commercial varieties. Commercial varieties of sugarcane usually are characterised by high lignin content and their productivity varies depending on variety type and the environmental factor (Masarin et al., 2011). The typical sugarcane productivity from South Africa ranges from 42.1 to 84.1 wet ton/hectare, with an average yield of 62.6 wet ton/hectare (Bezuidenhout and Singels, 2007). Therefore, the obtained results seem to be a positive gain toward developing and selection of most promising varieties with combined high agronomic productivity, high fermentable sugar content and release of sugars from pretreatment-hydrolysis of the fibre. Such selection would also be influenced by structural carbohydrates and ash contents.

4.5. Conclusions

Bagasse samples from different 115 varieties of sugarcane were evaluated for their responses to dilute acid pretreatment and enzymatic hydrolysis. The variability of fibre composition between varieties showed to be statistically significant. Most of precision breeding varieties were characterised by higher arabinoxylan, lower lignin and lower ash contents compared to varieties from classical breeding.

This work has clearly established how fibre composition variations could substantially impact sugar yields during pretreatment and enzymatic hydrolysis. However, the variation of xylose yields was not always consistent with the fibre composition, breeding technology or variety type. The contribution of variety type to digestibility variations was statistically significant for most of classical breeding varieties assessed, and was less obvious to many precision breeding varieties. The digestibility of most of precision breeding varieties was substantially higher than many classical breeding varieties. The findings obtained from this study have significant contribution to the sugarcane development with the aim of selecting sugarcane with highly hydrolysable fibres in conjunction with high biomass and sucrose yield per hectare to make lignocellulose to provide efficiency and economic benefits to the bio-ethanol process. Further, optimisation of the pretreatment processes for the preferred varieties is required to maximise the combined sugars yield.

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4.6. References

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Table 4-1: The local and imported varieties of sugarcane investigated in this study

Local	Local	Local	Imported	Imported	Imported
(1) N31	(62) 01K0016	(100) NCo376	(8) MZC74/275	(29) US66/5615	(51) 66N2008
(2) NCo376	(63) 01F2810	(101) 05TG004	(9) CP72/2086	(30) US82/40	(76) Co244
(3) N22	(64) 01F0152	(102) 05TG005	(10) Ja55/485	(31) US48/34	(77) Co285
(4) N21	(65) 01G1818	103-05TG007(103	(1) 1CP79/1658	(32) Pindar	(78) Co745
(5) N24	(66) 03T2530	(104) 05TG008	(12) CP70/1133	(33) POJ2364	(79) Q96
(6) N25	(67) 92E1109	(105) 05TG010	(13) CP70/321	(35) NM214	(83) R570
(7) N27	(68) 96M0058	(106) 05TG011	(14) CP68/1022	(37) Trojan	(84) POJ2725
(18) NCo310	(69) 98T1260	(107) 05TG012	(15) Q119	(38) IM76-237	(85) POJ2878
(34) NCo377	(70) 00F0884	(108) 05TG014	(16) Q135	(39) NG77-61	(86) KF70-190
(36) NCo376	(71) 99B0325	(109) 05TG015	(17) Q96	(40) IJ76-424	(87) LCP85-384
(52) 4G0025	(72) 00U1422	(110) 05TG016	(19) M124/59	(41) IK76-33	(88) POJ2714
(53)04G0098	(73) N46	(111) pHan-UGD1.1	(20) Uba	(42) IM76-248	(89) Co213
(54) 95F1099	(74) 01G1662	(112) pHan-UGD1.2	(21) Kassoer	(43) 04X0002	(90) N55/805
(55) 99F2004	(75) 96L1778	(113) 05TG017	(22) Yon-san-tan	(44) 04X0003	(92) Co205
(56) 05K0001	(80) N40	(114) 05TG018	(23) 57NG155	(45) 04X0022	(93) CP57/614
(57) 00F0379	(81) N37	(115) 05TG019	(24) B41227	(46) 04X0023	(94) NiN2
(58) 99T0269	(82) N41		(25) CB36/14	(47) 04X0026	(95) CB38/22
(59) 96L0167	(91) N36		(26) BJ5924	(48) 04X0033	(96) N52/219
(60) 00K2172	(98) N48		(27) Co213	(49) 04X0036	(97)Q158
(61) 01K0013	(99) N27		(28) US82/37	(50) 04X0052	

*numbers in parenthesis refers to varieties identification number

Table 4-2: Statistical summary of chemical composition and higher heating value of bagasse from 115 varieties of sugarcane

Statistics	Classical breeding							Precision breeding						
	<i>Glu</i> ^a	<i>Araxyf</i> ^a	<i>Lig</i> ^{a,b}	<i>Extr</i> ^{a,c}	<i>A</i> ^a	<i>TSC</i> ^a	<i>HHV</i>	<i>Glu</i> ^a	<i>Araxyf</i> ^a	<i>Lig</i> ^{a,b}	<i>Extr</i> ^{a,c}	<i>A</i> ^a	<i>TSC</i> ^a	<i>HHV</i>
Mean	36.7	26.0	20.1	7.7	1.8	62.6	18.4	36.8	29.1	17.8	6.9	0.9	65.9	18.3
Standard error	0.2	0.1	0.2	0.2	0.0	0.2	0.0	0.5	0.3	0.5	0.3	0.1	0.7	0.1
Standard Deviation	1.6	1.3	1.7	1.9	0.5	1.7	0.3	2.1	1.2	2.0	1.3	0.2	2.7	0.3
Skewness	0.0	0.0	-0.5	0.2	0.3	0.5	-0.1	-0.3	-0.1	0.6	-1.2	1.1	-0.8	0.2
Minimum	32.9	23.1	15.6	3.5	0.6	59.3	17.5	32.6	27.1	14.4	3.8	0.7	59.8	17.9
Maximum	40.7	28.5	23.1	12.4	3.4	68.0	19.2	40.7	31.0	22.2	8.5	1.4	69.2	18.8
Number of varieties	100	100	100	100	100	100	100	15	15	15	15	15	15	15

^aGlu, Araxyf, lign, extr, A, TSC are weight percentage of glucan, arabinoxylan, lignin, extractives, ash and total structural carbohydrates in dry biomass basis, respective.

HHV is the Higher Heating Value (MJ/kg)

^bLignin content was calculated as the sum of acid soluble and insoluble lignin,

^c Extractive content Include water and ethanol extractives

Table 4-3: Statistical summary of xylose, glucose and combined sugars yield of bagasse from 115 varieties of sugarcane. 100 varieties originated from classical breeding and 15 from precision breeding

Statistics	Classical breeding			Precision breeding		
	Xylose ^a	Glucose ^b	CSY ^c	Xylose ^a	Glucose ^b	CSY ^c
Mean (g/100g RM)	14.6	22.6	42.4	15.8	25.9	48
Standard error	0.2	0.5	0.5	0.5	1.2	1.2
Standard Deviation	2	5.2	5.5	1.8	4.7	4.6
Skewness	-0.1	-0.5	-0.4	-0.1	0	-0.2
Minimum (g/100 g RM)	8.8	7.4	27.3	11.6	18.9	39.9
Maximum (g/100 g RM)	20.4	32.9	53.3	19.7	33.5	55.2
Number of varieties	100	100	100	15	15	15

^aXylose yield after pretreatment at 180 °C, 0.5 (%w/w) for 15 min

^bGlucose yield after enzymatic hydrolysis of pretreated

^cCSY is the combined sugar yield (sum of all pentose and hexose sugars after pretreatment and enzymatic hydrolysis)

Table 4-4: Correlation coefficient (r) between chemical composition of bagasse from 34 varieties of sugarcane and total xylose yield, glucose yield and combined sugar yield. The glucose yield is at 15 FPU/g WIS

Component	Xylose yield	Glucose yield	Combined sugar yield
Ash	-0.0773	-0.6432*	-0.6327*
Extractives	-0.0578	-0.1169	-0.1185
Lignin	-0.0749	-0.6063a	-0.6466 ^a
Arabinoxylan	0.0108	0.3652 ^a	0.3044
Glucan	0.1468	0.4654 ^a	0.5582 ^a
Total carbohydrate	0.1074	0.6339 ^a	0.6621 ^a

^a correlations are significant at 95% confidence levels.

Table 4-5: Effect of variety type on pretreatment and enzymatic hydrolysis responses

Group	Variety	Composition of raw material (%)				Xylose yield (g/100g RM)					Glucose yield (g/100g RM)				
		Glu.	Arxyl	Lig.	Ash	1	2	3	4	5	1	2	3	4	5
1	20	35.3	26.5	22.8	1.3	12.7±0.1 ^b	7.0 ±0.7 ^b	12.3 ±0.2 ^a	14.9 ±0.1 ^b	14.5 ±0.4 ^a	15.3 ±1.0 ^b	20.4±1.7 ^b	19.5±0.2 ^b	19.8 ±0.3 ^b	22.9±1.0 ^b
	34	34.9	27.2	22.6	1.4	15.4±0.1 ^a	10.8±0.1 ^a	12.8±1.1 ^a	17.5±0.6 ^a	14.9±0.7 ^a	18.3±2.2 ^a	25.0±1.2 ^a	29.1±0.7 ^a	21.7±0.7 ^a	31.5±2.0 ^a
2	30	39.6	25.4	20.6	1.4	6.7±0.1 ^b	16.0±1.0 ^a	11.5±0.2 ^a	17.0±1.2 ^a	14.6±0.5 ^a	19.7±0.8 ^a	27.2±1.6 ^a	26.6±0.3 ^b	23.1±0.6 ^a	25.1±0.3 ^a
	58	38.9	25.5	20.0	1.4	10.7±0.3 ^a	9.6±0.4 ^b	12.8±0.1 ^b	17.5±0.9 ^a	13.9±0.8 ^a	14.1±1.2 ^b	19.6±1.8 ^b	30.6±1.0 ^a	20.2±0.1 ^b	24.3±0.9 ^a
3	57	38.0	25.1	17.4	2.0	13.3±0.8 ^a	9.8±0.9 ^a	12.0±0.3 ^a	17.1±0.5 ^a	15.6±0.2 ^a	12.7±1.5 ^b	22.6±1.1 ^a	18.8±0.2 ^b	18.8±0.7 ^b	21.2±0.7 ^b
	63	38.6	25.4	17.7	1.9	7.5±0.7 ^b	11.0±0.7 ^a	9.7±0.2 ^b	14.8±0.8 ^b	14.3±0.1 ^b	25.6±1.4 ^a	22.4±0.2 ^a	30.4±0.5 ^a	26.0±0.1 ^a	29.8±0.3 ^a
4	54	36.8	26.9	20.7	1.7	6.9±0.2 ^a	9.9±0.2 ^b	12.0±0.3 ^a	17.9±1.1 ^a	14.1±1.9 ^a	18.5±1.0 ^b	24.5±1.0 ^a	23.3±1.7 ^b	23.3±0.6 ^b	22.3±0.0 ^a
	94	36.7	27.3	19.3	1.8	7.6±1.2 ^a	11.3±0.1 ^a	8.3±0.6 ^b	12.8±1.2 ^b	16.2±0.8 ^a	23.5±1.7 ^a	18.9±1.1 ^b	28.3±1.5 ^a	26.3±0.7 ^a	20.8±0.4 ^b
5	88	35.6	25.7	20.2	2.5	8.3±0.1 ^b	9.6±0.8 ^a	9.7±0.6 ^a	17.0±0.2 ^b	15.6±1.0 ^a	18.0±1.3 ^a	26.3±0.9 ^a	27.8±0.4 ^a	21.1±0.7 ^a	24.3±0.4 ^a
	97	35.3	26.1	20.0	2.6	11.8±1.0 ^a	8.4±0.7 ^a	11.2±1.0 ^a	18.2±0.3 ^a	15.6±0.7 ^a	18.8±0.7 ^a	24.1±1.0 ^a	23.5±0.3 ^a	22.2±1.1 ^a	24.9±1.3 ^a
6	103	36.1	28.3	18.4	0.9	8.3±0.1 ^b	14.7±0.3 ^a	7.3±0.4 ^a	16.0±0.3 ^b	15.7±0.8 ^a	27.2±0.1 ^a	25.5±0.3 ^b	30.5±0.8 ^a	25.0±0.9 ^a	30.0±1.2 ^a
	105	36.3	27.3	19.1	0.8	16.2±0.3 ^a	14.9±0.1 ^a	6.3±0.1 ^a	18.1±0.4 ^a	16.3±0.4 ^a	24.6±0.5 ^b	27.0±0.6 ^a	30.0±0.5 ^a	23.6±0.7 ^a	28.7±1.0 ^a
7	106	38.1	29.5	16.6	0.9	17.2±0.7 ^a	14.2±1.1 ^a	11.8±0.4 ^a	16.1±0.3 ^a	15.9±0.3 ^a	25.4±0.4 ^b	29.8±0.4 ^a	33.8±0.1 ^a	25.0±0.8 ^b	29.1±0.3 ^a
	114	38.4	30.0	16.1	0.9	15.3±0.2 ^b	15.7±0.3 ^a	7.3±1.2 ^b	18.6±0.4 ^b	15.1±0.1 ^b	27.8±0.2 ^a	24.5±0.8 ^b	32.0±0.2 ^b	27.9±0.8 ^a	25.4±0.5 ^b

Glu. Arxyl. Lig. means glucan, arabinoxylan and lignin, respectively in the raw material

1, 2, 3, 4, and 5 are the pretreatment conditions (150°C, 0.96%, 15 min); (190°C, 0.07%, 15 min); (200°C, 0%, 10 min) (160°C, 0.96%, 15 min) and (180°C, 0.5%, 15 min)

Average ± standard deviation

The values in the column for each group having similar superscript letters do not differ between each other at a significance level of 0.05.

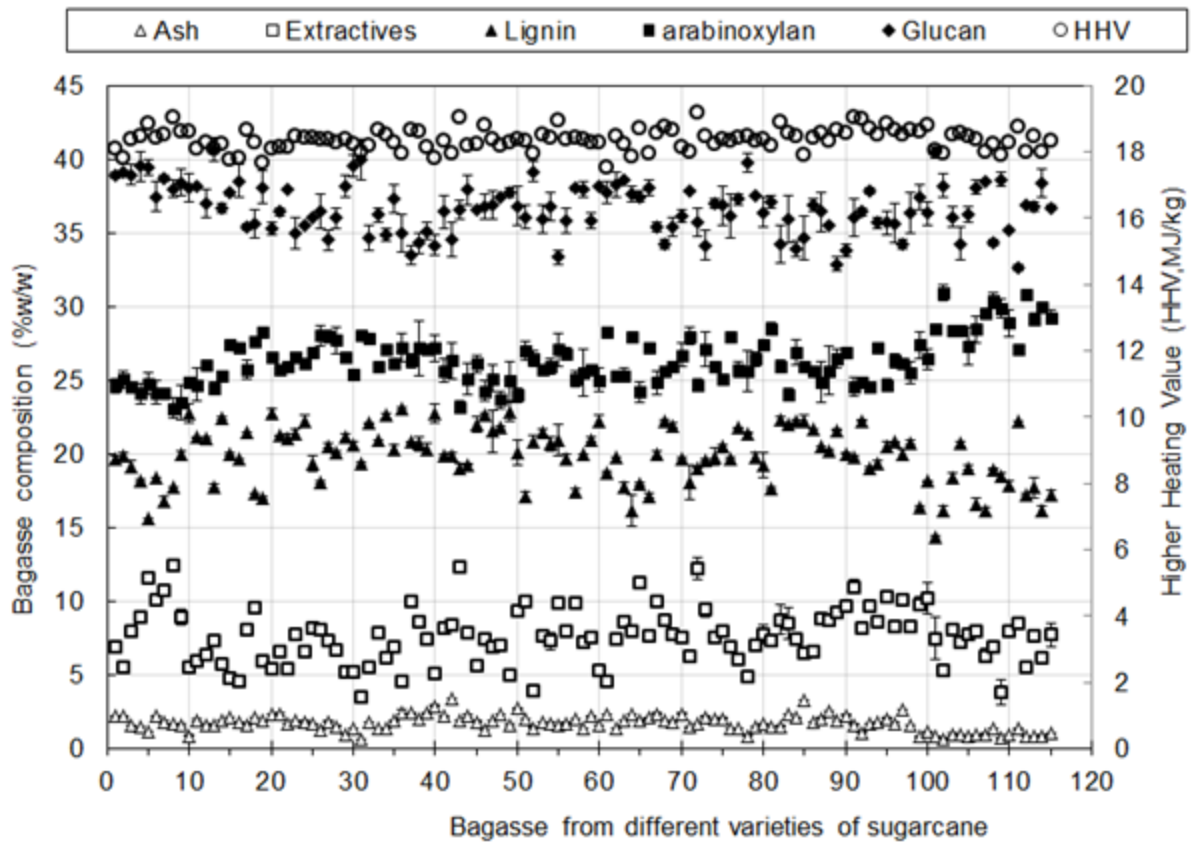


Figure 4-1: Average chemical composition and higher heating values of bagasse from 115 varieties of sugarcane. The error bars represents the variation of four replicates.

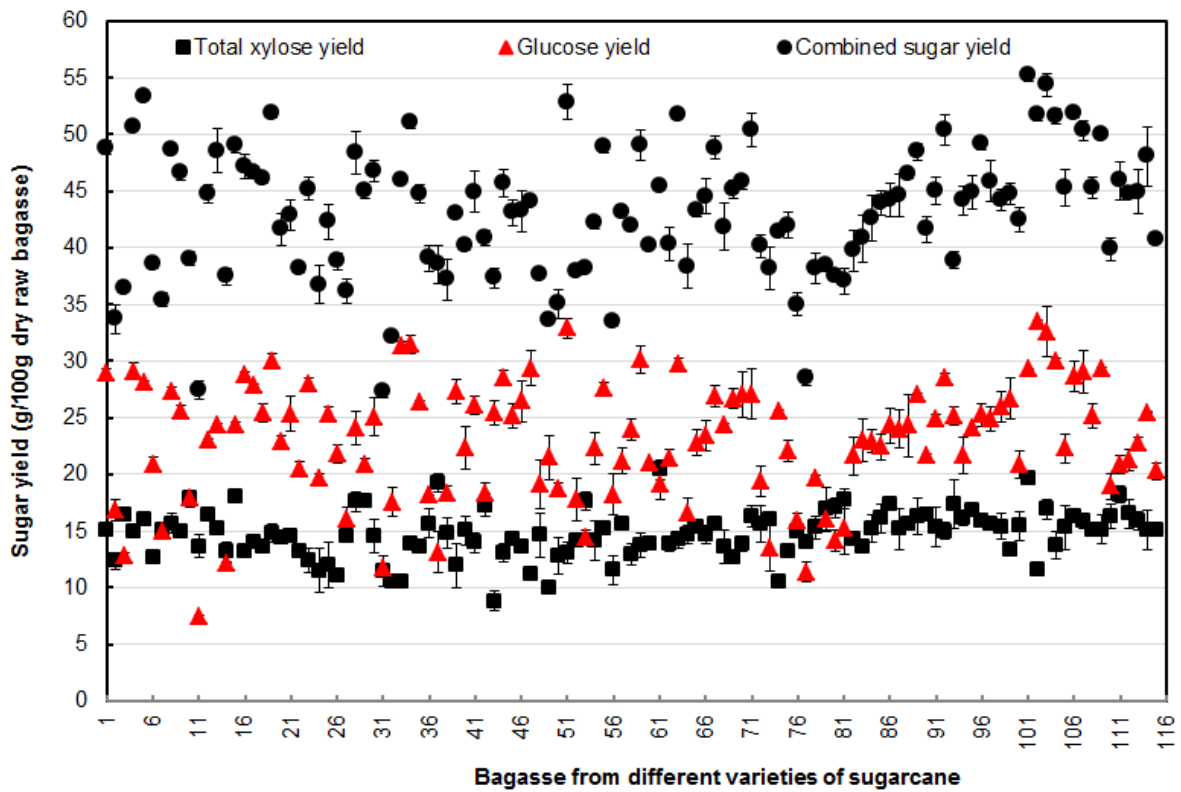


Figure 4-2: Variations of xylose, glucose, and combined sugar yields of bagasse from 115 varieties of sugarcane after pretreatment at 180°C, 0.5%w/w H₂SO₄ and 15 min and enzymatic saccharification at 15 FPU/g WIS.

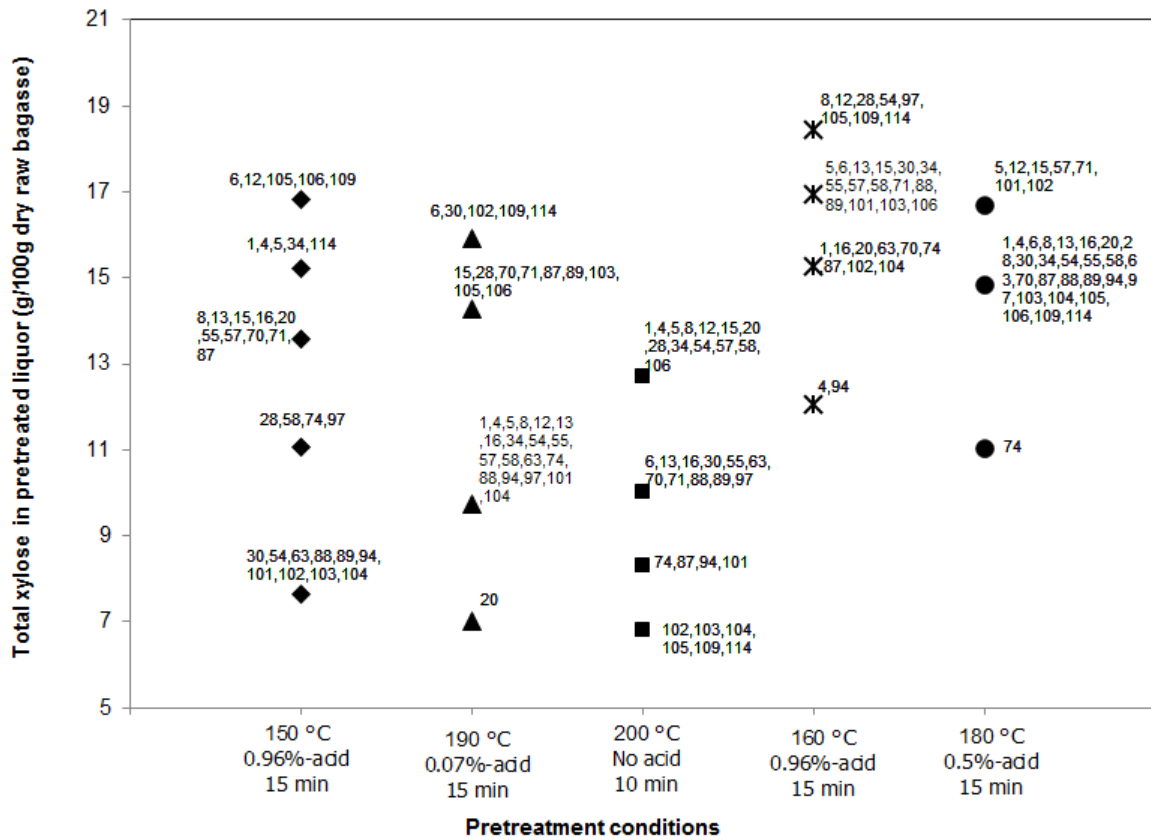


Figure 4-3: Average total xylose yields of bagasse from 34 varieties of sugarcane as the function of pretreatment conditions. Values for varieties listed in each group at a specific pretreatment condition are not significantly different to each other at 95% confidence interval.

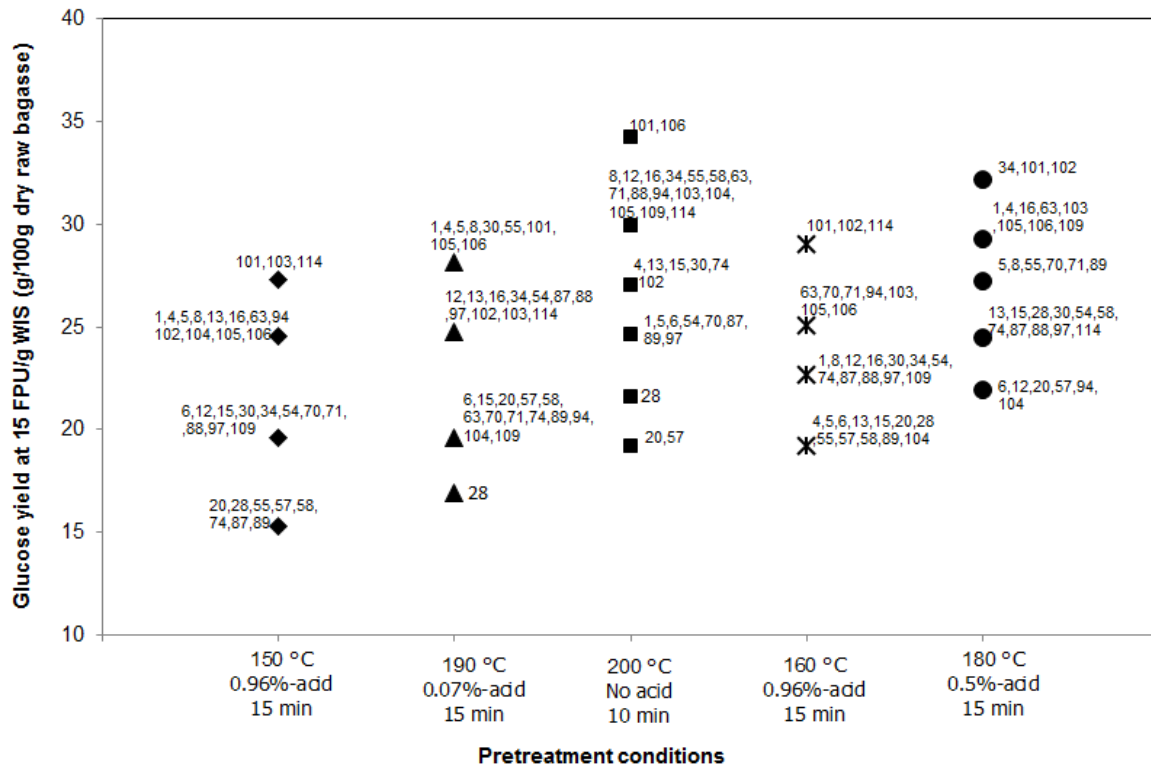


Figure 4-4: Average glucose yields at 15 FPU/g WIS of bagasse from 34 varieties of sugarcane at different pretreatment conditions. Values for varieties listed in each group at a specific pretreatment condition are not significantly different to each other at 95% confidence levels.

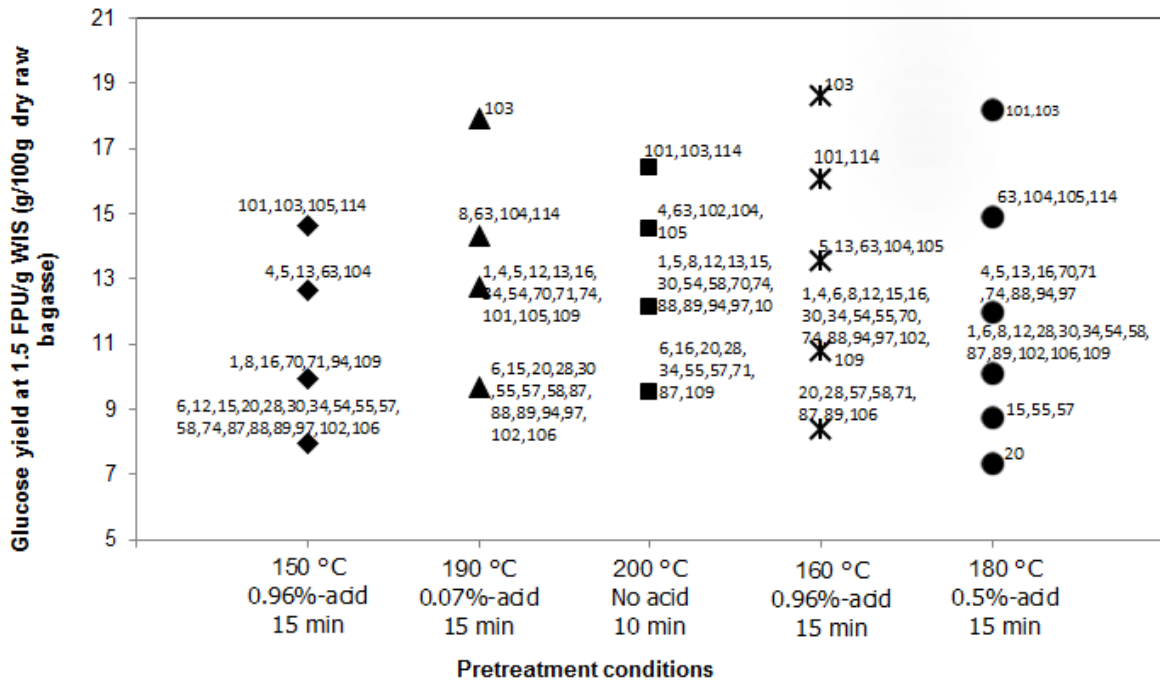


Figure 4-5: Average glucose yields at 1.5 FPU/g WIS of bagasse from 34 varieties of sugarcane different pretreatment conditions. Values for varieties listed in each group at a specific pretreatment condition are not significantly different to each other at 95% confidence interval.

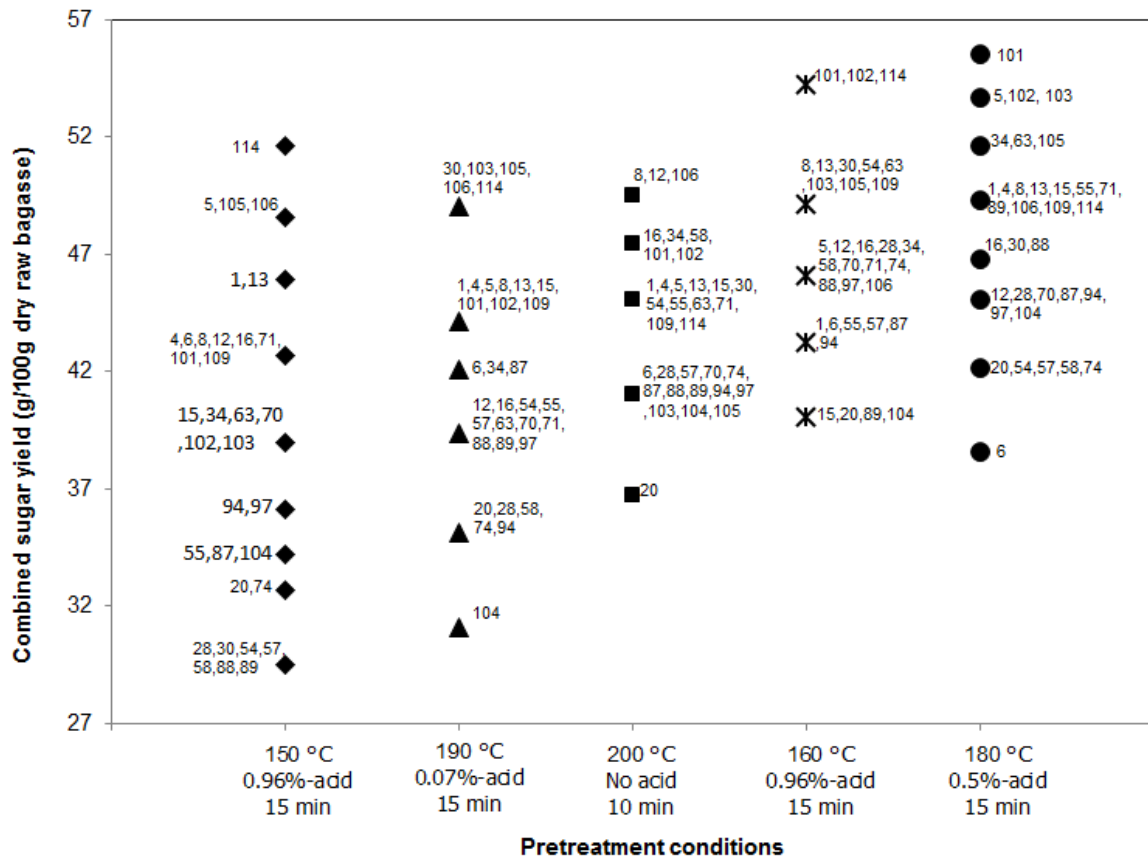


Figure 4-6: Average combined sugar yields of bagasse from 34 varieties of sugarcane as the function of pretreatment conditions. Values for varieties listed in each group at a specific pretreatment condition are not significantly different to each other at 95% confidence interval.

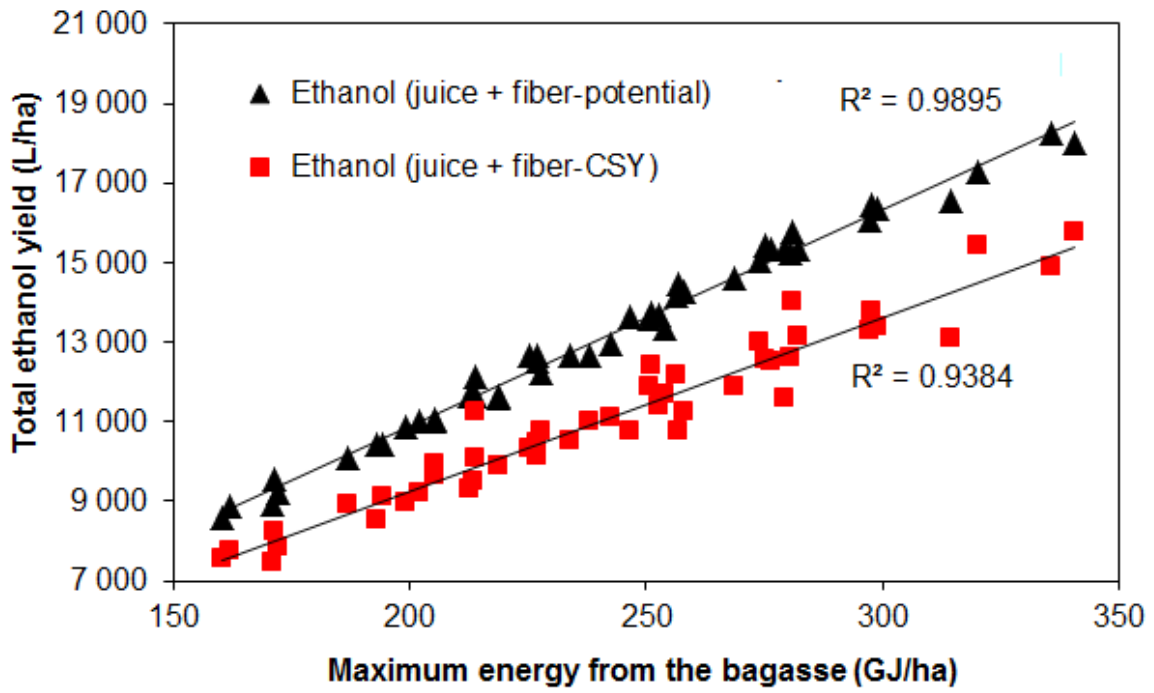


Figure 4-7: Correlation between total potential ethanol yield per unit hectare and the maximum energy from the bagasse per unit hectare for 48 varieties of sugarcane.

Chapter 5

5. Optimisation of dilute acid pretreatment for maximising combined sugar yield from sugarcane varieties with different chemical composition

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Title: “*Optimisation of dilute sulphuric acid pretreatment to maximise combined sugar yield from sugarcane bagasse for ethanol production*”

Authors: Yuda Benjamin, Hongbin Cheng and Johann Görgens

Objective of dissertation and summary of findings in present chapter

This chapter addresses **objective 2**. The preferred varieties of sugarcane (three from each breeding technology) were treated by varying temperature, acid concentrations and residence time in a central composite design (CCD) manner. The pretreated solids obtained were enzymatic hydrolysed to determine combined sugar yield. The results obtained were used to demonstrate (i) the importance of pretreatment optimisation on increasing sugar yield (ii) impact of cultivar selection on improving bioconversion and increasing sugar yield per harvested ton of bagasse. Furthermore, the results were used to preliminary assess the optimum pretreatment that can give maximum combined sugar yield from different varieties.

The results showed that optimisation improved CSY from 9 to 18% compared to the results obtained before optimisation (during screening). The differences in CSY among varieties at optimum conditions were significant (55.1-67.6 g/100 g dry raw material). It was found that the CSY could be increased up to 34.1% by selecting the best performing variety compared to industrial bagasse. It was also found that the difference in CSY between varieties was decreasing with the increase in pretreatment severity.

Candidate declaration

With regard to chapter 5 page numbers 105–141 of this dissertation, the nature and scope of my contribution were as follows.

Name of contribution	Extent of contribution (%)
Planning of experiments	60
Executing experiments	100
Interpretation of results	80
Writing the chapter	100

The following co-authors have contributed to chapter 4 page numbers 105–141 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Hongbin Cheng	hcheng@sun.ac.za	<ul style="list-style-type: none"> • Experimental planning • Reviewing the manuscript 	<p style="text-align: right;">10 30</p>
2. Johann Görgens	jgorgens@sun.ac.za	<ul style="list-style-type: none"> • Experimental planning • Interpretation of results to correlate with literature • Reviewing the manuscript 	<p style="text-align: right;">30 20 70</p>

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 105–141 in the dissertation,
2. no other authors contributed to chapter 5 page numbers 105–141 in the dissertation besides those specified above , and
3. potential conflicts of interest have been revealed to all interested parties and that any necessary arrangements have been made to use the material in to chapter 5 page numbers 105–141 of this dissertation.

Signature	Institutional affiliation	Date

Abstract

Increasing fermentable sugar yields per gram of biomass depends strongly on optimal selection of varieties and optimisation of pretreatment conditions. In this study, dilute acid pretreatment of bagasse from six varieties of sugarcane was investigated in connection with enzymatic hydrolysis for maximum combined sugar yield (CSY). The CSY from the varieties were also compared with the results from industrial bagasse. The results revealed considerable differences in CSY between the varieties. Up to 22.7% differences in combined sugar yield at the optimal conditions was observed. The CSY difference between the best performing variety and the industrial bagasse was 34.1%. High ratio of carbohydrates to lignin and low ash content favoured the release of sugar from the substrates. At mild pretreatment conditions, the differences in bioconversion efficiency between varieties were greater than at severe condition. This observation suggests that under less severe conditions the conversion efficiency was largely determined by the properties of the biomass. The results from this study support the possibility of increasing sugar yields or improving the conversion efficiency when pretreatment optimisation is performed on varieties with improved properties.

Key words: sugarcane bagasse, pretreatment, enzymatic hydrolysis, combined sugar yield, optimisation.

5.1. Introduction

Sugarcane represents a preferred crop for bioenergy (ethanol) production due to high biomass yields, and high fermentable sugar content [1]. However, the fibrous residue (bagasse) generated after sugar juice (mainly sucrose) extraction for ethanol production may also be used to increase the ethanol yield per ton of harvested cane. However, converting sugarcane bagasse (SB) that is recalcitrant to enzymatic hydrolysis into fermentable sugars requires a costly pretreatment process to make its structural carbohydrates more accessible [2,3]. One of the strategies of reducing pretreatment cost is to improve feedstock quality

(high structural carbohydrates content, and high convertibility) through crop development, and selection with the view of maximising ethanol output from both sugar juice, and bagasse per unit land.

The feedstock quality of sugarcane varieties can be improved through plant breeding by classical or genetic engineering, and both of these have shown the possibility of producing sugarcane lines which are less recalcitrant to bioconversion without affecting plant performance in controlled environmental conditions [4, 5]. In order to identify sugarcane varieties with improved potential for combined ethanol production from both sugar juice and bagasse, samples of varieties in the breeding program at South African Sugarcane Research Institute (SASRI) were screened. Of this collection, 100 varieties from classical breeding were selected on the basis of high biomass yields, while an additional 15 varieties from precision breeding (genetic engineering), aimed at increasing soluble sugar content, were also included. These 115 varieties were screened in terms of potential ethanol yields per hectare from both sugar juice, and bagasse, as reported previously [6]. After screening, the next step was pretreatment optimisation to fully demonstrate the advantage of variety selection on fermentable sugar yield from the bagasse.

Dilute sulfuric acid (DSA) pretreatment represents the most widely researched technology on different types of feedstocks ranging from agricultural residues to woody and herbaceous crops [2,7–9]. In this method, the soaked material was held at elevated temperature for a specific period of time. Hemicellulose was hydrolysed into the liquid fraction, leaving the solid material porous, enriched with cellulose and lignin [10]. The removal of hemicellulose weakens the carbohydrates–lignin matrix structure, thus increasing cellulose accessibility. Nevertheless, in severe conditions, pentose and hexose sugars may turn into non-sugar compounds [9]. Therefore, optimisation of pretreatment conditions is important for efficient conversion of lignocellulose material into fermentable sugars.

Optimisation of pretreatment conditions for either xylose recovery or cellulose digestibility has been actively researched [11–16]. Working on corncob, Cai et al. [17] found that the condition for the highest xylose yield was less severe compared with that for the maximum glucose yield after enzymatic saccharification. However, the condition for the

highest glucose yield also resulted in high sugar degradation. Other researchers have proposed a two-step process to minimise sugar degradation [10, 18]. The first step is performed at low temperature to target xylose and the other is conducted at high temperature for high glucose yield. However, such a suggestion raises the question of economics and energy costs.

Another approach is to find the pretreatment conditions that could maximise the combined sugar yield (CSY) (the total pentose and hexose sugars released after the combined pretreatment and enzymatic hydrolysis), while keeping the by-products formation as low as possible. Lloyd and Wyman [19], working with corn stover showed that the optimisation of xylose yield did not lead to maximum CSY. Similarly, the maximum glucose yield from enzymatic hydrolysis also failed to release the highest CSY. The conditions that provide maximum sugar yield are therefore a compromise between those for maximum xylose recovery and those for maximum glucose yields. However, to the best of the authors' knowledge, none of these research studies have considered the optimisation of the CSY of bagasse coming from different sugarcane varieties.

The objective of this study was to investigate the effects of the DSA pretreatment conditions (temperature, acid concentration, and reaction time) on the pretreatment and the enzymatic hydrolysis responses of the bagasse from the six selected varieties of sugarcane and one sample of industrial origin. The purpose was to identify varieties with reduced pretreatment requirements. To maximise CSY, the optimisation was performed according to a central composite design (CCD) under response surface methodology (RSM) as a statistical method.

5.2. Materials and methods

5.2.1. Raw materials and samples preparation

The bagasse samples from six varieties sugarcane used in the present study were supplied by SASRI. The varieties were developed through classical and precision breeding technologies. The precision breeding varieties were developed by down regulating

expression of an endogenous enzyme UDP glucose dehydrogenase as described elsewhere [20]. The feedstocks were sampled from mature sugarcane (12 months old) in an experimental field located at Mount Edgecombe (29.7000° S , and 31.0333° E), KwaZulu-Natal in November 2009. The genotypes were first planted field trial in 2006. This means that the bagasse evaluated in this study were from third ratoon crops. The varieties 99F2004⁵⁵, 00F0884⁷⁰, and 01G1662⁷⁴ were derived from classical breeding and 05TG004¹⁰¹, 05TG008¹⁰⁴, and 05TG018¹¹⁴ were derived from precision breeding. The superscripts 55, 70, 74, 101, 104, and 114 will be used to describe and discuss the genotypes further in the manuscript. The detailed on how these substrates were sampled, prepared and analysed for chemical compositions shown in Table 5-1 is reported elsewhere [6].

The industrial SB (labelled 120) was provided by TSB Sugar Mill in Malelane, Mpumalanga, South Africa. The sample was washed four times and each wash was collected and measured for residual sugar content. The washed bagasse was oven dried at 40°C for 72 hours, followed by milling them in a laboratory ultra-centrifugal mill model ZM200 basic (Resch GmbH, Germany). The milled sample had a moisture content of 5%. Prior to its use, the milled samples were sieved in a vibratory sieve shaker model AS200 basic (Resch GmbH, Germany) to obtain a representative particle size suitable for the raw material composition analysis and for the pretreatment studies. The particles retained between 425 and 825 µm were packed in plastic bags and then stored in a temperature and moisture-controlled room set at 20 °C and relative humidity of 65% until needed.

5.2.2. DSA pretreatment

DSA pretreatment was carried out in small tubular batch reactors, according to Yang and Wyman. [21]. Dry material (DM, 1.5 g) was soaked in 30 ml of DSA solution for 12 hours. Soaked samples were concentrated through filtering to a solid loading of 30% (w/v). The obtained wet biomass was loaded into the reactor and compressed by a metal rod to ensure uniform heat and mass transfer. The reactor was first submerged into a heating-up fluidised s, and bath set at 30 °C above the target temperature. The reactor was heated until the

target temperature was reached (approximately within 120 seconds), after which it was transferred into the second fluidised sand bath set at the target reaction temperature. After the reaction time was completed, the reactor was quenched by submerging into a cold water bath. After cooling, the whole slurry was mixed with 100 ml of distilled water and vacuum-filtered into a solid and a liquid fraction. One part of liquid fraction was analysed for major monomeric sugars (xylose, glucose, and arabinose); sugar degradation (furfural, and HMF); and acetic acid formation; and the other part was used to determine the total sugars in the pretreated liquor (monomers and oligomers) by post-hydrolysis as described below. The solid fraction was further washed in three washes (each wash with 100 ml) to raise the pH up to 5 prior to enzymatic hydrolysis, and is subsequently referred to as water insoluble solids (WIS).

5.2.3. Experimental design and optimisation

A CCD under RSM was selected to determine the relationship between temperature, acid concentration and time as the main pretreatment parameters. The experimental conditions were designed by Design Expert, version 8.0.2 (State Ease Inc., Minneapolis, MN, USA). Two-level, three-factor CCD was used to determine conditions leading to maximise the CSY as dependent response variable. The number of experiments was six at axial points, six replicates at centre point and eight at factorial points leading to 20 runs ($2^3 + 2 \times 3 + 6 = 20$). The independent variables in real values are shown in Table 5-2. The range and levels of independent variables were selected based on the results obtained after a preliminary study on all samples (data not shown). Variables in coded values were calculated by Design Expert. The values for the axial points, factorial points and centre point were (-1.682 at the lowest point and +1.682 at the highest point), (-1 and +1), and (0), respectively. The second order quadratic model with interactions in coded form was used to predict the optimal pretreatment condition maximum CSY as expressed in Eq. 1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y , X_1 , X_2 , , and X_3 stands for CSY, temperature, acid concentration, , and reaction time respectively in coded form; β_0 , β_1 , β_2 , β_3 ; β_{11} , β_{22} , β_{33} , β_{12} , β_{13} , , and β_{23} are regression coefficients estimated from the experimental data.

5.2.4. Enzymatic hydrolysis

The WIS fraction was subjected to enzymatic hydrolysis to evaluate the effect of the pretreatment on the enzyme accessibility. These experiments were conducted in 24 ml glass tubes. The tubes were loaded with 200 mg (dry weight) of WIS and 10 ml of 0.05 M citrate buffer (pH 4.8) with the enzyme solution. Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial contamination. Two commercial enzymes preparations were used: 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 enzyme activities of both undiluted enzymes were determined by applying analysis protocol described elsewhere [22]. Cellulase loading of 32.31 mg protein/g WIS (corresponding to 15 FPU/g WIS) of Spezyme CP supplemented with β -glucosidase of 2.02 mg protein/g WIS (equivalent to 15 IU/g WIS) was applied in all the experiments. Tubes loaded with the mixtures were placed in water bath shaker maintained at 50 °C with shaking at 90 rpm. Samples were withdrawn after 72 hours, prepared as described below and analysed for sugars by HPLC.

5.2.5. Post-hydrolysis

After the pretreatment, a 5 ml sample of the pretreatment liquor was taken to perform post-hydrolysis by using 72% sulfuric acid, according to NREL procedure [23], to determine the content of oligomeric carbohydrates. Finally the sample was prepared for sugars detection by HPLC as described below.

5.2.6. Chemical composition analysis methods

The NREL procedure described by Slutter et al. [24–26] was used for the chemical composition analysis after being consecutively extracted with water, and with 95% ethanol for 48 hours in total in a Soxhlet apparatus. For the pretreated material the same procedure was used, except that no water or ethanol extraction was carried out because of pretreatment to remove most of extractives. The acid soluble lignin of the pretreated material was not measured.

The concentration of glucose, xylose, arabinose and acetic acid from the liquid fractions resulting from untreated and pretreated materials compositional analysis, pretreated liquor, post-hydrolysis and enzymatic hydrolysis were quantified by HPLC on an Aminex HPX-87H Column equipped with 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 sulfuric acid and a flow rate of 0.6 ml/min. Sugars and acetic acid concentrations were measured with a RI detector (Shodex, RI-101) operated at 45 °C. Under these conditions, the column does not resolve xylose, galactose, and mannose. The presence of mannose, and galactose in untreated samples were checked on an Xbridge™ Amide column (4.6 x 250 mm, 3.5 µm particle size) equipped with an Xbridge™ Amide precolumn (Waters) at 30 °C, eluted at a rate of 0.7 ml/min with 0.05% ammoniumhydroxide in water (A), and 0.05% ammoniumhydroxide in 90% acetonitrile (B). Sugars were detected by a Varian 380-LC evaporative light-scattering detector. No mannose and galactose peaks were detected. Therefore, the xylose quantification obtained on an Aminex HPX-87H Column was accurate. The glucan, xylan, arabinan, and o-acetyl group contents were calculated as $(0.95 \times \text{cellobiose} + 0.9 \times \text{glucose})$, $0.88 \times \text{xylose}$, $0.88 \times \text{arabinose}$, and $0.683 \times \text{acetic acid}$, respectively [25].

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) 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 5mM

trifluoroacetic acid in water (A) and 5mM trifluoroacetic acid in acetonitrile (B). Separation was carried out by gradient elution from 5% mobile phase B, increasing to 11% B over 14 minutes and then increasing to 40% B over 3 minutes. The mobile phase composition was then kept constant at 40% for 2 minutes, followed by a decrease to 5% B over 5 minutes and ending with a final step of constant composition at 5% B for 4 minutes in order to equilibrate. HMF and furfural concentrations were measured with a Dionex Ultimate 3000 diode array detector at 215 and 285 nm.

5.2.7. Data and statistical analysis

The composition of raw material was performed in four replicates. The average values and standard deviation (average \pm standard deviation) was used to present the results. For the pretreatment and enzymatic hydrolysis the experiments were performed in duplicate and the average values were presented in the Tables 5-2, 5-3 and 5-4.

The Design Expert, version 8.0 (State Ease Inc., Minneapolis, MN, USA) was applied for the regression analyses, and was also used to find the optimum values of CSY. The fitness of the second order polynomial model obtained from the regression analysis was evaluated by coefficient of determination R^2 , and its statistical significance was checked by F-test at a probability ($p < 0.05$). The student's t-test was also performed to determine the statistical significance of the regression coefficients. The STATISTICA software, version 10 (Statsoft Inc., Tulsa, USA) was employed to generate the response surface plot.

The comparison of the responses of the SB samples was facilitated by employing factorial ANOVA. The significant differences in sugar yields among the samples were confirmed by Bonferroni's post hoc test. The hypothesis was accepted or rejected at 95% confidence interval. Likewise, the correlation coefficients were calculated using STATISTICA (software, version 10).

5.3. Results

5.3.1. Feedstocks chemical composition

The chemical composition of the bagasse samples obtained from classical breeding varieties (55, 70, and 74), and precision breeding varieties (101, 104, and 114) are summarized in Table 5-1. The industrial bagasse (120), obtained from sugar mill, was used as reference material. The samples showed considerable variations in chemical components. The values for glucan, xylan, arabinan, lignin, acetyl group, extractives, and ash in dry weight basis ranged from 34.1 to 40.7, 19.5 to 27.2, 1.3 to 2.7, 14.4 to 22.4, 2.8 to 3.2, 3.8 to 6.2, , and 0.8 to 2.0% as, respectively. The sum of all components measured varied between 90% and 96.5%. This could be attributed to components that were not quantified (i.e. methyl glucuronic acid) and some degradation of the sugars occurring during the acid hydrolysis. The sum of glucan, xylan and arabinan makes the measured total structural carbohydrate vary from 60.5 to 69.2%. This means that upon hydrolysis the potential sugar released as monomeric (glucose, xylose and arabinose) ranges between 67.7 and 77.6 g/100 g DM, which make these materials promising feedstocks for ethanol production.

5.3.2. Effect of pretreatment conditions on WIS composition

Table 5-2 shows the composition of the WIS after DSA pretreatment expressed as percentage of theoretical values. Acid soluble lignin was not measured after pretreatment because its amount was not significant. The recovered WIS was between 50.1 and 76.5 g/100 g raw material (RM) (RM) (Appendix B-2), with the lowest value being at the harshest condition (190 °C. 0.85% (w/w) for 15 minutes). The sum of glucan, acid insoluble lignin, and xylan accounted for 79.3 to 96.8% of the WIS recovery. Glucan was less hydrolysed in most of the pretreatment conditions. The glucan solubilisation ranged from 4 to 16% theoretical. Similarly, no significant differences in the acid insoluble lignin before and after pretreatment at many instances were observed, except at severe conditions where the acid insoluble lignin was higher than the initial values (up to 108% theoretical). This could be related to the lignin condensation phenomena when severe condition is applied as previously reported elsewhere

[18]. The Bonferroni's post-hoc test revealed that variety 101 presented the highest ratio of glucan/acid insoluble lignin, whereas the industrial SB (120) had the lowest ratio (Appendix B-3). This suggests that variety 101 could be more digestible than others.

Hemicellulose is the prime target for acid hydrolysis. Since xylan is a largest component of hemicellulose (81.2–84.5%), it was used to describe hemicellulose hydrolysis. Xylan remained in the WIS was decreasing exponentially as the pretreatment severity (temperature, acid concentration, and reaction time) was increasing (Table 5-2). Up to 98.4% of theoretical xylan was hydrolysed. Industrial bagasse showed slightly lower xylan solubilisation than the rest.

5.3.3. Effect of pretreatment conditions on xylose hydrolysate fractions

The composition hydrolysate liquor after pretreatment across conditions is presented in Table 5-3. Other components measured were glucose, arabinose, and acetic acid (Appendix B-2). However, xylose and by-products (furfural and HMF) were selected to analyse the effects of the pretreatment because xylose is the major sugar (56 to 88.7%) while furfural and HMF are important sugar degradation products.

Xylose yield as a sum of monomeric and oligomeric sugar after applying various different conditions of DSA pretreatment is summarized in Table 5-3. Xylose yields ranged from 6.9 for variety 55 (170 °C, 0.45%, 5 min) to 20.2 g/100 g RM for variety 101 (180 °C, 0.65%, 10). Figure 5-1 depicts how temperature, acid concentration, and reaction time determined xylose yields. A clear trend was observed of increasing temperature, acid concentration, and reaction time with increasing xylose yields to some extent (Figure 5-1a). Nevertheless, severe conditions resulted into a significant reduction in xylose yield (Figure 5-1d). The decrease in xylose yield at severe conditions suggests rapid destruction of xylose [27]. The highest yields were observed at 180 °C, 0.65%, 10 min for varieties 55, 70, 74, , and 101; , and 170 °C, 0.85%, 15 min for varieties 104 and 114. These conditions yielded 19.4, 19.4, 19.3, 20.2, 19.9, and 20 g/100 g RM, respectively, were corresponding to 69.4, 70.3, 70.8, 67.6%, 68.7, and 64.7% of xylose in native material (Table 5-1 and Table 5-3).

Alternatively, the highest yield from the industrial bagasse was found at 180 °C, 0.99%, 10 min (16.9 g/100g RM corresponding to 76.3% of theoretical) but it was significantly lower than the highest values obtained from the varieties (Table 5-3). Nevertheless, the precision breeding varieties (101, 104, and 114) exhibited improved xylose yield per gram of biomass than classical breeding (Figure 5-2).

As expected, furfural and HMF production increased with increasing in pretreatment severity (Table 5-3). The highest furfural and HMF yields were 4.37 and 0.49 g/100 g RM, respectively. HMF formation was directly correlated with glucose measured in the hydrolysate liquor (Appendix B-4). In the case of furfural, no correlation was observed with xylose lost during pretreatment. The lack of correlation suggests that xylose degraded into other compounds rather than furfural alone [11,28]. In general, varieties 70 and 114 showed higher HMF formation than others whereas samples 55, 104, and 120 showed the lowest. Additionally, the formation of furfural was much faster in variety 101 than the rest while SB 120 showed the least.

5.3.4. Effect of pretreatment conditions on enzymatic digestibility of WIS

The effectiveness of DSA pretreatment on cellulose digestibility was evaluated in terms of glucose yield (EH glucose) after enzymatic hydrolysis of the WIS and the results are presented in Table 5-4. Increasing temperature, acid concentration, and reaction time) significantly enhanced EH glucose yields (Figure 5-3). For example, 71.3% improvement in glucose yield for variety 70 was obtained when pretreatment temperature increased from 170 to 190 °C (Figure 5-3a). However, temperature was less important for glucose yield for variety 104 in many instances. As such, no significant difference in EH glucose yields were observed when the temperature was increased from 170 to 190 °C (Figure 5-3b-d).

Comparative analysis of EH glucose yields between varieties revealed considerably variations. Up to 86.8% differences in glucose yield was observed at the mild condition (170 °C, 0.45% for 5 min). Remarkably, under this condition (170 °C, 0.45% for 5 min), glucose yield achieved by the best performing variety (101) of 31.2 g/100 g RM was statistically

comparable to the maximum yield obtained by the poor performing varieties (70 , and 74) of 31.9–32.3 g/100 g RM or by the control (120) of 33.7 g/100 g RM. Ranking the varieties based mean EH glucose yields across experiments 1 to 8, the data clearly demonstrates how specific variety outperforms others (Figure 5-4). The yields differences between the varieties were in the order of 101> 1114> 55> 104> others (70, 74).

Figure 5-5 systematically compares glucose recovery (calculated as EH glucose yield divided by potential glucose in the WIS expressed as percentage) from best variety 101 vs. medium performing (55) versus industrial bagasse (control) across selected conditions. DSA pretreatment increased considerably the glucan conversion giving values from (43.2-72%) for the less severe conditions to 100% for the harshest conditions. Varieties 55 and 101 seemed to be more digestible than the industrial bagasse. At mild conditions, the differences in glucose recovery between the samples were bigger but decreased at severe conditions.

5.3.5. Statistical modelling of CSY and validation

The experimental results on CSY summarized in Table 5-4 were fitted into the quadratic model (Eq. 1) to quantitatively estimate the effect of each independent variable on the CSY. The statistical significant of each factor was determined by ANOVA, which revealed that all process parameters influenced the CSY. Table 5-5 shows the model coefficients and significance term for each sample. All linear increased the CSY, except the acid concentration for variety 70, which showed the negative effect. The rest of the models coefficients impacted CSY in a negative manner. This suggests that the increases of temperature, acid concentration, and reaction time do not always translate into high CSY. Furthermore, the p-value of the model was lower than 0.005, indicating that the model was significant (Appendix B-5). The lack of fit was not significant, which imply that the models reasonably predict the experimental data (Appendix B-5). Likewise, the determination coefficient (R^2) of the model was also high (0.91–0.98), showing that more than 90% of the result variability was attributed to the process variables.

The models were plotted in a three dimensional surface response to understand the effects of the independent variables on CSY (Figure 5-6). The plots represent the interactions of the two independent variables, while the third variable is held constant. The analysis of the surface responses for samples 55, 70, 74, and 120 showed that the increase of temperature and reaction time improved the CSY to some extent. However, further increase of these two variables resulted in the considerable reduction of the CSY. Similar plots on CSY were observed for varieties 101, 104, and 114, when acid concentration and reaction time were varied while temperature was kept constant at the centre point (180 °C).

The numerical optimisation process was conducted to determine the optimal condition leading to maximum CSY. The optimisation criteria were set according to maximization of EH glucose and CSY and to minimization of by-products formation. The maximisation of sugars was set higher level of importance compared to furfural and HMF formation. After the optimisation procedures the optimal pretreatment condition and the maximum CSY were identified (Figure 5-7). The predicted maximum CSY was validated by performing extra experiments in triplicates at the predicted best pretreatment conditions and results are also depicted in Figure 5-7. There was a good agreement between the model prediction and the experimental data. The total sugar recoveries obtained after optimisation were 84.9, 79.3, 78.8, 87.1, 82.4, 79.0, and 74.5% of theoretical for samples 55, 70, 74, 101, 104, 114, and 120 respectively. This show an increase of up to 4.6% compared the highest average values obtained before optimisation (Table 5-4).

The optimal condition for each variety was substituted into all models (Table 5-5) and the values obtained were compared to the maximum CSY (Figure 5-7). The optimal conditions for varieties 70 and 74 under predicted the CSY for varieties 55, 101, and 104 up to 10.7%. Conversely, the optimal conditions for varieties 55, 101, 104, and 114, accurately estimated the CSY for all varieties. The estimated yields were within the experimental errors (0.1–2.4%).

5.3.6. The effect of chemical composition on xylose and EH glucose yields

The influence of chemical compositions on xylose and EH glucose yields was estimated by calculating correlation coefficients across pretreatment conditions (Table 5-6). Lignin and ash content showed negative correlation with xylose and EH glucose yields. Xylan, arabinan, and acetyl content positively correlated with xylose and EH glucose yields.

5.3.7. Mass balance

Figure 5-8 depicts the overall mass balance of the solids and liquid fractions of the best performing variety (101) and the control (industrial bagasse) after DSA pretreatment at the centre point conditions (180 °C, 0.65%, for 10 min). This condition was taken as an example to evaluate the material balance because it was replicated six times (experiments 15 to 20) and results were statistically reproducible (Table 5-4, Figure 5-6). In addition, CSY obtained by this condition was very close to the maximum values (Figure 5-7). The industrial bagasse showed higher WIS recovery (68.1%) than that of variety 101 (59.2%) due to higher acid insoluble lignin. The overall mass losses were 10.8% for variety 101 and 9.3% for industrial bagasse. The possible reason for these lost mass could be the decomposition and degradation of xylan into other compounds than furfural, which were not measured by HPLC. In addition, this study did not quantify the acid soluble lignin in the liquid fractions after pretreatment. Other components such as ash and extractives were also not measured after the pretreatment. All this could contribute to the above losses. Despite the overall mass balance losses, the overall material balances of glucan, arabinan, and acid insoluble lignin of both samples were above 93%, showing that the analytical methods used were accurate.

5.4. Discussion

5.4.1. A combination of pretreatment optimisation and feedstock selection for increases sugar yields

Xylose yields after pretreatment (Table 5-3), EH glucose yields after enzymatic hydrolysis (Table 5-4), and combined sugar yields after pretreatment-hydrolysis (Table 5-4),

varied significantly, in part depending on the pretreatment condition, and the chemical composition, with the latter distinguished the varieties at the same pretreatment condition. Increasing pretreatment severity (temperature, acid concentration, and reaction time) led to improved sugar yield from the varieties (Figure 5-1, Figure 5-3 and Figure 5-6). However, severe conditions reduced the xylose yield as well as CSY due to excessive degradation of xylose to furfural (Table 5-3). The exponential accumulation of furfural with the increase of pretreatment severity has been reported elsewhere [29]. Conversely, severe conditions, particularly, high temperatures promoted to glucose yield/recoveries by producing highly digestible solids [18,27]. The effectiveness of DSA pretreatment method is primarily based on solubilisation of hemicellulose thereby increases the available surface area, making cellulose more accessible to enzymatic hydrolysis [2,10]. In addition, high temperature assists in weakening the structure of the biomass, which further increases digestibility [27].

Furthermore, varieties with lower lignin, lower ash, and higher structural carbohydrates content showed higher yields of xylose and EH glucose as well as CSY (Figure 5-2, Figure 5-4, Figure 5-7; Table 5-1 and Table 5-6). This favoured most of the precision breeding varieties over the majority of the classical breeding varieties. As such, most of the precision breeding varieties showed improved xylose yields (Figure 5-2), glucose yields (Figure 5-4) as well as CSY (Figure 5-7). Nevertheless, this is the first study to observe the influence of chemical composition on xylose yield [30–32]. Most of these correlations reported in literature were based on a single pretreatment condition. Similar conclusion could also be drawn in the current study by utilizing single pretreatment condition but that did not lead to strong correlation. The weaker relationship between chemical composition, and xylose yields could be related to sugar degradation.

To date, CSY after DSA pretreatment-hydrolysis of SB from South Africa mill has not been higher than 49.5 g/100 g RM [33], which was similar to 50.4 g/100g RM for industrial bagasse obtained in the present study. According to our results the CSY could be increased up to 34.1% by selecting the best performing variety (Figure 5-7). These results clearly demonstrate the importance of pretreatment optimisation and feedstock selection for increasing fermentable sugar yield per gram of biomass. With the latter is depending on

feedstock quality, i.e. high structural carbohydrates content, reduced lignin content and improved digestibility.

5.4.2. Feedstock quality determines bioconversion efficiency of biomass

Bioconversion trend observed for the recovery of glucose across pretreatment conditions was attributed to chemical composition differences among the varieties. The glucose recovery was higher for the samples with improved quality i.e. high ratio of carbohydrates: lignin and low ash content (Figure 5-5; Table 5-1 and Table 5-6). It was further observed that the differences in the recovery between the samples were greater at mild conditions than at severe conditions. This observation is in agreement with a most recent work on maize genotypes [34], which demonstrated that digestibility of corn stover at sup-optimal pretreatment was largely determined by the chemical composition features but its influence was less evident when increasing pretreatment severity.

Furthermore, from an economics point of view, the feasibility of cellulosic ethanol production at the industrial scale is conditioned to the efficient release of sugar (currently glucose) from lignocellulose biomass [35,36]. This favours severe conditions for maximum cellulose conversion (Figure 5-5). However, high pretreatment severity implies high energy or chemical demands. In addition, severe conditions results into lower fibre recovery in the process and xylose degradation (Figure 5-1 and Table 5-3). Biorefinery industry is currently seeking for a new solution that is able to reduce the production costs. Our results have demonstrated that high bioconversion efficiency could be obtained by applying less severe conditions (Figure 5-5). More recently, diverse studies on feedstock quality have proven that some of the intrinsic properties of the crop are heritable [37–39]. Therefore, the crop properties that caused the best performing variety to have reduced recalcitrance to pretreatment and enzymatic hydrolysis needs to be further investigated. Through this way, the processing costs might be reduced while increasing ethanol yield per gram of feedstock input.

5.4.3. Assessment of common optimal pretreatment conditions

One of the purposes of this study was to establish common optimal pretreatment conditions that could be applied during screening of sugarcane varieties. Previous optimisation of DSA pretreatment conditions determined that pretreatment severity around 3.5 was the best compromise between xylose recovery, and cellulose digestibility [40–42]. This fact also remained true for the optimal conditions for varieties 70 and 74. However, these conditions were slightly severe compared to best conditions (severity of 3.3–3.4) for other varieties, consequently, they underestimated maximum CSY for varieties 55, 101, 104, and 114, up to 10%. In contrast, the remaining four conditions (179 °C, 0.54%, 12 min; 177 °C, 0.7%, 10 min; 176 °C, 0.77%, 12 min, and 181 °C, 0.65%, 10 min) were appropriate for simulating maximum CSY from each variety. Therefore, any of these conditions can be applied for varieties screening, provided that experiments are conducted in a similar manner as that was used in the present study.

5.5. Conclusions

A combination of feedstock selection and pretreatment optimisation has the potential to improve conversion efficiency of the biomass and reduce pretreatment. In the present study, conversion efficiencies of bagasse from six varieties of sugarcane after DSA pretreatment and enzymatic hydrolysis were investigated. The results revealed considerable differences in sugar yields/recoveries among the varieties. The maximum CSY ranged from 55.1 g/100 g RM (78.8% of theoretical) to 67.6 g/100 g RM (87.1% of theoretical). The maximum CSY from industrial bagasse was only 50.4 g/100 g RM (74.5% of theoretical). Generally, high ratio of carbohydrate: lignin and reduced ash content favoured sugar yields from the samples. However, at severe condition, the bioconversion efficiency of the varieties was largely determined by the severity of the pretreatment. As such, the differences in glucose yield/recovery between varieties were smaller at severe condition than at mild condition. At mild conditions, glucose yield/recovery was higher to those varieties with higher substitution of structural carbohydrate, lower lignin and lower ash content. It was further established that

different sugarcane varieties had a common optimal pretreatment conditions for maximum CSY. Identification of such conditions is of high contribution to biofuels industry as single pretreatment condition can be applied during screening of sugarcane varieties.

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5.6. References

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Table 5-1: Chemical composition of bagasse from different varieties of sugarcane on a dry weight basis

Variety ID	55	70	74	101	104	114	120
Glucan	35.1 ± 0.4	36.1 ± 0.3	36.9 ± 0.6	40.7 ± 1.0	34.1 ± 1.0	38.3 ± 1.6	39.6 ± 0.6
Xylan	24.6 ± 0.5	24.3 ± 0.7	24.0 ± 0.2	26.3 ± 0.6	25.5 ± 0.3	27.2 ± 0.7	19.5 ± 0.3
Arabinan	2.5 ± 0.2	2.2 ± 0.1	1.5 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	1.3 ± 0.1
Lignin	(19.6 ± 0.6)	(20.4 ± 0.5)	(19.7 ± 0.5)	(14.4 ± 0.3)	(16.4 ± 0.3)	(16.1 ± 0.3)	(22.4 ± 0.2)
<i>Acid soluble</i>	2.8 ± 0.3	4.3 ± 0.2	2.7 ± 0.1	2.2 ± 0.3	1.2 ± 0.2	1.5 ± 0.4	2.2 ± 0.1
<i>Acid insoluble</i>	16.8 ± 0.1	16.1 ± 0.4	17.0 ± 0.7	12.2 ± 0.2	15.2 ± 0.1	14.6 ± 0.6	20.2 ± 0.2
Acetyl group	3.2 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	3.3 ± 0.2	3.2 ± 0.2
Extractives	9.9 ± 0.3	7.5 ± 0.1	7.5 ± 0.3	7.4 ± 0.9	7.3 ± 0.2	6.1 ± 0.2	5.0 ± 0.5
Ash	1.6 ± 0.1	1.8 ± 0.1	2.0 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.3 ± 0.3
Mass closure	96.5	95.2	94.5	95.0	90.0	94.4	92.3

Table 5-2: Recovery of glucose, xylose and acid insoluble lignin in the WIS after dilute acid pretreatment of different sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120) expressed as percentage of theoretical (content in the raw material).

Run	Variables ^a			Glucose							Acid insoluble lignin							Xylose						
	X ₁	X ₂	X ₃	55	70	74	101	104	114	120	55	70	74	101	104	114	120	55	70	74	101	104	114	120
1	170	0.45	5	96	95	95	96	95	95	95	96	98	98	99	97	98	100	24	28	23	30	19	24	30
2	190	0.45	5	94	95	92	94	94	95	92	99	99	101	101	98	99	100	10	8	6	12	9	7	12
3	170	0.85	5	96	93	93	93	96	95	96	96	98	99	98	97	99	101	12	14	13	8	10	13	20
4	190	0.85	5	92	90	92	92	93	91	94	96	99	101	103	98	103	100	4	6	4	5	4	4	7
5	170	0.45	15	95	93	91	94	95	95	95	99	98	99	102	100	101	102	11	12	11	11	13	12	20
6	190	0.45	15	93	92	90	94	93	92	93	101	103	101	102	97	100	103	3	5	4	3	4	4	7
7	170	0.85	15	92	91	92	93	94	92	93	99	99	99	104	97	100	103	7	7	8	8	7	8	12
8	190	0.85	15	84	85	84	87	87	87	90	105	107	105	115	107	106	107	2	2	3	2	2	2	4
9	163.2	0.65	10	93	96	95	93	94	95	93	96	99	99	107	98	101	99	11	17	14	26	15	15	20
10	196.8	0.65	10	87	91	85	88	87	87	89	108	103	99	108	105	102	108	2	2	2	2	3	3	4
11	180	0.31	10	92	96	92	93	96	96	91	96	101	99	104	104	100	102	4	10	10	11	11	13	16
12	180	0.99	10	88	91	91	93	93	91	90	100	99	99	98	96	99	104	2	6	5	5	4	5	7
13	180	0.65	1.6	94	95	94	95	95	93	93	98	104	98	99	97	101	102	11	32	22	24	12	26	30
14	180	0.65	18.4	91	91	91	91	94	91	92	106	103	101	98	103	100	103	3	5	5	2	4	5	7
15	180	0.65	10	93	94	91	94	94	93	87	98	99	99	98	98	99	98	5	7	6	6	6	7	13
16	180	0.65	10	91	91	91	93	93	94	92	99	99	100	101	99	98	100	2	6	7	5	6	7	10
17	180	0.65	10	92	93	92	94	94	91	90	98	99	99	99	95	99	99	4	6	6	6	7	6	10
18	180	0.65	10	91	93	92	94	92	91	88	97	98	100	100	96	98	106	5	5	6	6	6	7	11
19	180	0.65	10	95	93	91	95	94	92	88	97	99	99	99	97	97	101	4	6	6	7	7	7	10
20	180	0.65	10	94	94	89	94	94	93	90	98	98	101	101	98	99	107	3	5	7	6	7	7	8

^a Pretreatment conditions applied in the study, runs 1 to 8, 9 to 14, and 15 to 20 represents factorial points, star points, and center points, respectively,

X₁, temperature (°C); X₂, acid concentration (%w/w); X₃, reaction time (min).

Table 5-3: Xylose yield, xylose recovery and combined furfural and MMF yields in the hydrolysate liquor after dilute acid pretreatment sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120) at various conditions

Run	Xylose yield (g/100g RM)							Xylose recovery (% of theoretical)							Furfural , and HMF formation (g/100g RM)						
	55	70	74	101	104	114	120 ^a	55	70	74	101	104	114	120	55	70	74	101	104	114	120
1	6.9	11.4	8.3	14.1	13.7	11.6	10.0	24.7	41.3	30.4	47.2	47.3	37.5	45.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1
2	11.8	16.3	17.5	18.5	13.1	18.3	12.1	42.2	59.0	64.2	61.9	45.2	59.2	54.6	0.4	0.6	0.5	0.8	0.6	1.0	0.8
3	12.8	14.6	15.3	18.4	18.9	15.0	14.3	45.8	52.9	56.1	61.6	65.2	48.5	64.5	0.1	0.2	0.4	0.8	0.3	0.2	0.2
4	16.8	15.4	12.1	17.7	17.4	18.2	14.3	60.1	55.8	44.4	59.2	60.0	58.9	64.5	1.1	1.2	1.1	1.6	1.3	2.0	0.9
5	16.9	14.1	13.7	17.5	15.8	14.4	15.3	60.5	51.1	50.2	58.6	54.5	46.6	69.0	0.4	0.5	0.4	1.0	0.6	0.5	0.6
6	14.7	10.3	12.5	13.6	16.3	15.3	9.8	52.6	37.3	45.8	45.5	56.3	49.5	44.2	1.9	2.6	2.8	3.1	2.4	3.0	3.5
7	14.0	17.2	13.6	17.9	19.9	20.0	13.1	50.1	62.3	49.9	59.9	68.7	64.7	59.1	0.5	1.0	1.1	1.3	1.0	1.2	0.8
8	11.3	9.8	10.7	11.9	13.2	13.2	9.9	40.4	35.5	39.2	39.8	45.6	42.7	44.7	2.5	4.7	4.6	4.3	4.6	4.8	4.0
9	17.6	11.2	12.7	18.9	18.6	17.0	13.7	63.0	40.6	46.6	63.2	64.2	55.0	61.8	0.2	0.1	0.7	0.3	0.3	0.3	0.3
10	15.0	11.1	11.6	14.1	11.3	12.9	8.6	53.7	40.2	42.5	47.2	39.0	41.7	38.8	3.4	2.3	4.4	4.3	4.5	3.1	4.3
11	12.4	16.6	12.3	15.6	16.1	16.7	13.8	44.4	60.1	45.1	52.2	55.6	54.0	62.3	0.3	0.5	0.5	0.5	0.7	0.6	0.4
12	15.0	10.1	13.6	16.4	13.2	16.8	16.9	53.7	36.6	49.9	54.9	45.6	54.4	76.3	1.7	1.0	1.9	2.0	1.5	1.9	1.6
13	8.9	11.0	7.4	12.6	14.9	13.2	12.4	31.8	39.8	27.1	42.2	51.4	42.7	56.0	0.3	0.1	0.1	0.1	0.2	0.1	0.0
14	17.0	9.3	10.1	16.0	15.3	16.0	12.7	60.8	33.7	37.0	53.5	52.8	51.8	57.3	1.7	1.8	1.8	3.0	2.0	2.0	2.0
15	19.1	18.3	18.5	20.0	18.2	18.8	15.8	68.3	66.3	67.8	66.9	62.8	60.8	71.3	1.2	1.1	1.5	1.4	1.2	1.2	1.2
16	18.4	16.1	18.1	19.8	19.0	19.5	15.9	65.8	58.3	66.4	66.3	65.6	63.1	71.8	1.1	0.9	1.8	1.4	1.2	1.3	1.0
17	19.3	19.4	19.3	19.6	19.1	19.7	16.0	69.0	70.3	70.8	65.6	65.9	63.7	72.2	1.3	1.1	1.6	1.3	1.3	1.3	1.1
18	19.4	18.7	18.5	19.4	19.0	19.1	16.5	69.4	67.7	67.8	64.9	65.6	61.8	74.5	1.2	1.2	1.5	1.3	1.3	1.3	1.4
19	19.4	19.0	18.9	20.2	19.2	18.9	16.5	69.4	68.8	69.3	67.6	66.3	61.1	74.5	1.2	1.2	1.6	1.4	1.0	1.1	1.0
20	19.4	19.2	19.1	20.0	18.9	19.0	16.7	69.4	69.5	70.0	66.9	65.2	61.5	75.4	1.3	1.2	1.5	1.3	1.4	1.1	1.5

Table 5-4: Yields of glucose after enzymatic hydrolysis and combined sugar (sum of glucose, xylose, and arabinose) after dilute acid pretreatment and enzymatic hydrolysis, , and combined sugar recovery (combined sugar yield divided by maximum sugar content in percentage) from sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120).

Run	EH glucose yield (g/100g RM)							^a Combined sugar yield (g/100g RM)							^b Combined sugar recovery (% theoretical)						
	55	70	74	101	104	114	120	55	70	74	101	104	114	120	55	70	74	101	104	114	120
1	21.1	16.7	20.4	31.2	22.8	20.9	18.1	32.8	34.6	33.0	51.0	42.2	38.4	32.3	47	49	47	66	60	50	48
2	29.4	28.6	25.6	35.9	28.4	34.7	30.6	44.9	49.9	49.4	60.2	46.8	59.5	46.8	64	71	71	78	67	78	69
3	25.3	22.6	21.4	34.4	26.2	28.1	21.0	43.2	42.7	42.1	58.9	50.9	48.7	39.5	62	61	60	76	73	64	58
4	32.6	29.8	29.8	37.2	25.4	32.0	30.0	54.3	49.7	46.4	62.0	48.4	57.1	48.6	78	71	66	80	69	75	72
5	27.2	25.8	24.7	34.7	29.5	29.4	25.0	49.9	45.1	43.5	58.2	50.8	49.2	45.4	71	64	62	75	73	64	67
6	35.2	32.3	31.9	39.0	29.3	35.9	32.6	55.3	47.4	51.0	58.5	50.8	57.5	45.7	79	67	73	75	73	75	68
7	28.4	26.6	30.8	38.1	29.6	32.9	28.0	47.0	52.7	50.8	62.6	54.9	59.2	45.1	67	75	73	81	79	78	67
8	31.8	26.4	28.4	35.2	32.2	32.5	29.9	51.3	44.2	47.7	55.3	53.1	55.2	43.3	74	63	68	71	76	72	64
9	23.8	23.5	24.4	32.5	25.3	26.3	23.0	47.7	39.6	42.4	58.5	50.4	49.8	40.5	68	56	61	75	72	65	60
10	34.0	29.9	30.5	39.0	32.2	37.9	33.7	57.1	47.4	50.0	60.6	51.5	58.4	46.5	82	67	71	78	74	77	69
11	29.4	28.6	29.5	35.4	29.3	28.1	26.6	46.2	51.7	46.0	57.2	51.2	50.8	45.5	66	74	66	74	73	67	67
12	31.2	31.1	30.7	39.7	34.9	35.9	29.0	51.9	46.4	49.8	62.1	53.9	58.7	49.8	74	66	71	80	77	77	74
13	23.6	18.6	21.3	30.4	23.0	23.8	19.2	38.6	36.4	32.9	49.2	43.2	44.0	36.5	55	52	47	63	62	58	54
14	32.2	30.3	29.4	36.6	32.3	36.8	29.4	54.7	45.1	44.6	58.6	52.5	58.5	45.5	78	64	64	76	75	77	67
15	33.5	30.6	27.6	38.4	32.9	36.6	27.4	58.0	55.0	52.1	64.7	56.6	62.3	48.2	83	78	74	83	81	82	71
16	32.5	28.4	26.6	39.5	32.4	35.3	29.5	56.6	50.5	51.0	65.4	56.7	60.6	49.5	81	72	73	84	81	79	73
17	34.7	30.3	27.7	39.2	31.4	35.5	29.6	59.9	56.5	53.5	66.1	57.4	62.2	50.0	86	80	76	85	82	82	74
18	34.0	29.2	27.2	38.9	30.4	34.6	28.6	59.3	54.4	51.8	64.4	55.3	60.1	50.6	85	77	74	83	79	79	75
19	34.1	29.2	28.0	39.6	31.2	35.0	28.6	59.0	54.3	52.7	66.0	56.3	60.0	49.5	85	77	75	85	81	79	73
20	32.9	29.5	26.8	39.6	32.3	35.8	28.3	57.9	55.0	52.0	66.9	57.4	61.3	49.5	83	78	74	86	82	80	73

^a refers to the sum of all sugar (glucose, xylose, and arabinose) obtained after pretreatment and enzymatic hydrolysis

^b refers to the combined sugar yield divided by theoretical sugar in the native material expressed as percentage

Table 5-5: Regression coefficients of the mathematical models of combined sugar yield from sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120, probability from the ANOVA-table , and determination coefficient.

	55	70	74	101	104	114	120
b ₀	58.48	54.22	52.14	65.56	56.65	61.07	49.54
b ₁	3.58 (<0.0001)	2.44 (0.0038)	2.84 (<0.0001)	0.56 (0.2313)*	0.17 (0.5854)*	3.65 (<0.0001)	2.36 (<0.0001)
b ₂	1.63 (0.0033)	-0.03 (0.9616)*	1.29(0.0043)	1.32 (0.0130)	1.57(0.0004)	2.00 (0.0016)	0.72 (0.0360)
b ₃	4.05 (<0.0001)	1.70 (0.0260)	3.15 (<0.0001)	1.41 (0.0093)	2.70 (<0.0001)	3.08 (<0.0001)	2.02 (<0.0001)
b ₁₁	-2.43 (0.0002)	-3.50 (0.0003)	-1.79 (0.0004)	-1.93 (0.0011)	-2.08 (<0.0001)	-2.23 (0.0006)	-2.14 (<0.0001)
b ₂₂	-3.62 (<0.0001)	-1.54 (0.0351)	-1.18 (0.0062)	-1.89 (0.0013)	-1.51 (0.0005)	-2.51 (0.0002)	-1.04 (0.0051)
b ₃₃	-4.48 (<0.0001)	-4.47 (<0.0001)	-4.42 (<0.0001)	-3.92 (<0.0001)	-3.17 (<0.0001)	-3.36 (<0.0001)	-3.02 (<0.0001)
b ₁₂	-0.25 (0.6683)*	-1.91 (0.0484)	-2.95 (<0.0001)	-1.84 (0.0094)	-1.11 (0.0193)	-2.92 (0.0007)	-0.95 (0.0351)
b ₁₃	-1.69 (0.0124)	-3.07 (0.0048)	-2.18 (0.0008)	-2.28 (0.0026)	-0.48 (0.2517)*	-3.09 (0.0005)	-3.13 (<0.0001)
b ₂₃	-3.35 (0.0001)	-0.92 (0.3044)*	-0.39 (0.4160)*	-0.91 (0.1440)*	-0.50 (0.2384)*	0.17 (0.7823)*	-1.46 (0.0038)
R ²	0.98	0.91	0.97	0.94	0.96	0.96	0.97

The values in the parentheses indicate probability from the ANOVA-table.

*The values which were not significant at 95% confidence level

Table 5-6: Correlation coefficient (r) between chemical components and xylose or EH glucose yields across pretreatment conditions.

Conditions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Component	Correlations between component and xylose yield														
Glucan	0.262	0.452	0.089	0.003	0.172	-0.387	-0.108	-0.161	0.016	-0.094	0.082	0.646	0.123	0.049	-0.167
Xylan	0.400	0.592	0.402	0.646	0.151	0.748*	0.761**	0.747*	0.539	0.733*	0.473	0.062	0.191	0.435	0.934**
Arabinan	0.355	0.013	0.284	0.838**	0.354	0.783**	0.753*	0.723*	0.640	0.619	0.513	-0.165	0.422	0.602	0.697*
Lignin	-0.445	-0.583	-0.290	-0.678*	-0.010	-0.637	-0.761**	-0.808**	-0.526	-0.422	-0.566	-0.397	-0.447	-0.526	-0.557
Acetyl	0.232	-0.217	0.176	0.662	0.573	0.573	0.289	0.661	0.814**	0.261	0.098	0.832**	0.549	0.900**	0.079
Extractives	-0.390	-0.163	0.177	0.132	0.380	0.381	-0.119	0.049	0.244	0.159	-0.362	-0.350	-0.500	0.243	0.616
Ash	-0.763**	-0.111	-0.627	-0.801**	-0.528	-0.526	-0.689*	-0.736*	-0.790**	-0.757**	-0.584	-0.565	-0.866**	-0.722*	-0.236
	Correlations between component and glucose yield														
Glucan	0.364	0.688*	0.383	0.699*	0.359	0.673*	0.620	0.338	0.555	0.645	0.350	0.326	0.386	0.359	0.371
Xylan	0.527	0.390	0.701*	0.267	0.656	0.378	0.559	0.475	0.573	0.422	0.518	0.773	0.622	0.775**	0.671*
Arabinan	0.456	0.275	0.537	0.026	0.531	0.116	0.141	0.436	0.257	0.265	0.237	0.551	0.396	0.600	0.584
Lignin	-0.360	-0.670*	-0.632	-0.273	-0.588	-0.446	-0.602	-0.568	-0.507	-0.694*	-0.208	-0.720*	-0.515	-0.876**	-0.661
Acetyl	0.358	0.642	0.513	0.230	0.517	0.377	0.412	0.833**	0.338	0.774**	0.082	0.444	0.514	0.624	0.613
Extractives	0.173	-0.272	0.147	0.167	0.086	0.129	-0.071	0.067	0.016	-0.161	0.341	0.028	0.249	0.018	0.188
Ash	-0.677*	-0.780**	-0.749*	-0.213	-0.802**	-0.357	-0.606	-0.835**	-0.638	-0.777**	-0.350	-0.774**	-0.635	-0.773**	-0.740*

Correlations coefficients marked with * means significant at the 0.1 level and ** at the 0.05 level

The results of experiment 15, is the average of run 15 to 20.

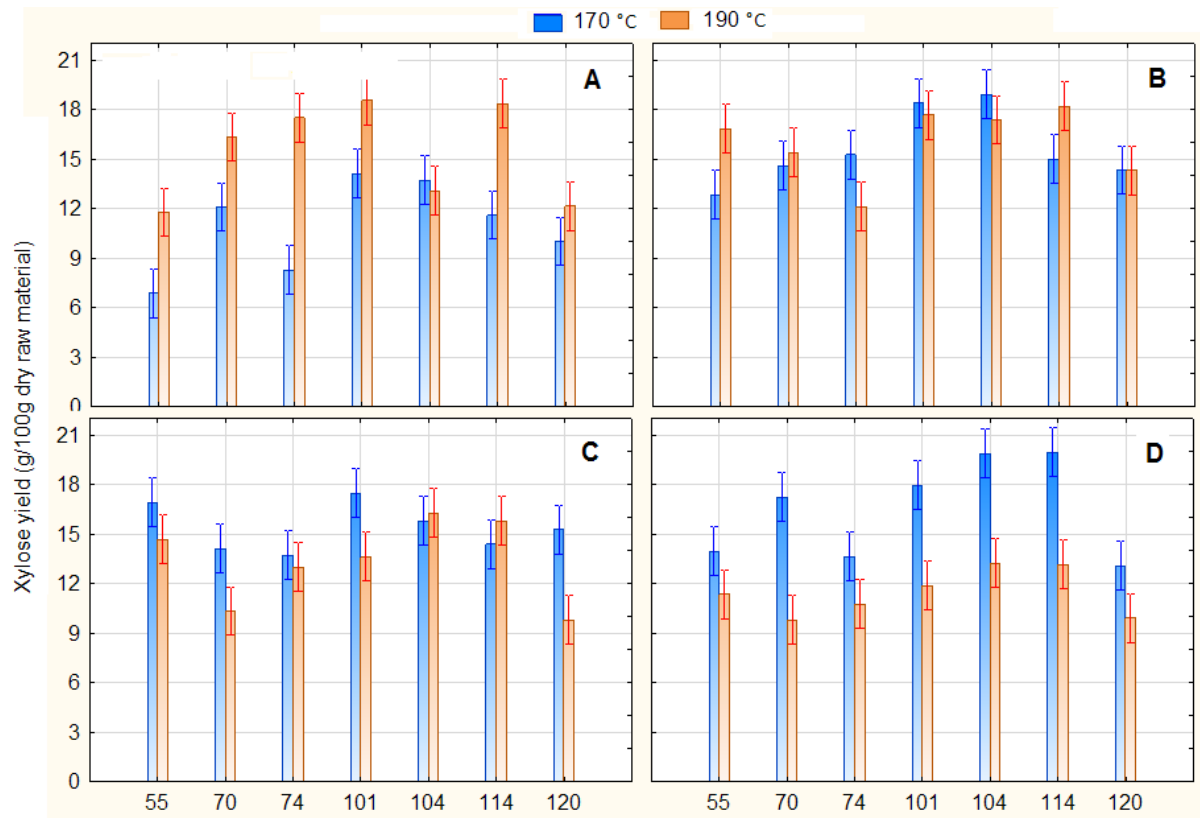


Figure 5-1: The effects of temperature, acid concentration, and reaction time on xylose yield from different sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120). (A) 0.45 (%w/w) for 5 min, (B) 0.85 (%w/w) for 5 min, (C) 0.45 (%w/w) for 15 min, and (D) 0.85 (%w/w) for 15 min. The error bars denote 95% confidence intervals.

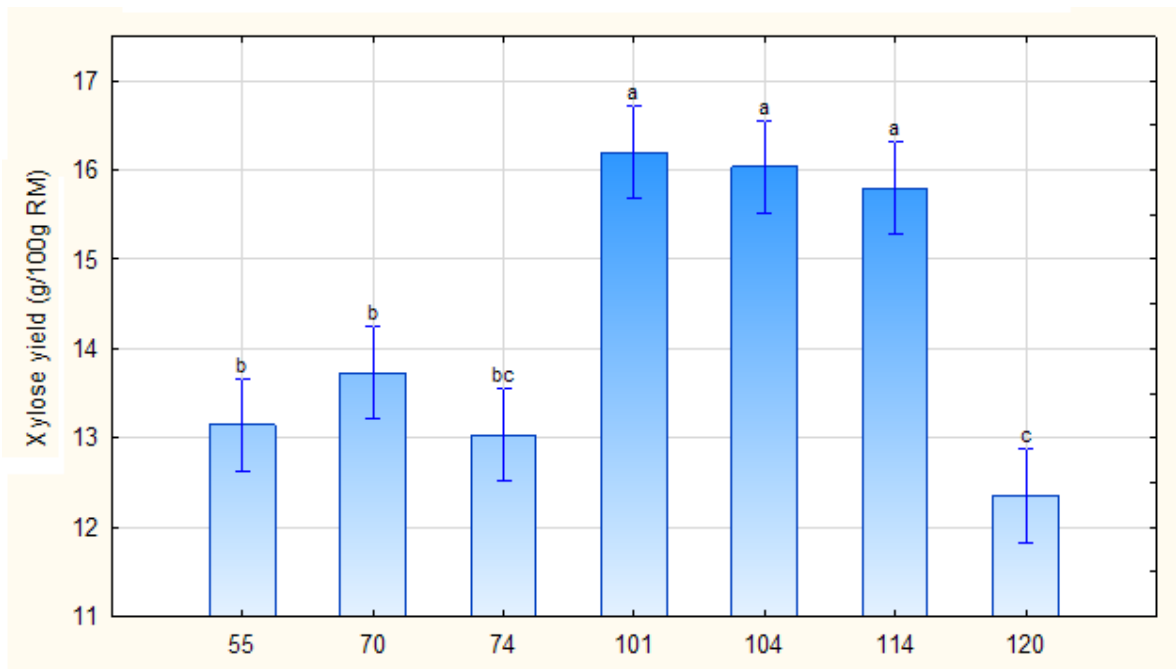


Figure 5-2: Comparison of xylose yields from different sugarcane bagasse samples (55, 70, 74, 101, 104, 114, and 120) after the dilute sulfuric acid pretreatment. The yields are based mean values of all factorial points. The columns with similar insulated letters do not differ between each other at a significance level of 0.05.

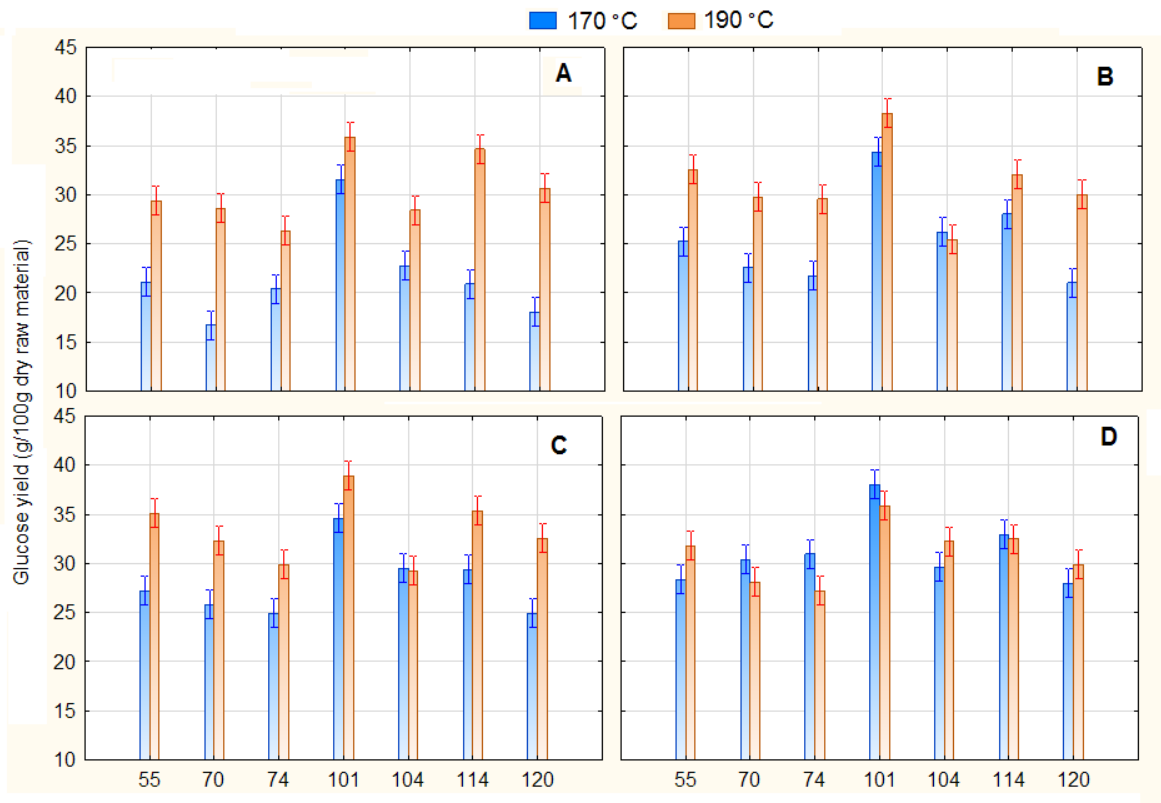


Figure 5-3: Glucose yields as the function of temperature after enzymatic hydrolysis of different sugarcane bagasse (55, 70, 74, 101, 104, 114 , and 120) for (A) 0.45%w/w sulfuric acid and 5 min, (B) 0.85 (%w/w) and 5 min (C) 0.45 (%w/w) and 15 min (D) 0.85 (%w/w) and 15 min applied in the pretreatment. The error bars denote 95% confidence intervals.

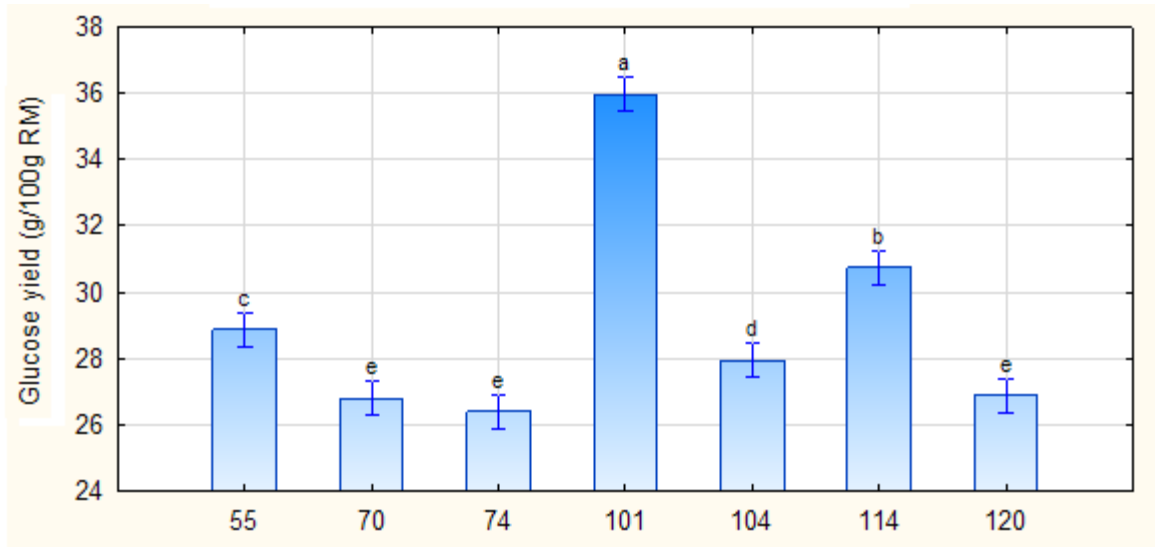


Figure 5-4: Comparison of glucose yields from different sugarcane bagasse (55, 70, 74, 101, 104, 114, and 120) after enzymatic hydrolysis of dilute sulfuric pretreated materials. The yields are based mean values of all factorial points. The vertical error bars denote 95% confidence intervals.

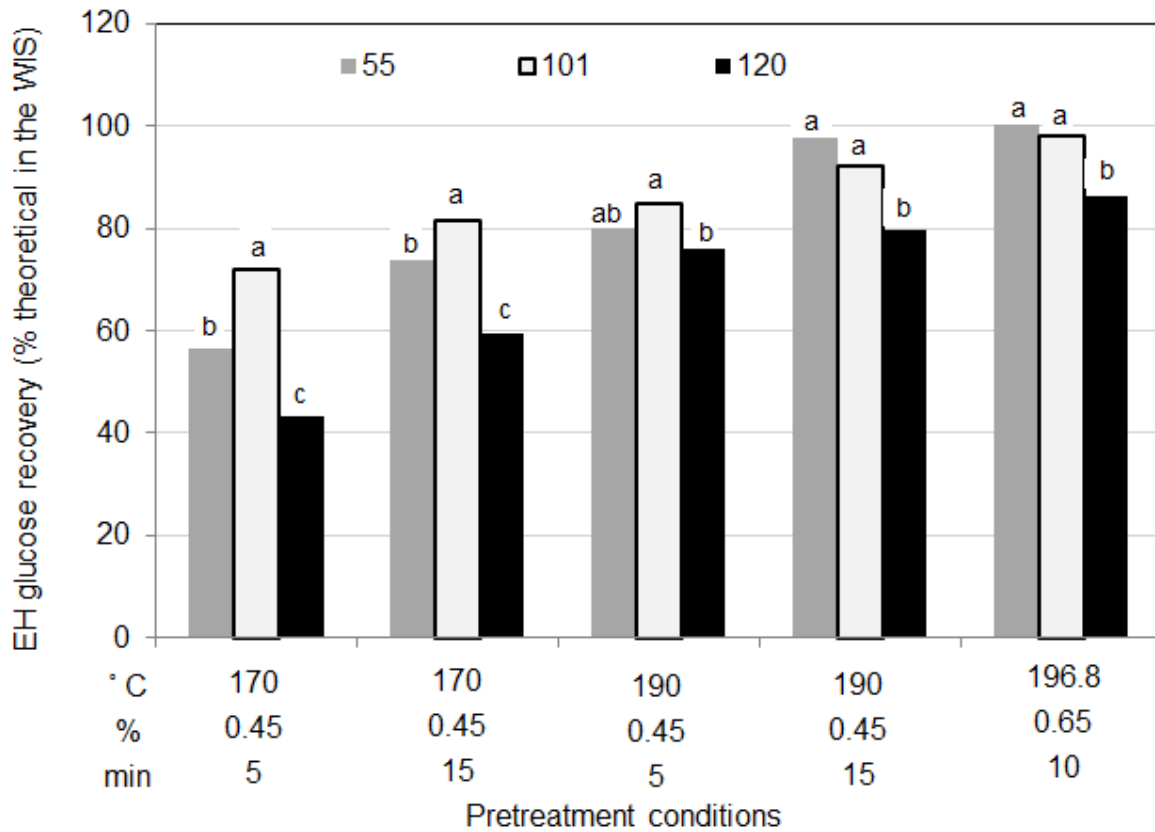


Figure 5-5: Bioconversion of different bagasse samples (55, 101, and 120) across selected dilute acid pretreatment conditions of increasing severities. EH glucose recovery was calculated as percentage of potential glucose in the WIS. Inserted letters (a, b, c) means values with different alphabet differed significantly at $p < 0.05$.

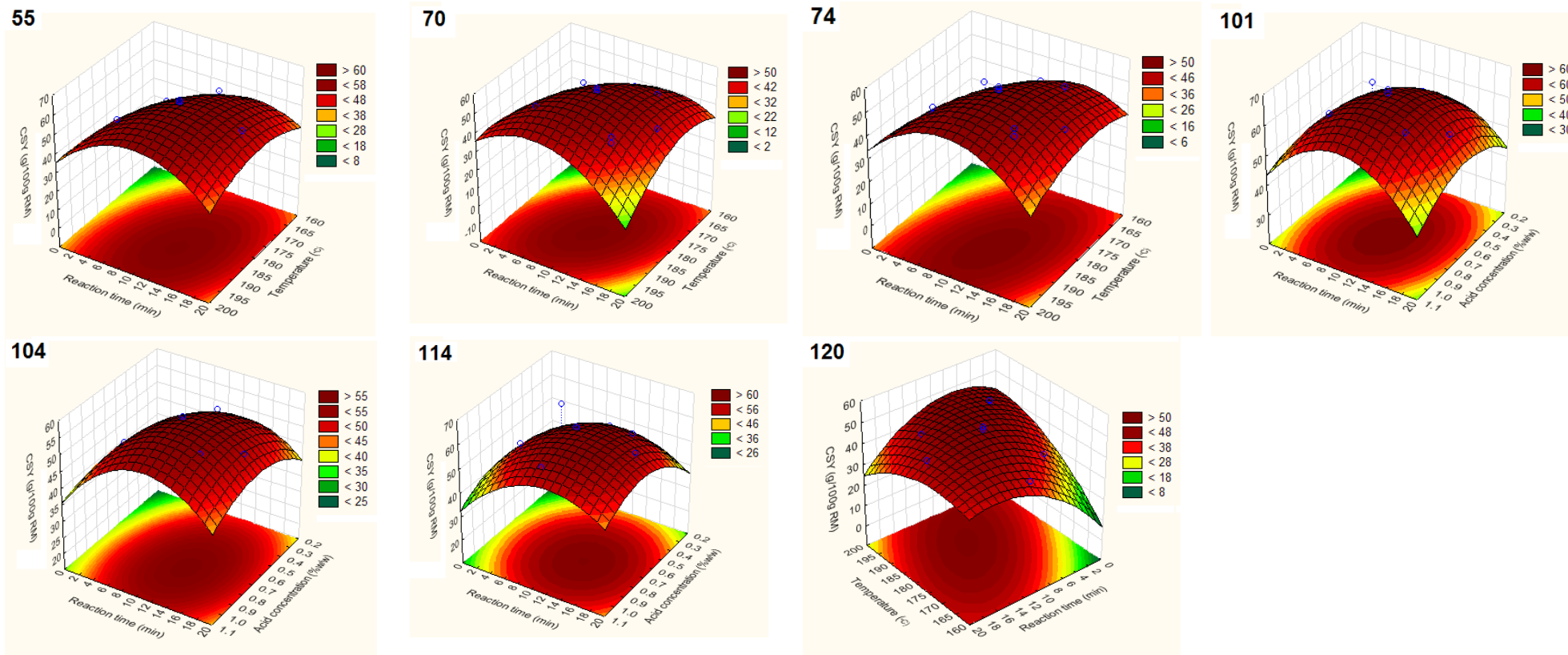


Figure 5-6: The response surface plot showing:the influence of temperature and reaction time on the combined sugar yield from sugarcane bagasse with identification 55, 70, 74, and 120; the influence of acid concentration and reaction time on the combined sugar yield from sugarcane bagasse with identification 101, 104, and 114

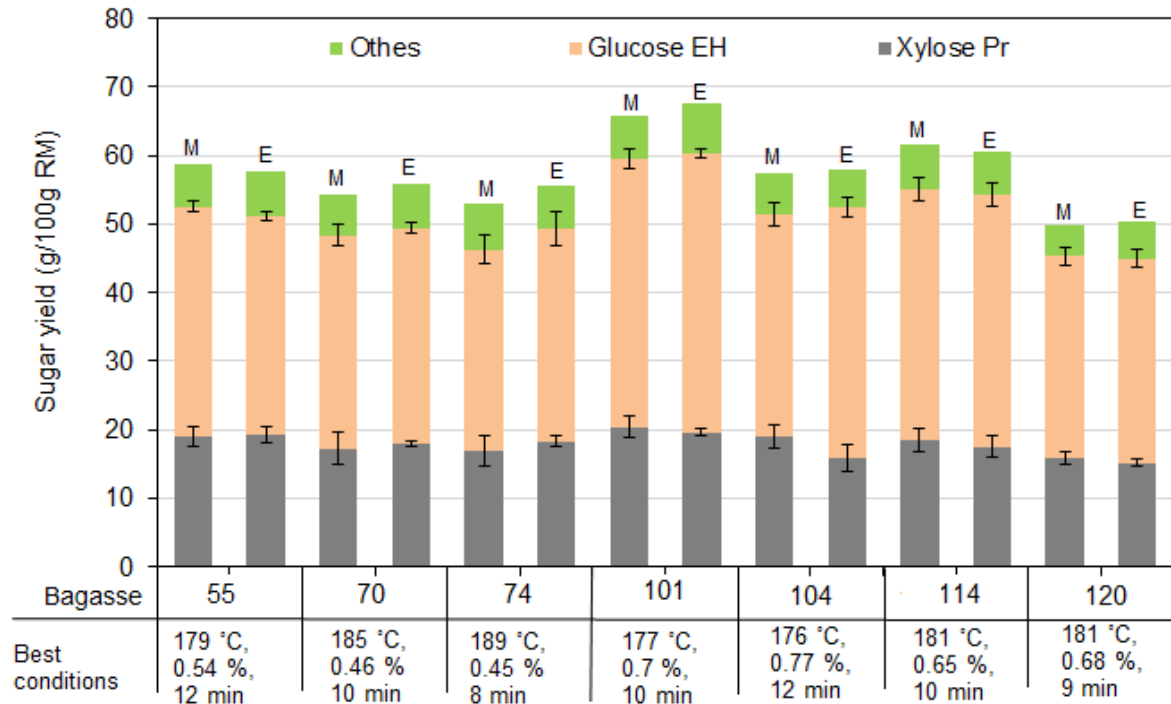


Figure 5-7: The predicted condition for the model optimisation, predicted values (M) and the validation experimental values (E) of combined sugar yields from different sugarcane bagasse. Xylose Pr. is xylose yield after pretreatment and Glucose EH is glucose yield after enzymatic hydrolysis. Others stand for the sum of glucose, arabinose (after pretreatment) and xylose after enzymatic hydrolysis.

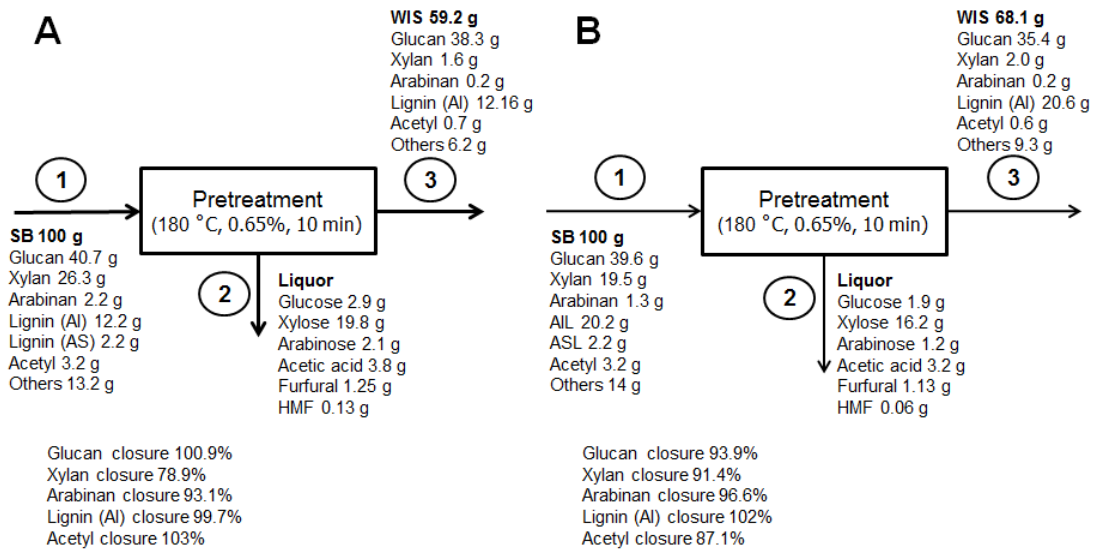


Figure 5-8: The overall mass balance of liquid and solid fractions obtained after dilute sulfuric acid pretreatment at center point conditions (180 °C, 0.65%-acid for 10 min): (A) best performer variety (101) , and (B) industrial bagasse (120). AS and AI refers to as acid soluble, and acid insoluble, respectively.

Chapter 6

6. Impact of cultivar selection and process optimisation on ethanol yield from different varieties of sugarcane

The adopted version was submitted to Biotechnology for Biofuels for publication in November, 2013 with the following details:

Title: “*Impact of cultivar selection and process optimisation on ethanol yield from different varieties of sugarcane*”

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Objectives of dissertation and summary of findings in present chapter

This chapter addresses objectives 2, 3 and 4. The pretreatment was carried out using the bench scale (1 litre). However, only classical breeding varieties and industrial bagasse were investigated. Precision breeding varieties were not evaluated due to the lack of materials availability caused by prolonged drought observed in 2010 (discussed in chapter 7). CCD approach was used to study the effects of temperature and residence time while the acid concentration was kept constant for all experiments. The combined sugar yields from the samples obtained after pretreatment and enzymatic hydrolysis were then used to develop the mathematical models that were applied to predict the optimum yield for each substrate. The mathematical models were later used to establish the range of pretreatment conditions “area in common” where 95% of the maximum combined sugar yield could be obtained. The optimal pretreatment condition was used to generate pretreated materials for Simultaneous Saccharification and Fermentation (SSF) process.

The SSF was performed on the unwashed pressed-slurry at different solid loadings and enzyme dosages, and at different feeding strategies (batch and fed-batch). The results obtained were used to evaluate the impact of cultivar selection and pretreatment optimisation on ethanol yield in an integrated manner. The correlations between chemical composition and hemicelluloses degree of substitution, with the responses (sugar and ethanol yield) were also evaluated.

The results showed that the combined sugar yield at the optimum conditions varied from 50.3 to 65.8 g/100 g dry raw material, corresponding to 74.4 and 92.3% of theoretical for industrial bagasse and variety 55. This means that the combined sugar yield could be improved up to 33% by selecting the best performing varieties. It was further observed that the pretreatment conditions with temperature ranged from 184 to 200 °C and varying residence time to provide a severity factor between 3.51 and 3.96 was observed to be the area in common where 95% of Max. CSY could be obtained. Significant higher ethanol concentration after Simultaneous Saccharification and Fermentation (SSF) of the pretreated bagasse was also observed from the best performing varieties (48.6–51.3 g/l) compared to industrial bagasse (38.3 g/l). The overall assessment of the cultivars showed greater improvement in combined ethanol yields per hectare (71.1–90.7%) for the best performing varieties with respect to industrial sugarcane.

Candidate declaration

With regard to chapter 6 page numbers 142–184 of this dissertation, the nature and scope of my contribution were as follows.

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

The following co-authors have contributed to chapter 6 page numbers 142–184 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Maria P. García-Aparicio	garcia@sun.ac.za	• Experimental planning	10
		• Assisted in interpretation of results and literature	30
		• Extensive reviewing the chapter	60
2. Johann Görgens	jgorgens@sun.ac.za	• Experimental planning	30
		• Interpretation of results to correlate with literature	10
		• Reviewing the chapter	40

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 142–184 in the dissertation,
2. no other authors contributed to chapter 6 page numbers 142–184 in the dissertation besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that any necessary arrangements have been made to use the material in to chapter 6 page numbers 142–184 of this dissertation.

Signature	Institutional affiliation	Date

Abstract

BACKGROUND (objective): The development of “energycane” varieties of sugarcane is underway, targeting the use of both sugar juice and bagasse for ethanol production. The current study evaluated a selection of such “energycane” cultivars for combined ethanol yields from juice and bagasse, by optimisation of dilute acid pretreatment optimisation of bagasse for sugar yields.

RESULTS: Significant variations were observed in sugar yields (xylose, glucose and combined sugar yield) from pretreatment-hydrolysis of bagasse from different cultivars of sugarcane. Up to 33% difference in combined sugar yield between best performing varieties and industrial bagasse was observed at optimal pretreatment-hydrolysis conditions. Significant improvement in overall ethanol yield after Simultaneous Saccharification and Fermentation (SSF) of the pretreated bagasse was also observed from the best performing varieties (84.5–85.6%) compared to industrial bagasse (74.8%). The ethanol concentration showed inverse correlation with lignin content and the ratio of xylose to arabinose, but it showed positive correlation with glucose yield from pretreatment-hydrolysis. The overall assessment of the cultivars showed greater improvement in combined ethanol yields per hectare (71.1–90.7%) for the best performing varieties with respect to industrial sugarcane.

CONCLUSIONS: These results suggest that the selection of sugarcane variety to optimize ethanol production from bagasse can be achieved without adversely affecting juice ethanol and cane yield, thus maintaining first generation ethanol production levels, while maximising second generation ethanol production.

Keywords: sugarcane varieties, pretreatment optimisation, enzymatic hydrolysis, SSF, ethanol.

6.1. Introduction

Sugarcane represents a preferred crop for the production of bio-ethanol, which is the widely used biofuel in the world today [1], due to high biomass yields and high fermentable sugar content [2]. Integration of first and second generation technologies for ethanol production from both sugarcane juice and the lignocellulosic residue (bagasse) could improve the sustainability and economics of the process, thereby increasing ethanol yield per ton of harvested sugarcane [3]. However, the recalcitrance of the lignocellulose requires a more complex processing technology compared to the juice, to obtain the fermentable sugars. The biochemical production of ethanol from lignocellulose basically involves the subsequent steps of pretreatment, enzymatic hydrolysis and fermentation. Although numerous advances have been made towards cellulosic ethanol in the last decades, its production at large scale is still hampered by pretreatment and enzyme costs [4,5]. Reduction of production costs can be obtained through optimisation of the different steps in an integrated manner [3,6], since each step has an impact in the following. The direct use of pretreated material at high solids loading as substrate during simultaneous saccharification and fermentation is considered a promising strategy to reach at least 4% (v/v) ethanol in the fermentation broth [7]. Problems associated with inhibitors and mixing at high solids loading can be alleviated by using the pressed pretreated material and fed-batch feeding during the SSF [8].

One aspect that has received less attention is the impact of feedstock properties on the operational conditions and economics of the production process. It has been demonstrated that variations in feedstock lead to different process requirements, even for similar biomass or varieties of the same species [6]. Therefore, further reduction of global cost could be obtained through crop development and selection of varieties with advantageous traits including agronomic properties (high biomass, sugar and fibre production per hectare) and, in the case of lignocellulosic residues, being more amenable to conversion to monomeric sugars through pretreatment-hydrolysis, often related to high

structural carbohydrates content, reduced lignin content and improved digestibility. These aspects are referred to as “feedstock quality” in the present study.

Biomass yield and composition, and ultimately sugar and ethanol yields, vary depending on various factors such as variety (genotype), year, harvest period [9] and location [10]. Several studies have proven the negative correlation between cellulose digestibility with lignin [11–13] and ash [14] contents, whereas it is improved by carbohydrate content. Selection of varieties with fibres with high ratio carbohydrate: lignin and reduced ash content would be beneficial to maximise sugars and ethanol yield, provided that other agronomic traits are not compromised. Up to 26% difference in the sugar yield were observed from straw of different cultivars of wheat when applying a standard hydrothermal pretreatment followed by enzymatic hydrolysis [14]. Similar differences have been found for feedstocks with reduced lignin content in ethanol yield during SSF of alkali pretreated corn stover [15] and dilute acid pretreated sorghum bagasse [12]. A more recent work evaluated the impact of genotype of maize on sugar yield when the maize forage was pretreated under different severities of dilute acid [16]. It was observed that samples with higher cellulose, reduced lignin and highly substituted hemicelluloses provided significantly higher sugar yields (90 versus 180 g/Kg for a combined severity factor of 0.95), but the differences among varieties were reduced by increasing the severity of pretreatment.

The feedstock quality of sugarcane varieties can be improved through classical breeding or precision breeding (genetic engineering), and both of these have shown the possibility to produce sugarcane lines that are less recalcitrant to bioconversion, without affecting plant performance in controlled environmental conditions [13,17]. In this context, the present study evaluated the responses of different sugarcane varieties from classical breeding at various stages of the conversion process: Altering pretreatment severity, altering enzyme requirements and the eventual ethanol yield during SSF, and overall ethanol yield considering agronomic data (l ethanol/ha) [18]. In a previous study, 115 varieties from the breeding program at South Africa Sugarcane Research Institute

(SASRI) were screened in terms of potential ethanol yields per hectare from both sugar juice and bagasse [6,19]. Out of the 115 cultivars, the bagasse of 3 preferred varieties from classical breeding were selected for further optimisation together with an industrial bagasse for comparison purposes. In the present study, each bagasse sample was firstly subjected to different pretreatments conditions to determine those that provide the maximum combined sugar yield, while minimizing byproduct formation. This optimisation was done by central composite design (CCD) varying the temperature and time for a fixed acid loading. Subsequently the pressed materials pretreated under optimum conditions were used as substrate to carry out SSF with two different enzyme loadings. Additional fed-batch SSF experiments were conducted in order to obtain ethanol concentration of at least 40 g/l. Finally, the overall ethanol yield (l/ha) considering biomass yield and ethanol from the juice and the bagasse was also calculated, to identify preferred varieties for bio-ethanol production.

6.2. Materials and Methods

6.2.1. Raw material and sample preparation

The sugarcane varieties were developed by South African Sugarcane Research Institute (SASRI) through classical breeding towards higher biomass yield [6]. The experimental field trial was conducted at SASRI, Mount Edgecombe, KwaZulu Natal (latitude: 29.7000° S; longitude: 31.0333° E). The genotypes were first planted in the field in 2006. The genotypes used in this study were from 5th ratoon. The plants were rain fed and no fertilizer was used. The genotypes were 99F2004⁵⁵, 00F0884⁷⁰ and 01G1662⁷⁴ and all of them had a South African origin. The superscripts (55, 70 and 74) were used for varieties identification. The industrial sugarcane bagasse (labelled 120) was provided by TSB Sugar Mill in Malelane, Mpumalanga, South Africa.

To obtain the bagasse, 20 to 30 of cane stalks (not less than 6 kg) per clone per plot were randomly cut from the experimental field in September 2011 (8 months old plants). The stalks were shredded and then blended with water (1.5 kg of sample and 3 litres of

water) for twenty minutes. Thereafter, the finely crushed shredded canes from the blending jar were washed with water (400g of sample and 1 litre of water) three times and each wash was collected and measured for residue sucrose and other soluble sugars. In the case of industrial bagasse, the sample was washed three times (200g and 500 ml of water) and each wash was analysed for residual sugar content.

The remaining fibres after washing were pressed to reduce water content and dried at 40 °C for four days until reach a moisture content of 6%. The samples, mixed and sieved in a vibratory sieve shaker model AS200 basic (Resch GmbH, Germany) to obtain a representative particle size suitable for the composition analysis and for the pretreatment studies. The particles retained between 600 and 1000 µm (ref) were used for composition analysis and for the pretreatment [20]. The samples were quarter sampled and then packed in zipped plastic bags and stored in a temperature and moisture controlled room until needed.

6.2.2. Dilute acid pretreatment

Dilute acid pretreatment was conducted in 1000 ml Hastelloy C276 Parr reactor with a magnetic driven turbine agitator (Model 4540, Parr Instrument Company, Moline, Illinois). The surface and internal temperatures of reactor were monitored with two thermocouples type Pt. RTD class B (Omega Moline, Parr Instrumentation company) connected controller (Model 4848B, Parr Instrument Company, Moline, Illinois). The vessel was loaded with 60g (dry weight) and 600ml of sulphuric acid solution (0.5 %w/w), sealed and stirred at 250 rpm via 4848B controller. The vessel was heated using 4 kW fluidized sand bath (Model SBL-2D, Techne Co., Minneapolis, MN) coupled with a temperature controller (Model TC-8D, Techne, Minneapolis, MN), previously heated to 350 °C. The reaction time of pretreatment initiated once the target temperature was reached. At the end of the reaction time the vessel was quenched by submerging it into cold water. When the temperature of 100 °C was reached (within 4 min), the vessel was opened.

The pretreated material (slurry) was characterised in terms of total solids, water soluble solids, water insoluble solids and pH [21]. For analytical purposes, the slurry was vacuum-filtered into solid and liquid fractions. The solid fraction was further washed three times with deionized water, each wash with 300 ml. The remaining solids, referred to as Water Insoluble Solid (WIS), were weighed to calculate the insoluble solid recovery. The chemical composition of the WIS was determined as described in section 2.7. Likewise, the pretreatment liquor and wash liquor were analysed for oligomeric and monomeric sugars, sugar degradation products (furfural and HMF), acetic acid and formic acid.

6.2.3. Experimental design

The dilute acid pretreatment optimisation was based on a two level, two factors central composite design (CCD), employed to investigate the effect of temperature and reaction time at fixed acid loading on different responses. The CCD was composed of eleven runs with four experiments at axial points, three replicates at centre point and four at factorial points, and was repeated for each bagasse sample included in the study. The acid loading and solids loading were kept constant at 0.5% (w/w) and 10% (w/v), respectively. The range of pretreatment conditions were selected based on previous studies [22]. The pretreatment experiments were performed in a random order. Xylose yield after pretreatment, glucose yield after enzymatic hydrolysis (EH glucose) and combined sugar yield (CSY) were considered as responses for the model. Experimental data were analysed using Design Expert, version 8.0.2 (State Ease Inc., Minneapolis, MN, USA). The software determines which independent variables have significant effects on the process responses by analysis of variance (ANOVA). The experimental data were fitted into the quadratic model as described in Eqn. 1 below.

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_{ii}^2 + \sum_{i=1}^{n-1} \sum_{j=1}^n \beta_{ij} x_i x_j \quad (1)$$

Where Y is estimated value of the response; n is the number of independent variables; β_0 is an intercept, β_i , β_{ii} and β_{ij} stand for coefficients for linear, quadratic and

interaction of two independent variables; X_i , X_i^2 and X_iX_j refers to as linear, quadratic and two way interaction effects, respectively.

The equation obtained for the combined sugar yield was used in Matlab 8.1 (R2013a) to construct contour plots representing the pretreatment conditions (temperature and residence time) that provide 95% of the maximum combined sugar yield for each of the bagasses.

6.2.4. Enzymatic hydrolysis

The WIS fraction of pretreated bagasse samples was subjected to enzymatic hydrolysis to evaluate the effect of the pretreatment and differences between sugarcane varieties on the hydrolysis of bagasse. These experiments were conducted in 250 ml Erlenmeyer flasks. The flasks were loaded with 1 g (dry weight) of WIS and 50 ml of 0.05 M citrate buffer (pH 4.8) with the enzyme solution, to give a solids loading of 2% (w/v). Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial growth. Two commercial enzymes preparations were used: Spezyme CP (Genencor-Danisco, Denmark) with cellulase activity of 65 FPU/ml and Novozym 188 (Novozymes A/S, Denmark) with β -glucosidase activity of 995 IU/ml. Enzyme activities were determined according to Ghose [23]. Cellulase loading of 0.2308 mL/ g WIS (15 FPU/g WIS) of Spezyme CP supplemented with β -glucosidase of 0.01508 mL/g WIS (15 IU/g WIS) was applied in all the experiments. Flasks loaded with the mixtures were placed in water bath maintained at 50 °C with shaking at 90 revolutions per minute. Samples were withdrawn after 72 h and prepared for analysis as described below.

6.2.5. Yeast and culture medium

Saccharomyces cerevisiae MH1000 was used in the SSF experiments [24]. The yeast strain was stored at -80 °C in the presence of 30% glycerol in vials and transferred to agar plates prior to use. The pre-inoculum was grown in 250 ml Erlenmeyer flask containing 50 ml of mineral media (20 g.l⁻¹ yeast extract, 7.5 g.l⁻¹ (NH₄)₂SO₄, 3.4 g.l⁻¹

KH_2PO_4 , 0.8 g.l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml trace element solution, 0.05 g.l^{-1} $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.5 g.l^{-1} and Citric acid, and 20 g.l^{-1} glucose) [25] for 24 h at a temperature of $30 \text{ }^\circ\text{C}$ with agitation speed of 150 rpm. A sample of this starting culture was transferred to a 1L Erlenmeyer flask with 300 ml of preconditioning medium (mineral media with 20% (v/v) of pretreatment liquor) with an initial optical density (OD) of 0.2. The preconditioning media was incubated at $30 \text{ }^\circ\text{C}$ and 150 rpm until it reached an OD of 4.5-5.5 (approximately after 16-18 h). The preculture was harvested by centrifugation at 8000 rpm for 5 min (Model Z366, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was discarded and the pellet was washed with PBS solution (containing 8.01 g.l^{-1} , NaCl; 0.2 g.l^{-1} , KCl; 1.78 g.l^{-1} , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 0.27 g.l^{-1} , KH_2PO_4 ; adjust pH to 7.4 with the addition of 3M KOH) and centrifuged again. The washing with PBS solution was repeated 3 times. The final pellet was diluted in PBS to obtain the selected inoculum size to start the SSF (5 g/l wet cells equivalent to 1.34 g/l dry cells).

The media were sterilized by autoclaving while the pretreated liquor was sterile filtered ($0.22 \text{ } \mu\text{m}$ Stericup, Millipore, Billerica, MA).

6.2.6. Simultaneous saccharification and Fermentation (SSF)

The pressed-slurry from optimum pretreatment conditions (maximum combined sugar yield) was used as substrate of SSF experiments. The slurry was pressed to a final moisture content of 59–63% using a 50 ton shop press with gauge model TDR NO. 55002 (Northern Tool and Equipment Company, USA) set at 5 MPa. The SSF was conducted in batch and fed-batch regime. Two loadings of enzymes containing the mixture of Cellic Ctec2 (cellulase) and Cellic Htec2 (endoxylanase) kindly provided by Novozymes (A/S, Denmark) were applied under the batch process but only single dosage was used for the fed-batch process. These loadings were selected based on enzymatic hydrolysis optimisation of sugarcane bagasse obtained from previous study [26]. The densities of Cellic Ctec2 and Cellic Htec2 were 1.09 and 1.22 g.ml^{-1} , respectively.

Samples were withdrawn periodically and analysed in HPLC for sugars, ethanol, glycerol and by products as described below.

6.2.6.1. Batch SSF with different enzyme dosage

The batch SSF was performed at a solid loading of 10% (w/w) at 35°C with 150 rpm for 5 days. Batch SSF were conducted in 250 ml Erlenmeyer flask with a final working weight of 200 g. The unsterilized pressed-slurry was supplemented with mineral media without glucose and the pH was adjusted by adding 3 M KOH. After adjustment of pH to 5, Cellic Ctec2 and Cellic Htec2 were added at two loadings. The first loading was 0.15 ml of Cellic Ctec2/g pretreated material (dry basis) and 0.0167 ml of Cellic Htec2/g pretreated material. For the second enzymes loading, Cellic Ctec2 and Cellic Htec2 were added 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 temperature of 35 °C. Thereafter the inoculum was added at a concentration of 5 g /l of wet cells (corresponding to approximately 1.34 g/l dry cells).

6.2.6.2. Fed-batch SSF

The fed-batch experiments were conducted in 1L bioreactor (BioFlo110, New Brunswick Scientific Co., Inc., NBS) with a final working weight of 0.6 kg at a set temperature of 35 °C and 150 rpm. The reactor containing the mineral medium without glucose was autoclaved at 121 °C for 15 min. The experiment was started by adding the pretreated material (un-sterilized) in order to give an initial 2% (w/w) of solids loading. Cellic Ctec2 and Cellic Htec2 were added to give the final enzyme dosage of 0.15 ml/g pretreated material and 0.213 ml/g pretreated material, respectively. The yeast cells were added after one hour of pre-saccharification to give a final concentration of 5 g /l of wet cells. The pH was maintained at 5 by controlled addition of 3 M KOH. The substrate was loaded twice daily (2% w/w each) until the final loading of 16% was reached.

6.2.7. Chemical Analyses

The carbohydrates and lignin contents of the extracted-free raw materials and WIS were determined by the laboratory analytical procedures (LAPs) proposed by the National Renewable Energy Laboratory (NREL). [27–29]. The sugar and by-products concentration of pretreatment liquor were analysed by HPLC. The pretreatment liquor was subjected to a mild acid hydrolysis to convert the sugars in oligomeric form into monomers [30]. The difference in concentration before and after the hydrolysis was assumed to be in oligomeric form.

Monomeric sugars, acetic acid, formic acid, ethanol and glycerol were determined by 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 a RI detector (Shodex, RI-101) operated at 45 °C. Since xylose and galactose, and mannose-arabinose co-eluted in the H-column, additional HPLC analysis were conducted. For these analysis the HPLC system was equipped with an Xbridge™ Amide column (4.6 x 250 mm, 3.5 µm particle size) and a Xbridge™ Amide precolumn (Waters) set at 30 °C using 0.05% ammoniumhydroxide in water (A) and 0.05% ammoniumhydroxide 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. Since galactose and mannose contents were minimal, the quantification provided by the Aminex HPX-87H Column was considered accurate.

The glucan, xylan, arabinan, o-acetyl group contents in raw material and WIS were determined by applying a conversion factor: as $(0.95 \times \text{cellobiose} + 0.9 \times \text{glucose})$, $0.88 \times \text{xylose}$, $0.88 \times \text{arabinose}$ and $0.683 \times \text{acetic acid}$, respectively [28].

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) 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). Separation was carried out by gradient elution from 5% mobile phase B, increasing to 11% B over 14 minutes and then increasing to 40% B over 3 minutes. The mobile phase composition was then kept constant at 40% for 2 minutes, followed by a decrease to 5% B over 5 minutes and ending with a final step of constant composition at 5% B for 4 minutes in order to equilibrate. HMF and furfural concentrations were measured with a Dionex Ultimate 3000 diode array detector at 215 nm and 285 nm.

6.2.8. Statistical analysis, severity and ethanol calculation

The chemical compositions of untreated materials were calculated as the average values and standard deviations (average \pm SD). One-Way-Analysis of Variance (ANOVA) was employed to evaluate the statistical significance of yield differences between various bagasse samples. The hypothesis was accepted or rejected at 95% confidence interval. The combined severity factor (CSF = $\log R_o'$) was calculated based on Eqn. 2 [31] while ethanol yield per hectare was estimated according to Eqn. 3.

$$\log R_o' = \log \left(t \cdot \exp \left[\frac{(T_H - 100)}{14.75} \right] \right) - \text{pH}_{\text{out}} \quad (2)$$

Where, “ t ” is reaction time in minutes, “ T_H ” is the reaction temperature in °C, 100 is the reference temperature and “ pH_{out} ” is the pH of the pretreated liquor.

$$E_Y = X \times C_Y \times T_C \times \eta_S \times (1000 / \rho_E) \quad 3$$

Where E_Y , ethanol yield ($\text{L}\cdot\text{ha}^{-1}$); X , is sugars content in both juice and bagasse (%cane); C_Y is the cane yield ($\text{ton}\cdot\text{ha}^{-1}$); T_C , stoichiometric conversion factor (0.538 for sucrose and 0.5111 for other sugars); η_S , conversion efficiency of substrate to ethanol when the fermentation employs MH1000 yeast strain; and $1000/0.789$ (density of ethanol at 20 °C, $\text{g}\cdot\text{ml}^{-1}$).

6.3. Results

6.3.1. Chemical composition of biomass

The chemical composition of the bagasse samples obtained from classical breeding (55, 70, 74) are summarized in Table 6-1. The industrial bagasse (120), obtained from the sugar mill, was used as reference material. The bagasse samples differed slightly in their chemical composition. The values for glucan, xylan, arabinan, acetyl groups, acid insoluble lignin, ash and extractives ranged from 37.4 to 39.6%, 19.5 to 23.3%, 1.3 to 2%, 2.2 to 3%, 1.3 to 1.9% and 4.3 to 5%, respectively. The sum of all components measured ranged from 91.3% to 93%. This could be attributed to components that were not quantified (i.e. methyl glucuronic acid) and some degradation of the sugars occurring during the acid hydrolysis [13].

One way ANOVA analysis (Appendix C-1) indicated that xylan and acid insoluble lignin (AIL) were the only components that were significantly different between the various bagasse samples, at a significance level of 0.05 (*P*-values of 0.009 and 0.003 for the xylan and AIL, respectively). The varieties obtained from classical breeding presented significantly higher xylan (21.6-23.3%) than the industrial bagasse (19.5%). Regarding the AIL, the variety 74 presented values similar to the industrial bagasse (20.2%). Overall, varieties 55 and 70 had the highest amount of total structural carbohydrates (63.6 and 61.6%, respectively) and the lowest lignin content (17.1-17.3%). Assuming a conversion of 0.511 g of ethanol/ g sugar, the theoretical ethanol yield that could be obtained ranged from 398.2 to 419.8 L/dry ton for the industrial bagasse and variety 55, respectively.

6.3.2. Dilute H₂SO₄ pretreatment and enzymatic hydrolysis

The effect of feedstock properties in pretreatment requirements was evaluated. A CCD was applied to evaluate the influence of temperature and residence time on sugars recovery in the different fractions of pretreated material. The impact of pretreatment conditions on the digestibility of the WIS was also studied. Finally, the combined sugar

yield (CSY) from the combined pretreatment-hydrolysis process was determined considering sugars solubilisation in the pretreatment liquor and sugar released during enzymatic hydrolysis. The pretreatment conditions required to provide the maximum CSY were compared among the different varieties.

In order to quantitatively predict the effect of each independent variable on the responses (xylose, EH glucose and combined sugar yields), regression analysis was performed according to the quadratic model (Eqn. 1) to fit the responses as function of the experimental conditions. The statistical significance of each factor was determined by ANOVA.

6.3.2.1. Effect of pretreatment on sugar recovery and inhibitors formation

The recovery of the main sugars, glucose and xylose, in the liquid and solid products from different conditions of dilute acid pretreatment are listed in Table 6-2, for the bagasse from varieties 55, 70, 74 and 120 included in the present study. It is worth to note that the industrial bagasse (variety 120) required more severe conditions of pretreatment to obtain the maximum combined sugar yield. As expected, most of the glucose was retained in the WIS (86-97.3%), while xylose was the main component in the pretreatment liquor (contained 53-85.3% of the xylose in raw material). Nevertheless, glucose solubilisation increased with the severity of pretreatment reaching a maximum of 11.9% recovered in the pretreated liquor (Run 6, variety 70). This trend was also observed for the industrial bagasse, but the values of glucose in the liquor were lower (2.5-7.7%) in spite of the most severe conditions within the CCD. Similarly, xylose recovery in the pretreatment liquor of bagasse 120 was lower (36.4-78.6%) compared to that of varieties 55, 70 and 74 (53-85%). However, the xylose recovery in the WIS was higher in 120 (4.1-12.6%) for most of the range of pretreatment conditions tested.

Hemicellulose removal from the bagasse fibres is considered as parameter of the effectiveness of dilute acid pretreatment on accessibility [32] but depending on its

severity, the solubilized sugars can be further degraded into furans. Statistical analysis was used to evaluate the effect of temperature and residence time on recovery of the hemicellulose, specifically xylan in the form of oligomeric and monomeric xylose, in the pretreatment liquor. The equations for the total xylose recovery in the pretreatment liquor for each variety (Table 6-3, Eqns. 4-7) were used to draw contour plots (Figure 6-1). Both temperature and reaction time impacted xylose yield in the pretreatment liquor in a negative manner for all varieties in the range of pretreatment conditions investigated. However, it can also be observed that varieties had different pretreatment requirements. For example variety 55 required lower temperature and shorter residence time (186°C for 5 minutes) than the 120 (190 °C for 8 minutes) to attain its maximum xylose recovery in the liquor (82.5 and 78.5% for the varieties 55 and 120, respectively).

Another parameter desirable for fermentation processes is the presence of sugars in monomeric form. The amount of xylose recovered in liquid fraction in monomeric or oligomeric (xylo-oligomers, XOS) form is depicted in Figure 6-2. No XOS were detected in the pretreatment liquor for the most severe pretreatment conditions applied to bagasse from varieties 74 and 120. The maximum XOS yield, about 14 g/100 g RM, was obtained for the variety 55 for the lowest CSF (0.96), which corresponded with 64% of the total xylose in the liquor. Although higher severities resulted in higher proportion of xylose in monomeric form, these conditions also increased xylose degradation into by products (Figure 6-2). Up to 59.5% of theoretical xylose was degraded (variety 120, CSF of 2.16). Nonetheless, the levels for furfural (3.6 g/100 g RM) and formic acid (0.2 g/100 g RM), degradation product from xylose and furfural respectively [33], did not account for all the xylose lost, similar to previous reports on optimisation of dilute acid pretreatment [34].

Regarding the total amount of inhibitors present in the pretreatment liquor, similar values were observed for the four substrates evaluated. Acetic acid, originated through hydrolysis of the acetyl groups of the hemicelluloses [35], was the inhibitor present in the liquor at the highest concentration. The acetyl hydrolysis into acetic acid increased with severity of the pretreatment, and it maxed out at CSF of 1.98 and 2.16 for classical

breeding varieties and industrial bagasse, respectively (Appendix C-2). Under these conditions, the acetyl group hydrolysed was more than 96-98.4% of theoretical. Another difference observed among varieties was the presence of HMF at concentrations ranging from 0.091 to 0.782 g/100 g RM in the pretreatment liquors from the varieties 55, 70 and 74, while for the industrial bagasse the highest concentration was 0.218 g/100 g RM (Appendix C-2).

6.3.2.2. Effect of pretreatment on enzymatic hydrolysis of washed pretreated solids

As mentioned earlier, the undesired feedstocks properties, present in differential of the same feedstock, can require increasing severity in process requirements (pretreatment and enzymatic hydrolysis). In an attempt to study the effect of feedstock-pretreatment combination on enzyme susceptibility, the WIS fraction of each variety-pretreatment combination was subjected to enzymatic saccharification. Enzymatic hydrolysis of the untreated materials was included for comparison.

The cellulose conversion of untreated materials was less than 30%. The differences in the recalcitrance of the bagasse of the different varieties could already be observed on the EH of the untreated material. The untreated bagasse from varieties 55 and 74 provided between 6 and 9% greater cellulose conversion than that of the variety 74 and industrial bagasse. Dilute acid pretreatment considerably increased the glucan conversion compared to untreated bagasse, giving values from 48.6-66.4% for the less severe conditions to 100% for the harshest conditions (Table 6-4). The differences in digestibility observed between varieties 55-70 and 74-120 were more evident after applying the pretreatment. The varieties 55-70 seemed to be less recalcitrant, requiring severities of about 1.6 to reach a digestibility higher than 80%, while the bagasse from variety 74 and industry (120) needed severities of at least 1.9 to reach digestibilities close to 80%.

Although more severe pretreatment conditions generally improve the accessibility of the fibres during enzymatic hydrolysis, it is normally at the expense of xylose degradation

and lower fibre recovery in the pretreatment. The glucose yield from the fibres considering the insoluble solids recovery of the pretreatment and the glucose released during enzymatic hydrolysis was evaluated statistically. The effect of pretreatment conditions on glucose yield (g/100 g RM) is represented in contour plots (Table 6-4, Eqns. 8-11) in Figure 6-3. As expected, the highest yields of glucose were obtained at higher temperatures than those for maximum xylose yield (Figure 6-1). It was also found that the EH glucose yield for variety 74 could only be determined by the linear effects of temperature and residence time. Similarly to what was observed for xylose yields, the varieties 55 and 70 required less severe conditions to reach the maximum glucose yield compared to the other varieties.

6.3.2.3. Combined sugar yield

The sugars solubilized in the pretreatment liquor together with those released during enzymatic hydrolysis subsequent to pretreatment were used to determine the combined sugar yield (CSY, Table 6-4). The range of pretreatment conditions evaluated gave 9 to 15% differences in the CSY between the less and more harsh pretreatment conditions. The highest value of CSY was obtained for the central point for all the varieties evaluated (190°C for 10 minutes for the varieties 55, 70 and 74; 195°C for 9 minutes for industrial bagasse). The highest CSY were obtained for varieties 55 and 70 with respective average values of 65.5 and 63.7 g per 100 g dry material, respectively, which corresponds with 91.9 and 92.2% of sugars present in the raw material, respectively. Interestingly, the conditions that gave the highest CSY also generated lower concentration of inhibitors than those that gave the highest EH glucose yield (Figure 6-3). The concentrations of furfural (0.5 to 1 g/l), HMF (0.1 to 0.3 g/l), acetic acid (1 to 1.5 g/l) and formic acid (0.04-0.06 g/l) determined at these conditions (for the highest CSY) were under the threshold toxicity for *Saccharomyces cerevisiae* reported previously (0.3, 1.2, 2-6, 0.8 g/l for HMF, furfural, acetic acid and formic acid, respectively) [36,37].

The mathematical models for combined sugar yield containing the significant terms in coded form are also summarized in Table 6-3 (Eqns. 11-15). The statistical significance of each model was determined by ANOVA, which revealed that the models were significant, while the lack of fit was insignificant ($p < 0.05$) (Appendix C-3). The models were further validated performing additional experiments at the optimum conditions identified by the model (Table 6-3). The experimental CSY values differed less than 2% from the predicted values. These conditions were therefore selected to generate substrate for SSF experiments.

The maximum CSY values varied for the different varieties, but there was a common range of pretreatment conditions providing the maximum CSY for all varieties. Equations 11-14 (Table 6-3) were used in the Matlab programme in order to represent the pretreatment conditions that will reach 95% of the maximum combined sugar yield for each of the bagasses (Figure 6-4). It could be observed that variety 55 had a narrower set of conditions giving the maximum CSY followed by variety 70, 120 and 74. The area in common for the 4 samples evaluated is observed in the range of conditions defined by the intersection region (ABCB), which were between 184 and 200 °C for temperature and varying residence time to give a severity factor between 3.51 and 3.96.

6.3.3. Effect of pretreatment on SSF of unwashed pretreated solids

The batch SSF was performed on the unwashed pressed-slurry from optimum pretreatment conditions for maximum CSY, at a solids loading of 10%. The slurry was pressed up to a final moisture content of 59–63%. The use of the pressed-slurry presents some advantages for the process such as the avoidance of both washing step and loss of sugars. Some of the sugars remain soaked in the fibres, providing extra fermentable sugars for fermentation.

The time course for glucose, xylose and ethanol concentrations during batch SSF of dilute acid treated samples for the two enzyme dosages at a solids loading of 10 % are illustrated in Figure 6-5. All four substrates showed similar profiles. The initial glucose

(from the pretreatment liquor and the glucose generated during the prehydrolysis) was rapidly consumed within the first 8 h and remained at values close to zero until the end of SSF in the case of low enzyme dosage (Figure 6-5A), or until 100 h when the higher enzyme dosage was used (Figure 6-5B). As a result, ethanol concentrations were gradually increasing until these periods. The xylose, however, remained constant for the entire process.

The highest final ethanol concentrations were attained when using the varieties 55 and 70 for both enzyme dosages (Figure 6-5). No significant differences in ethanol yields were observed between variety 55 and 70, and between variety 74 and bagasse 120. The highest ethanol concentrations at low enzymes loading (0.15 ml of Cellic Ctec2/g pretreated material and 0.0167 ml of Cellic Htec2/g pretreated material) were 27.1, 29.3, 22.8 and 23.1 g/l for varieties 55, 70, 74 and bagasse 120, respectively (Figure 6-5A). These corresponded to ethanol yields of 70.4%, 74.8%, 59.3% and 61.4% of theoretical maximum, based on glucose content in each bagasse sample. As expected, increasing the enzyme dosage resulted in higher ethanol concentrations. Ethanol concentrations of 33.0, 33.1, 29.1 and 28.1 g/l, corresponding with ethanol yields of 84.5%, 85.6%, 79.9%, and 74.8% of the maximum theoretical yield based on glucose in the raw material for varieties 55, 70, 74 and bagasse 120, respectively, were obtained (Figure 6-5B). It is worth to note that a higher enzyme dosage was required for varieties 74 and 120 in order to obtain similar ethanol concentrations to those obtained at the lower enzyme dosage for varieties 55 and 70.

In an attempt to reach at least 40 g/l of ethanol [38], a fed-batch strategy was adopted for SSF to increase the dry mater concentration to 16% (w/w), while avoiding mass transfer limitations. Figure 6-6 depicts concentrations of xylose, glucose and ethanol for all four substrates during the fed batch SSF. Similarly to batch SSF, the ethanol concentration progressively increased while the residual glucose concentration remained almost zero for the first 76 h of SSF. However, glucose levels at the beginning of the SSF were higher than those were anticipated. This suggests that the addition

glucose came from Ctec2/Htec2 enzymes [39]. However, this does not affect the differences in ethanol yields among varieties. The level of xylose slightly increased during SSF probably due to the residual xylose in liquor soaked in the fibres and/or the xylan-degrading enzyme present in cocktails combinations. Ethanol concentrations higher than 40 g/l were reached only for varieties 55 and 70 after 68 h of SSF. The highest ethanol concentrations were 51.3 and 48.6 g/l, which correspond to yields of 77 and 74.3% of theoretical maximum based on glucose in the raw material for varieties 70 and 55, respectively. For varieties 74 and industrial bagasse 120, the highest ethanol concentrations were 37.1 and 38.3 g/l, which were equivalent to 57.3% and 61.4% of the theoretical maximum.

6.3.4. Correlations between lignin, xylose: arabinose ratio and EH glucose yield with ethanol yield

The impact of lignin content and ratio of xylose: arabinose on the ethanol concentration/yield was estimated by calculating coefficient of determination between them. In addition, relationship between the EH glucose yield and ethanol concentration was also established. The correlation was based on the highest ethanol concentration obtained by each substrate as depicted in Figure 6-7. As for the case of EH glucose yield (Table 6-4), most of the variation in ethanol concentration/yield was largely attributed to differences in lignin content between the varieties. Strong inversely correlation ($R^2=0.9098-0.9901$) between lignin content and ethanol concentration was observed (Figure 6-7A). The study observed inverse correlations ($R^2=0.611-0.7375$) between ethanol concentration and the ratio of xylose: arabinose (Figure 6-7B), but as expected, the ethanol concentration was strongly positive correlated with EH glucose yield ($R^2=0.7555-0.9244$) (Figure 6-7C).

6.3.5. Estimation of combined ethanol yield

The integration of second generation ethanol production from the bagasse into a first generation ethanol production from sugarcane juice is considered a feasible strategy for

industrial implementation, due to potential for integration of process unit operations such as feedstock handling, fermentation, distillation and energy utilities (steam, electricity). Moreover, the economics of the global ethanol production is highly influenced by agronomic properties of the cultivars such as biomass and juice yield. In this context, the combined ethanol yield for each variety of sugarcane was estimated considering agronomic data, ethanol production from the sugar juice (based on Eqn. 3) and ethanol production from the bagasse based on the results obtained in this study.

Table 6-5 summarizes the agronomic properties and values for ethanol yields for the different varieties of sugarcane [19]. The average cane productivity, content of soluble sugars in the juice and fibre content for the industrial sugarcane were obtained from literature, and were assumed to be 65 wet ton/hectare, 0.13g/g cane and 0.13g/g cane, respectively [40]. Conversion efficiency of the sugar from the juice of all varieties in the present study was assumed to be 85% [41], and the ethanol yield was calculated on the basis of cane yield and sugar content on the juice (Table 6-5). The ethanol yield obtained from bagasse from classical breeding varieties, was calculated based on bagasse yield per hectare (Table 6-5), and the conversion efficiency was obtained experimentally (highest ethanol yield obtained under the fed-batch SSF, 57.3–77%). Additionally, the potential ethanol yield from bagasse was calculated taking into account the extra ethanol that could be produced if the xylose (pretreatment liquor) was also fermented assuming a conversion efficiency of 0.36 g ethanol/g xylose consumed [42].

Differences in the combined and potential maximum ethanol yields were observed between varieties, with varieties from classical breeding (55, 70 and 74) being superior to industrial sugarcane. Variety 70 showed higher combined ethanol yield (from both juice and bagasse), whereas variety 55 was superior in terms of ethanol from the bagasse. Juice ethanol yields ranged 4,969 to 9,431 l/ha. The ethanol yields could be increased by 30–41% (6,479-12,273 l/ha), by combining first and second generation technology. Moreover, the combined ethanol yield was 1.33-1.47 higher than that of the juice (6,479-

12,641 l/ha) if the xylose recovered in the pretreatment liquor (at optimal conditions for maximum CSY) was also fermented.

6.4. Discussion

The combination of cultivar selection and process optimisation have the potential to enhance sugar conversion efficiency and increase ethanol output per feedstock and further reduce pretreatment severity and enzyme requirement, thereby reducing operating cost [13,14,22].

The bagasse from varieties 55 and 70 presented better response to pretreatment in terms of xylose recovery (Table 6-2), enzyme digestibility (Table 6-4) and therefore, combined sugar yield (Table 6-4). Likewise, these varieties had the highest ethanol yield (conversion efficiency) and final concentrations. This superior performance could be attributed to differences in chemical composition and structure between the samples (Table 6-1 and Figure 6-7). For example, varieties from classical breeding (55, 70 and 74) presented higher xylan content than industrial bagasse, but no strong correlation between xylan content and xylose recovery was determined for many instances (Appendix C-4). Whereas some other studies on herbaceous biomass indicated also insignificant correlation between xylose recovery and xylan content (transgenic switchgrass and alfalfa [43] as well as forage sorghum [12]), other studies revealed lowest xylose recovery for those varieties with higher xylan content (silvergrass, [44]). These weak correlations could be related to xylose degradation during pretreatment and/or differences in the xylan structure. In fact, the feedstocks evaluated in this study could be clustered in the pairs 55-70 and 74-120 according to the degree of arabinose substitution of the xylan backbone (ratio xylose: arabinose of 11.7-13.2 and 15.5-15 for the varieties 55-70 and 74-120, respectively).

In terms of EH glucose yield, higher digestibility of the pair 55-70 was also obtained even when no pretreatment was applied. This could be attributed to the lower lignin content compared to the pair 74-120. It is well known that the lignin matrix inhibits the

cellulases, acting not only as structural barrier but also by unproductive binding of enzymes, thus leading to lower cellulose digestibility [45]. Moreover, the digestibility obtained after the different pretreatment conditions presented a negative correlation with the ratio xylose to arabinose. It has been hypothesized that hemicelluloses with lower degrees of substitution are more likely to re-bond to the cellulose during mild dilute acid pretreatments [16]. This point is further supported by the higher recovery of xylan in the WIS from industrial bagasse compared to the classical breeding varieties (Table 6-2) for most of the pretreatment conditions evaluated.

However, the differences in cellulose digestibility between the best and poor performing varieties did not decrease when increasing the severity. This observation differs from the results found in previous studies when the pretreatment was conducted in a tubular reactor [12,16,22]. This could be due to the fact that the range of pretreatment conditions evaluated was close to the optimum for CSY.

As indicated in Table 6-3 and Table 6-4, the pair 55-70 provided the greater CSY at less severe pretreatment conditions than the industrial bagasse. Moreover, the conditions for the maximum CSY did not lead to substantial sugar degradation compared to those conditions that gave the highest glucan conversion (Table 6-3). This observation suggests that the conditions for highest CSY is of more benefit to ethanol production than maximising glucose yield, as more than 90% of theoretical sugar was recovered with the best performing varieties. The preferred varieties pretreated under optimum conditions provided up to 33% increment of CSY compared to industrial bagasse. Nevertheless, despite the different pretreatment requirements between varieties (Figure 6-1, Figure 6-3 and Figure 6-4; Table 6-3 and Table 6-4), the bagasse from the 4 varieties presented a range of conditions in common (temperature 184-200 °C and varying residence time to give the severity factor between 3.51 and 3.96) where the maximum CSY could be obtained (Figure 6-4). Although further research is needed for confirmation, this finding can constitute a promising tool to select optimum conditions without the pretreatment optimisation according to variety.

As expected, SSF process with the pair 55-70 resulted in higher ethanol concentration and ethanol yield for all the SSF processes evaluated. However, a fed-batch strategy was required in order to reach more than 40 g/l. Moreover, it appears that these feedstocks had less enzyme requirement probably due to the lower lignin content and higher branched xylan (Table 6-1 and Figure 6-7). Interestingly, the preferred varieties (55 and 70) in terms of sugars and ethanol yields efficiency (Table 6-3; Figure 6-5 and Figure 6-6) also showed higher combined ethanol per unit land compared to the industrial sugarcane (Table 6-5). The projected combined ethanol yield for these preferred varieties of “energycane” was almost twice of that observed for industrial sugarcane. These results suggest that ethanol yield per hectare can be improved through crop development and integrated conversion approach (“whole plant”, use of pressed-slurry, SSF).

6.5. Conclusions

The present study provides evidence of the impact of cultivar selection and process optimisation in sugar conversion efficiency and ethanol output per feedstock. Experimental results show that varieties with reduced lignin content and highly substituted xylan resulted in higher sugar and ethanol yields with milder pretreatment conditions and reduced enzyme dosage, which in turn could reduce the operating cost without detriment in ethanol production from the juice.

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6.6. References

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Table 6-1: Chemical compositions of bagasse from different varieties of sugarcane.

Values are given in % of dry matter

Component	55	70	74	120
Carbohydrates				
Glucan	38.3 ± 0.5	37.4 ± 0.7	38.1 ± 1.0	39.6 ± 0.6
Xylan	23.3 ± 0.4	22.5 ± 0.1	21.6 ± 0.6	19.5 ± 0.3
Arabinan	2.0 ± 0.1	1.7 ± 0.2	1.4 ± 0.2	1.3 ± 0.1
Lignin				
Acid soluble	3 ± 0.4	2.9 ± 0.4	2.8 ± 0.4	2.2 ± 0.1
Acid insoluble	17.3 ± 0.4	17.1 ± 0.2	19.5 ± 0.3	20.2 ± 0.2
Acetyl	3.3 ± 0.1	3.0 ± 0.2	2.8 ± 0.1	3.2 ± 0.2
Extractives	4.3 ± 0.9	4.8 ± 1.1	4.3 ± 0.1	5.0 ± 0.5
Ash	1.5 ± 0.1	1.9 ± 0.2	1.2 ± 0.1	1.3 ± 0.3
Mass closure	93.0	91.3	91.7	92.3

Table 6-2: Recovery of glucose and xylose in the WIS and in the hydrolysate after the dilute acid pretreatment as the percentage of theoretical value (content in raw material). The acid concentration at all pretreatment conditions was kept constant at 0.5% (w/w)

Run	Conditions				Glucose (%)								Xylose (%)							
	Temp (°C)	Time (min)	pH ^a	logR ₀ '	WIS				Liquor				WIS				Liquor			
					55	70	74	120	55	70	74	120	55	70	74	120	55	70	74	120
1	180(185)	5(4)	2.11(2.20)	0.94(0.91)	95	96	97.3	97	4.9	3.9	2.5	2.5	11	10	11	11	78.5	67	66.8	65.9
2	200(205)	5(4)	2.05(2.11)	1.59(1.58)	91	90	93.9	95	8.2	9.1	5.5	4.6	7.2	5.1	4.5	5.9	71.7	73	74.7	65.7
3	180(185)	15(14)	2.07(2.09)	1.46(1.56)	92	90	94	93	7.3	8.5	5.5	5.7	6.4	8.2	4.1	8.6	79.2	72	65.7	60.6
4	200(205)	15(14)	2.06(2.08)	2.06(2.16)	89	86	88.8	90	10.6	11	8.2	7.7	3.4	5.1	3.7	4.1	61.8	53	54.1	36.4
5	176(181)	10(9)	2.13(2.14)	1.10(1.20)	95	95	96.9	97	3.8	4	2.7	3.2	5.3	15	14	13	66.7	76	69.4	74.4
6	204(209)	10(9)	2.08(2.10)	1.98(2.06)	91	86	89.6	93	7.7	12	8.5	5.4	4.9	4.3	3.7	7.2	62.5	58	59.6	46.3
7	190(195)	3(2)	2.06(2.15)	1.07(0.95)	95	95	96.2	97	4	5.8	3.6	2.7	6.8	7.4	8.2	13	84	74	69.4	62.8
8	190(195)	17(16)	2.06(2.08)	1.82(1.92)	89	89	91.3	94	9.6	11	8.1	7	4.2	7.4	4.9	8.1	67.2	71	67.8	47.5
9	190(195)	10(9)	2.08(2.12)	1.57(1.63)	90	91	92	93	7.7	9.4	6.1	5.5	4.5	7.8	5.3	8.6	75.4	85	82.6	78.6
10	190(195)	10(9)	2.09(2.10)	1.56(1.65)	93	92	94	95	7.3	8.7	6.6	5.2	4.9	7	5.7	7.7	78.2	83	77.3	75.9
11	190(195)	10(9)	2.07(2.11)	1.58(1.64)	93	89	91.6	95	7.5	9.8	6.5	5	4.9	7.4	5.7	7.2	78.4	79	77.1	74

The conditions and values in parenthesis were employed for bagasse 120 to have a better response to pretreatment

^a The values showing the pH for varieties 55, 70 and 74 is the average of the three substrates

Table 6-3: Coefficient of determination, optimal conditions and maximum values according to the mathematical model for different optimisation criteria

Model (in coded form)	R ²	Optimal cond.		Max. Values	
		Temp.	Time	Pred.	Val.
4 $X_{55}=77.15-3.77T-4.12t-5.64T^2$	0.85	186	5	82.5	Na
5 $X_{70}=82.33-4.80T-2.50t-6.15Tt-8.51T^2-5.94t^2$	0.91	187	9	82.9	Na
6 $X_{74}=78.99-2.20T-2.99t-4.88Tt-7.56T^2-5.50t^2$	0.88	188	9	79.3	Na
7 $X_{120}=76.18-8.00T-7.02t-6.01Tt-8.06T^2-10.66t^2$	0.97	190	8	78.5	Na
8 $G_{55}=38.05+3.63T+1.79t-2.73T^2-2.91t^2$	0.98	193	10	38.7	Na
9 $G_{70}=34.99+2.73T+1.44t-2.61Tt-2.61T^2$	0.98	197	6	36.1	Na
10 $G_{74}=26.54+3.24T+2.63t$	0.88	194	15	30.4	Na
11 $G_{120}=27.67+2.25T+2.85t-1.39Tt$	0.97	198	14	31	Na
12 $CSY_{55}=65.47+2.16T+1.04t-4.90T^2-2.91t^2$	0.96	189	10	65.3	65.8
13 $CSY_{70}=63.73+1.80T+1.26t-4.55Tt-5.29T^2-1.96t^2$	0.99	188	11	63.5	64.5
14 $CSY_{74}=52.67+3.05T+2.54t-2.70Tt-2.85T^2-2.44t^2$	0.95	189	12	53	52.7
15 $CSY_{120}=49.24+0.35T+1.64t-2.82Tt-2.19T^2-2.08t^2$	0.92	192	12	49.7	50.3

X, G and CSY stands for xylose recovery after pretreatment (% of theoretical), glucose yield after enzymatic hydrolysis (g per 100g dry material) and combined sugar yield (g per 100g dry material); subscripts (55, 70, 74 and 120) stands for substrates; T and t represents temperature and reaction time in coded form.

Optimal conditions (Optimal cond.): Temp. is temperature (°C) and time (min); acid concentration was kept constant at 0.5% (w/w).

Pred. and Val. stands for the maximum values predicted by the model and those obtained experimentally, respectively.

Na means not determined.

Table 6-4: Yield of glucose after enzymatic hydrolysis, glucan digestibility, overall glucose recovery and combined sugar yield after pretreatment and enzymatic hydrolysis of different samples of sugarcane bagasse. The acid concentration at all pretreatment conditions was kept constant at 0.5% (w/w).

Run	Enzymatic hydrolysis								^b Overall glucose recovery (%)				^c Combined sugar yield (g/100 g RM)			
	Glucose yield (g/100 g RM)				^a Digestibility (%)											
	55	70	74	120	55	70	74	120	55	70	74	120	55	70	74	120
Untreated	11.7	12	9.2	8.9	27.5	28.6	21.7	20.2	-	-	-	-	15.7	17	13	12.5
1	26.7	25	21	20.8	66.4	63.6	50.5	48.6	68.1	65.0	56.7	50.0	54.3	48	40.6	39.6
2	33.9	36	29	29	87.6	97.0	71.9	69.3	86.9	96.3	73.2	70.5	59.6	62	51.8	47.6
3	30.6	34	27	29.6	78.6	91.6	68.4	72.3	79.9	91.4	70.9	72.7	58.6	60	48.8	48.3
4	36.5	35	31	32.2	96.6	97.1	81.1	81.1	96.3	96.3	80.3	81.8	60.3	55	49.3	45
5	27.4	26	20	24.5	67.5	64.8	49.7	57.5	68.1	65.0	52.0	59.1	51.5	51	42.2	45.3
6	38.7	33	31	29.6	100.0	92.9	81.5	72.6	98.7	91.4	82.7	72.7	58.7	55	51.2	44
7	29.9	33	21	24.3	74.3	84.5	50.8	56.8	75.2	86.6	52.0	59.1	58	58	42.4	42.4
8	37.5	36	30	31.9	99.2	97.2	76.8	77.1	98.7	96.3	78.0	79.5	62.3	62	52.7	47.4
9	38	35	28	27.7	99.3	92.4	70.9	67.4	96.3	93.9	70.9	68.2	64.8	65	53.3	50.3
10	37.8	35	28	27.4	95.4	92.8	70.1	65.8	96.3	93.9	73.2	68.2	65.5	64	52.6	49.1
11	38.4	35	28	27.3	97.4	94.4	71.2	65.6	98.7	93.9	70.9	68.2	66.1	63	52.1	48.3

^a Digestibility was calculated as glucose yield divided by the potential glucose in the WIS expressed in percentage

^b Overall glucose recovery is the sum of glucose obtained after pretreatment and enzymatic hydrolysis divided by potential glucose in the native material expressed in percentage

^c Combined sugar yield is the sum of glucose, xylose and arabinose obtained after pretreatment and enzymatic hydrolysis

Table 6-5: Cane yield, sucrose content, potential sugars content in juice and bagasse and ethanol yield from different varieties of sugarcane (55, 70, 74 and 120)

	55	70	74	120
Cane yields (wet ton/ha)	105.5 ^a	108 ^a	105.5 ^a	65 ^b
Sugar juice content (kg/ton wet cane)	149 ^a	163 ^a	149 ^a	140 ^b
Bagasse content (kg/ton wet cane)	147 ^a	127 ^a	161 ^a	133 ^b
Ethanol yield (L/ha)				
¹ Juice ethanol	7 731	9 431	8 305	4 967
² Bagasse ethanol (glucose + xylose)	3 605(431)	3 210(368)	3 049(380)	1 660(148)
³ Combined (juice + bagasse)	11 336	12 641	11 354	6 627

^a The cane yield, sugar juice and bagasse content for varieties 55, 70 and 74 were provided by SASRI, and they represent the average of two harvests (2009 and 2011).

^b The cane yield, sugar juice and bagasse content for sample 120 were obtained from the literature according to the average values of the industrial sugarcane.

¹Juice ethanol is the ethanol that can be produced by fermentation of juice sugars (sucrose, glucose and fructose).

²Bagasse ethanol is the ethanol that can be produced by fermentation of the xylose obtained after pretreatment and SSF of the pretreated bagasse. Values in parenthesis are ethanol yields from xylose.

³Combined ethanol yield is realistic total ethanol that can be obtained from the sugar juice and bagasse.

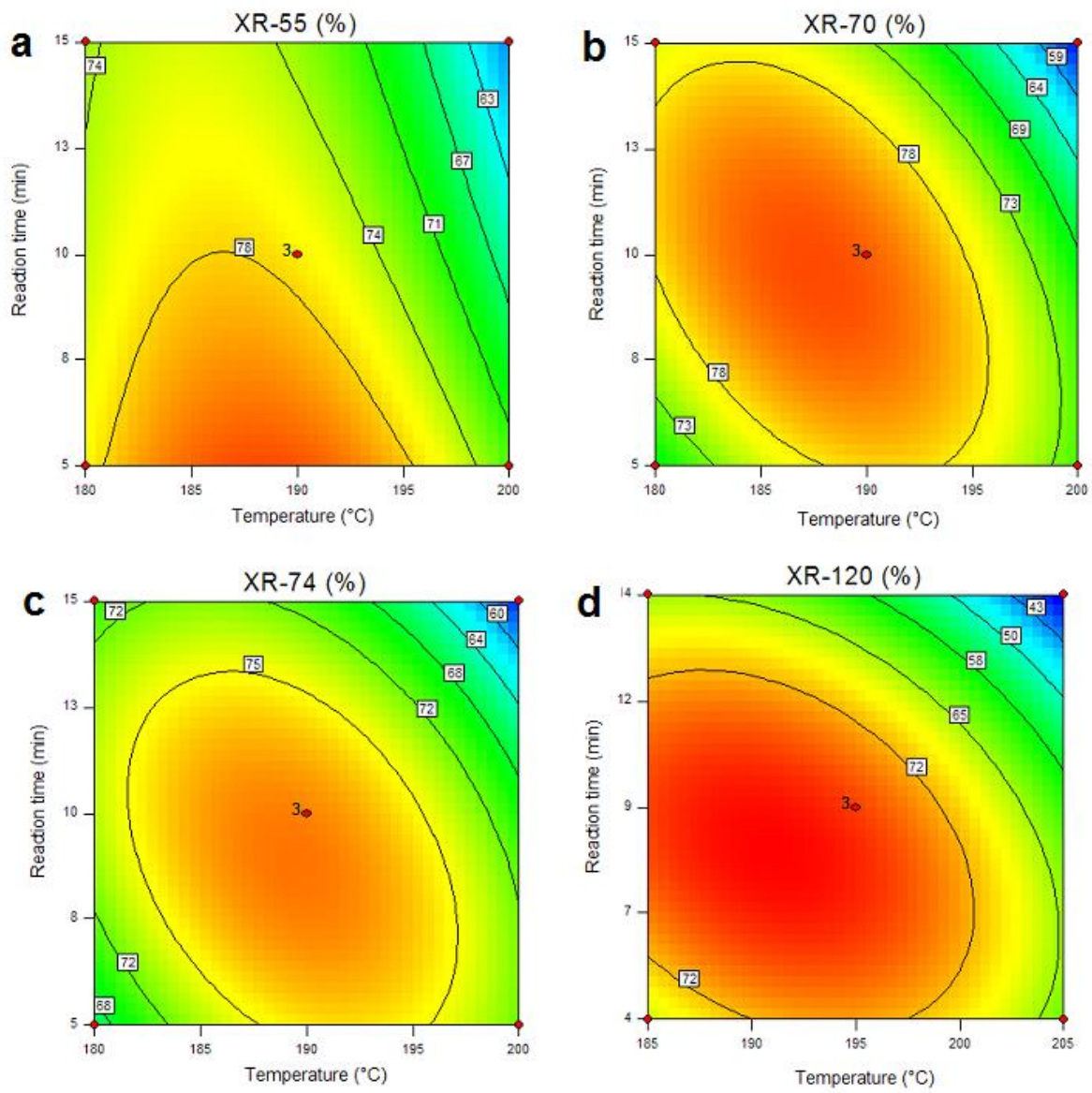


Figure 6-1: Contour plots for xylose recovery in the hydrolysate liquor for bagasse from (a) variety 55, (b) variety 70, (c) variety 74, and (d) industrial bagasse 120 as a function of pretreatment temperature and reaction time. The acid concentration was fixed at 0.5% (w/w).

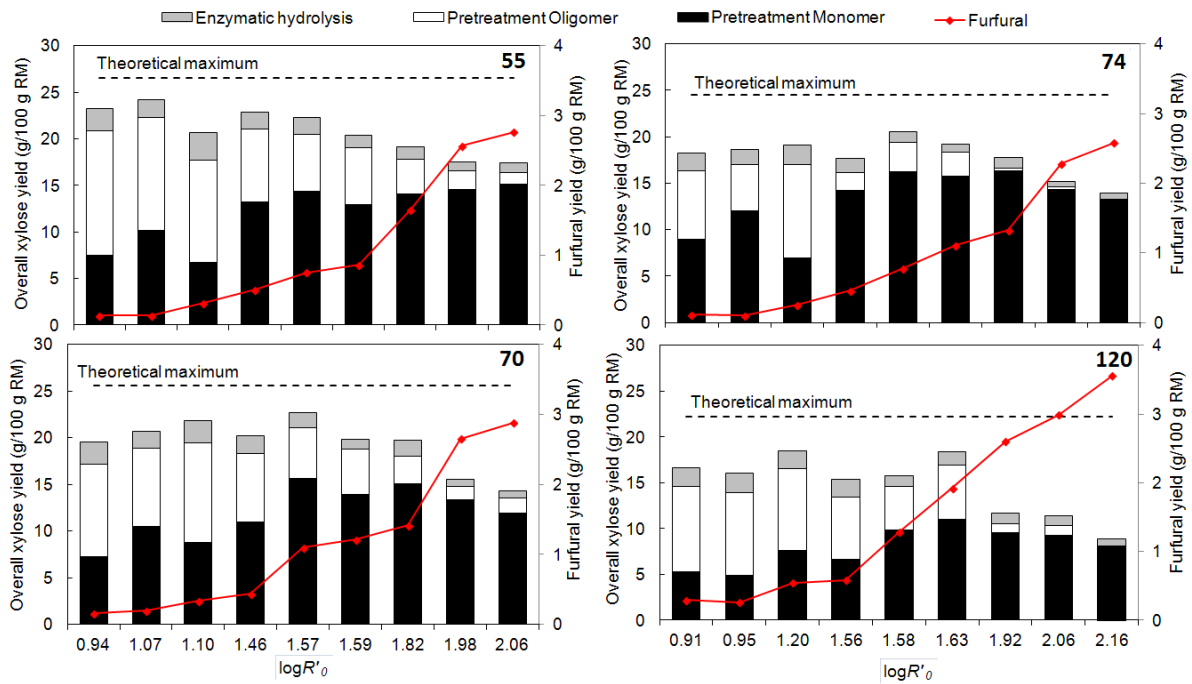


Figure 6-2: Overall xylose yield after pretreatment (as monomer and oligomer) and enzymatic hydrolysis and furfural formation after pretreatment of sugarcane bagasse samples (55, 70, 74 and 120) as the function of combined severity factor. The theoretical maximum for each feedstock is also indicated by discontinuous lines. Varieties 55, 70 and 74 were pretreated with similar conditions.

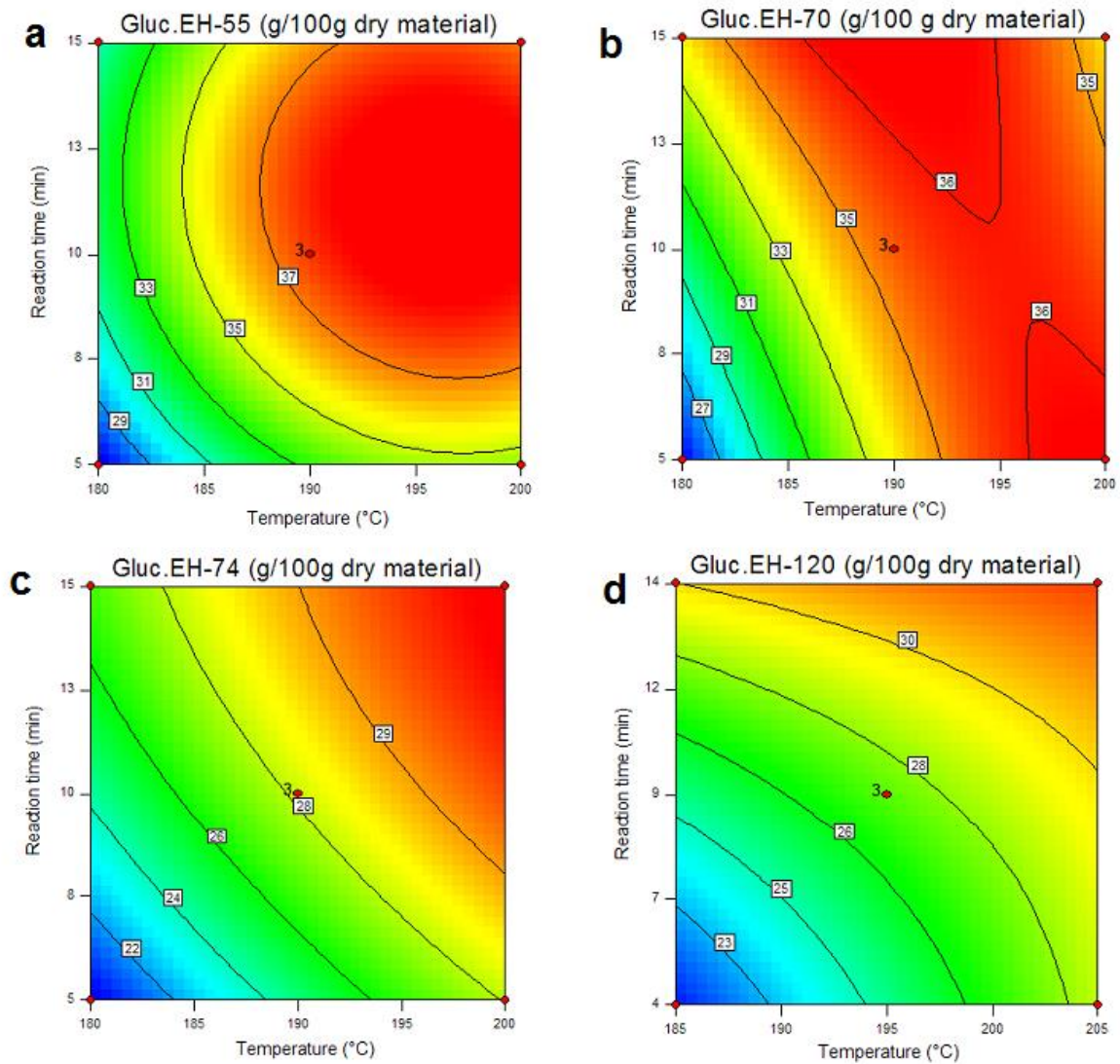


Figure 6-3: Contour plots for glucose yield from enzymatic hydrolysis (g/100 g raw material) showing influence of temperature and reaction time for: (a) variety 55, (b) variety 70, (c) variety 74, and (d) industrial bagasse 120. The acid concentration was fixed at 0.5% (w/w).

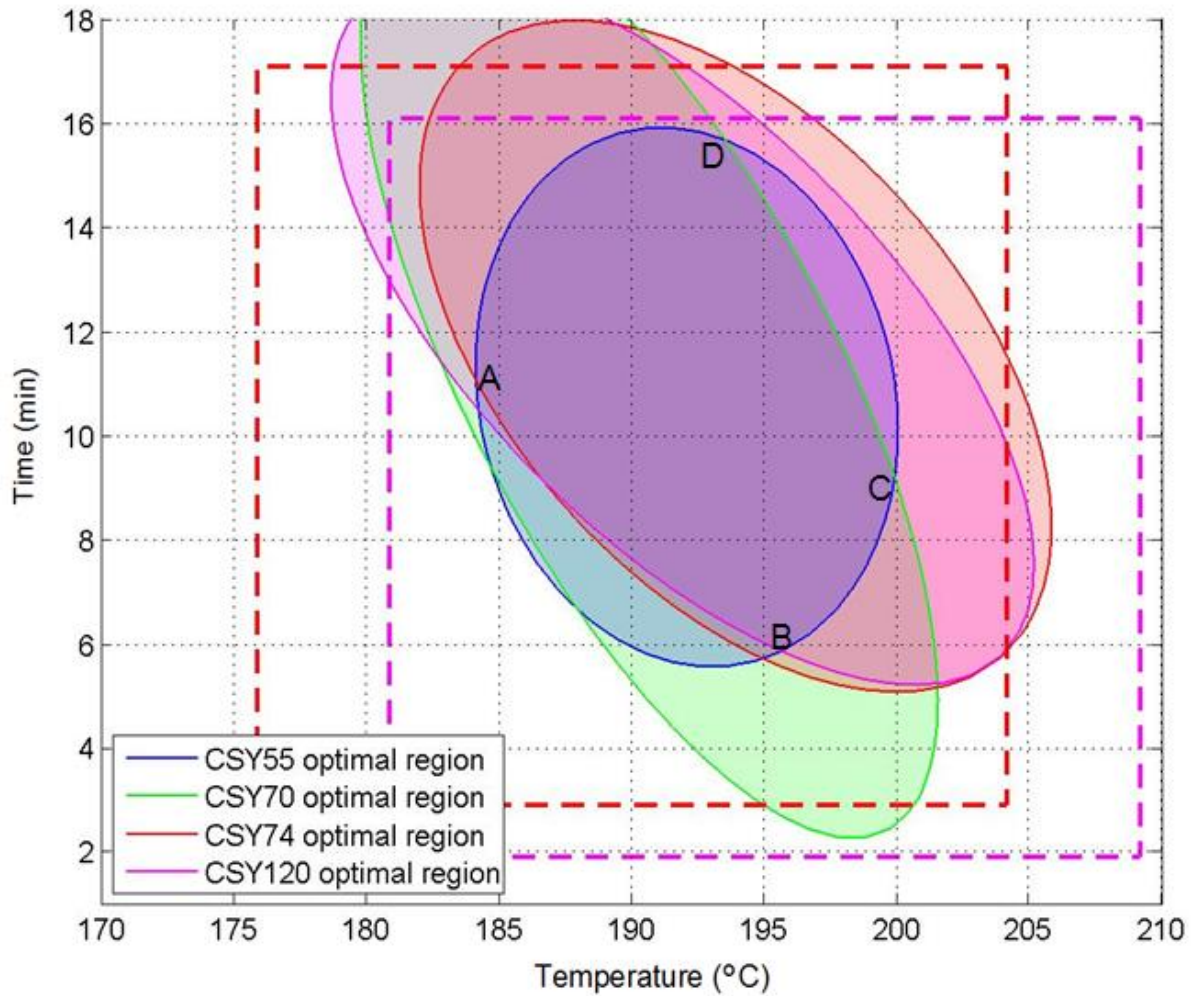


Figure 6-4: Contour plots representing the pretreatment conditions (temperature and reaction time) that provide 95% of the maximum combined sugar yield from bagasse (equations 11 to 14) of different sugarcane varieties. The dotted lines represent the input range of the independent variables for the cultivars: red for varieties 55, 70 and 74; pink for bagasse 120.

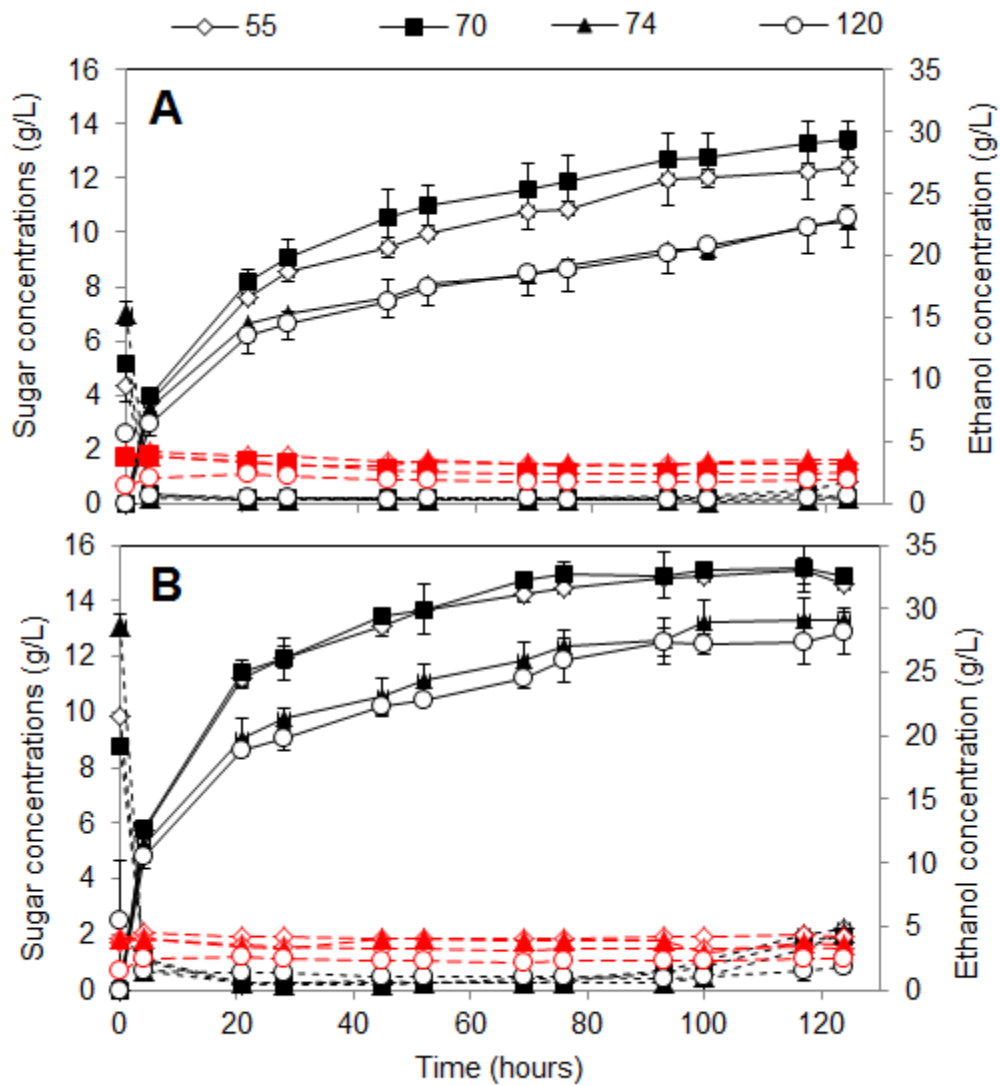


Figure 6-5: Glucose consumption (in dotted lines in black), xylose (dashed lines in red) and ethanol concentrations (in solid lines) during batch SSF of dilute acid pretreated sugarcane bagasse. Conditions: solid loading 10% (w/v), (A) 0.15 mL of Cellic Ctec2/g WIS and 0.0167 mL of Cellic Htec2/g WIS (B) 0.15 mL of Cellic Ctec2/g WIS and 0.213 mL of Cellic Htec2/g WIS. Samples 55, 70 and 74 were pretreated at 190 °C, 0.5% (w/w)-acid for 10 min and sample 120 was pretreated at 195 °C, 0.5% (w/w)-acid for 9 min.

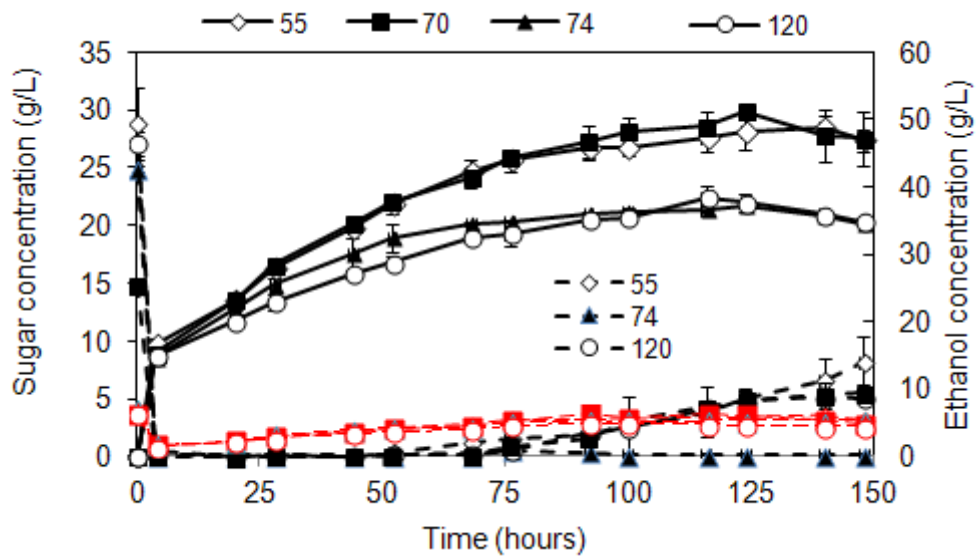


Figure 6-6: Glucose consumption (in dotted lines in black), xylose (dashed lines in red) and ethanol concentrations (in solid lines) during batch SSF of dilute acid pretreated sugarcane bagasse samples at 16% (w/w) solid loading and at enzyme dosage of 0.15 mL of Cellic Ctec2/g WIS and 0.213 mL of Cellic Htec2/g WIS. Samples 55, 70 and 74 were pretreated at 190 °C, 0.5% (w/w)-acid for 10 min and sample 120 was pretreated at 195 °C, 0.5% (w/w)-acid for 9 min.

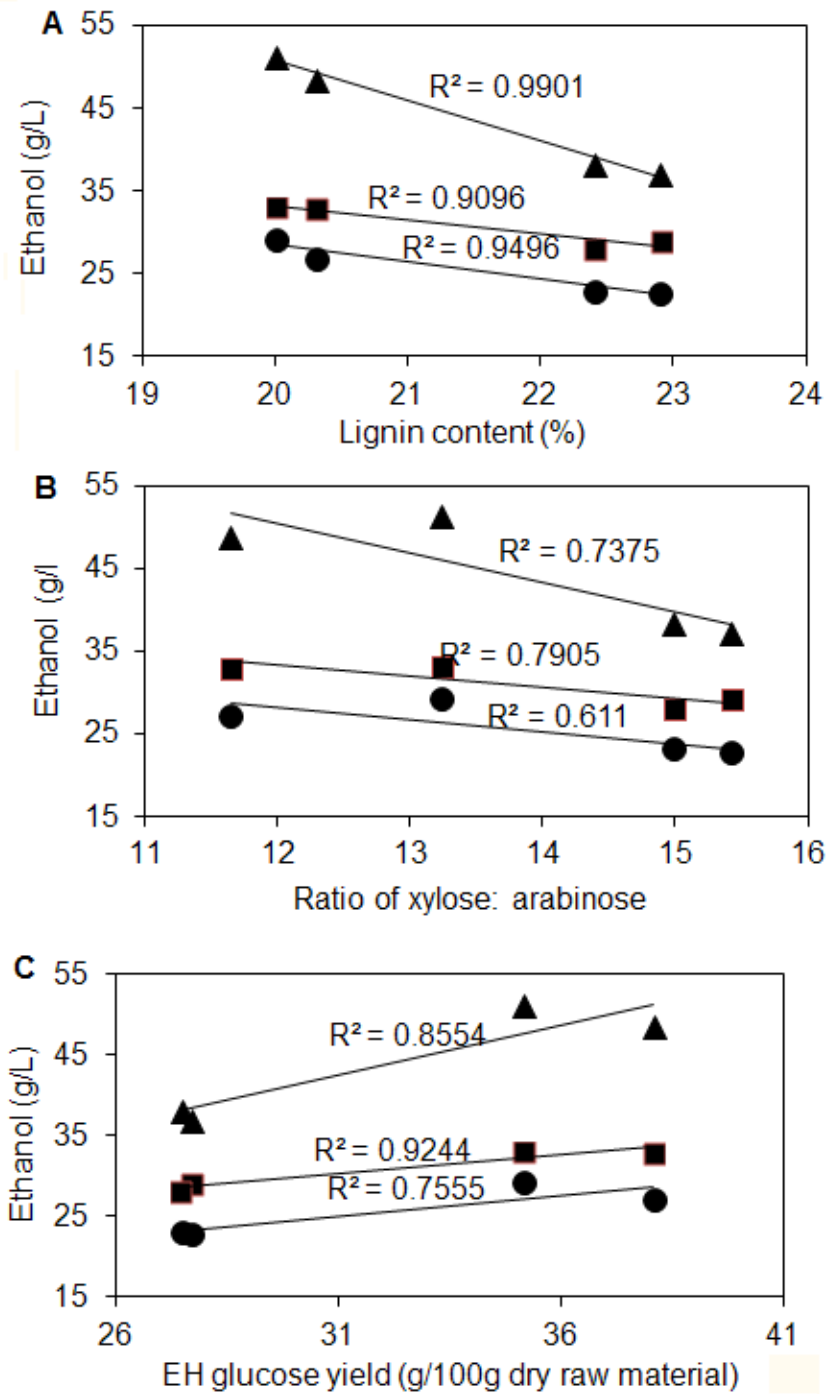


Figure 6-7: Correlations between the highest ethanol concentration during SSF of bagasse samples and (a) lignin content (b) ratio of xylose to arabinose (c) EH glucose yield at the pretreatment condition showed the highest combined sugar yield. Circle (●) and rectangular (■) makers represent ethanol concentration at low and high enzymes loadings for batch process whereas rectangular (▲) maker shows the ethanol concentration during fed-batch process.

Chapter 7

7. Comparison of chemical composition and ethanol yields of sugarcane varieties from different harvests

The adopted version was submitted to Industrial Crops and Products for publication in November 2013 with the following details:

Title: “*Comparison of chemical composition and ethanol yields of sugarcane varieties from different harvests*”

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Objective of dissertation and summary of findings in present chapter

In this chapter, the performance of the selected varieties in terms of agronomic properties, chemical composition and processability of bagasse, and combined ethanol yields (from bagasse and juice) across multiple harvests were compared (objective 5). This was achieved by considering agronomic data (cane yield, juice sugar, and bagasse content) and combined sugar yields obtained experimentally and estimated combined ethanol per hectare for the preferred varieties gathered between 2009 and 2011 (three harvests).

The results showed that the best performing varieties in terms of agronomic properties and bagasse processability was not maintained from one harvest to the other. The differences between varieties were more important than across harvests. It was further observed that the precision breeding varieties were hampered by prolonged severe drought

Candidate declaration

With regard to chapter 7 page numbers 185–218 of this dissertation, the nature and scope of my contribution were as follows.

Name of contribution	Extent of contribution (%)
Planning of experiments	70
Executing experiments	100
Interpretation of results	80
Writing the chapter	100

The following co-authors have contributed to 7 page numbers 185–218 of this dissertation.

Name	e-mail address	Nature of contribution	Extent of contribution
1. Shailesh V. Joshi	shailesh.joshi@sugar.org.za	<ul style="list-style-type: none"> • Provided field data • Reviewing the chapter 	100 50
2. Johann Görgens	jgorgens@sun.ac.za	<ul style="list-style-type: none"> • Experimental planning • Interpretation of results to correlate with literature • Reviewing the manuscript 	30 20 50

Signature of candidate:.....

Date.....

Declaration by co-authors

The undersigned hereby confirm that

4. the declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to chapter 7 page numbers 185–218 in the dissertation,
5. no other authors contributed to 7 page numbers 185–218 in the dissertation besides those specified above, and
6. potential conflicts of interest have been revealed to all interested parties and that any necessary arrangements have been made to use the material in to 7 page numbers 185–218 of this dissertation.

Signature	Institutional affiliation	Date

Abstract

Potential ethanol yields from the sugarcane “whole plant” approach depend on cane yield, soluble sugar content in the juice and fibre quality. All of these components can be influenced by many factors such as genotypes, environmental conditions and cultivation parameters. In this study the agronomic properties, chemical composition, sugar and ethanol yields from both sugar juice and bagasse of selected sugarcane varieties were compared for different harvests. Results showed wide variations in agronomic properties (cane yield, contents soluble sugar in juice and insoluble structural carbohydrates), sugar released from pretreatment-hydrolysis and ethanol yields from juice and bagasse. Combined ethanol yields ranged from 2016 to 14063 L/ha. The differences were greater among the varieties than across the harvests, except for precision breeding varieties due to sub-optimal rainfall in the observed preceding year. Prolonged severe drought also affected the performance of all varieties represented by lower and intermediate lignin content for cane yield compared to that had highest lignin content. Therefore, an attempt to reduce lignin content in the bagasse, to reduce processing requirements for ethanol production, should also target to improve crop tolerance toward severe drought conditions.

Key words: varieties, sugarcane, harvest, chemical composition, pretreatment, enzymatic hydrolysis, ethanol.

7.1. Introduction

Sugarcane (*Saccharum spp hybrids*) represents one of the major crops planted in tropical and subtropical countries. Its main distinguishing features include high biomass yield, high sucrose content (Somerville et al., 2010), high efficiency in assimilating solar energy (Tammissola, 2010), and high water requirements in terms of litres per kilogram of aerial biomass comparable to many other annual grain or sugar crops (Tammissola, 2010). Sugarcane has been used for ethanol production with Brazil leading production (Goldemberg, 2008). During the harvest of sugarcane, leaves, tops and trash are left in cane

field while the sugarcane stalks are transported to the mills, crushed to extract juice (sugar juice) for ethanol production (Gullett et al., 2006). Along with the fibrous residue (bagasse) following juice extraction, the left over harvest residue (leaves, tops and trash) have potential to be converted to ethanol in the “whole plant” conversion strategy. However, bagasse and harvest residues differ in physical nature and process requirements (Ferreira-Leitão et al., 2010; Krishnan et al., 2010). These differences in the process requirements, suggest that separation of the two residues is important during processing for optimal ethanol production. Therefore, this study was limited to bagasse.

Presently only syrup is fermented to ethanol. Converting bagasse to ethanol requires specialized enzymatic hydrolysis and fermentation processes, but is required to further increase ethanol production from sugarcane per unit per unit land (Wyman, 2007). However, prior to enzymatic hydrolysis of the residues, a costly pretreatment process is required to make its structural carbohydrates accessible to enzymes (Sun and Cheng, 2002). The chemical composition of bagasse determines in part the quality of the feedstock and has direct impact on processing costs (Masarin et al., 2011). Enrichment for structural carbohydrates content (cellulose and hemicellulose) and low lignin content is favourable for ethanol production, while high lignin content does not suit ethanol fermentation because lignin impedes ethanol yield (Isici et al., 2008).

Water, soil, fertilizers, diseases and temperature factors can affect the agronomic properties of sugarcane (cane yield, sucrose content and content of fibrous components). These factors have direct impact on the combined ethanol yield from sugar juice and bagasse (Basnayake et al., 2012; Patel, 1985; Singels et al., 2011; Tammissola, 2010; Waclawovsky et al., 2010). Water is often a limiting factor for sugarcane production, therefore, timely irrigation plays a vital role during the period of water deficiencies (Tammissola, 2010). The needs for irrigation can be minimised by developing sugarcane varieties that are drought resistance. This will also improve water utilization efficiency.

Moreover, chemical compositions of the bagasse from sugarcane varieties may vary depending on the genotype (Masarin et al., 2011). Studies on other types of energy feedstocks such as sweet sorghum (Zhao et al., 2009), switchgrass (Kim et al., 2011) and

winter triticale (Kučerová, 2007) have shown that the chemical composition of the biomass can vary depending on genotype, location, year, maturity, harvests, environmental and cultivation parameters. However, limited information is available on the variability in agronomic properties combined with chemical composition of bagasse and combined ethanol yield of sugarcane varieties from different harvests. The variation in properties between harvests should be considered in feedstock (genotype) selection for combined ethanol production from both sugar juice and bagasse.

To identify novel varieties of sugarcane with improved properties for combined ethanol production from both sugar juice and bagasse, 115 varieties from the breeding program at South Africa Sugarcane Research Institute (SASRI) were screened. Of these samples 100 varieties originated from classical breeding and 15 varieties were from precision breeding (genetic engineering). These varieties were screened in terms of combined properties of potential ethanol yields per hectare from both sugar juice and bagasse and bagasse processability, as reported previously (Benjamin et al., 2013), including optimisation of pretreatment conditions to maximise fermentable sugar yields from bagasse (Benjamin et al., 2014). In the present study, preferred varieties were compared using sugarcane materials from different harvests, to assess the impact of seasonal variations on the combined ethanol yield, from sugar juice and bagasse. Ethanol yields from sugar juice were estimated from soluble sugar content in the juice based on literature, while ethanol yields from bagasse were determined by pretreatment-hydrolysis-fermentation experiments. Such multi-harvest assessment of raw materials is expected to identify the most promising variety for ethanol production from the sugarcane plant.

7.2. Materials and methods

7.2.1. Raw material and samples preparation

The sugarcane varieties used in the present study were developed by South African Sugarcane Research Institute (SASRI) through classical and precision breeding technologies. The precision breeding varieties were developed by down-regulating

expression of an endogenous enzyme UDP glucose dehydrogenase as described elsewhere (Bekker, 2007). The experimental field trial was conducted at the SASRI, Mount Edgecombe, KwaZulu Natal (latitude: 29.7000° S; longitude: 31.0333° E). The varieties had South Africa origin and were first planted field in 2006. This means that the varieties evaluated in this study were from 3rd and 5th ratoon crops. Clones were planted in 35m plot with 3 replications, with a row spacing of 1m and plants to plant spacing of 0.5m. The crop was grown in rainfed condition throughout the experiment. In total six varieties were included in the study, where varieties 99F2004⁵⁵, 00F0884⁷⁰ and 01G1662⁷⁴ were derived from classical breeding and 05TG004¹⁰¹, 05TG008¹⁰⁴ and 05TG018¹¹⁴ were derived from precision breeding. The superscripts 55, 70 74, 101, 104 and 114 will be used to describe and discuss the varieties further in the manuscript.

Twenty stalks samples per variety per plot were cut from the experimental field at the time of harvest and were used to determine the cane yield, juice sugar content (sucrose, glucose and fructose), fibre contents and others important measurements as described by standard millroom analysis (Anonymous, 2009).

For chemical characterization and pretreatment-hydrolysis study of the bagasse, 20 to 30 stalks were used. The stalks were shredded and then blended with water (1.5 kg of sample and 3 l of water) for 20 min. Thereafter, the finely crushed shredded canes from the blending jar were washed with water three times and each wash was collected and measured for residue sucrose and other soluble sugars. The remained fibre was pressed to reduce water content and was dried at 40 °C for four days until dry. The average moisture content of the materials after drying was about 6%. Prior to its use, the milled sugarcane bagasse was sieved to obtain a representative particle size suitable for the raw material composition analysis and for the pretreatment studies. The particles retained between 425 and 825 µm were packed in zipped plastic bags. The prepared samples were stored in a temperature and moisture controlled room set at 20 °C with a relative humidity of 65% up until processed.

7.2.2. Dilute sulphuric acid pretreatment of bagasse

Dilute sulphuric acid pretreatment of bagasse samples was carried out in a small tubular reactor (18 cm long and 1.27 cm internal diameter), according to Yang and Wyman. (2009). Dry Material (1.5 g) was soaked in 30 ml of dilute sulphuric acid solution for 12 h. Soaked samples were concentrated through filtering to a solid loading of 30% (w/v). The obtained wet biomass was loaded into the reactor and compressed by a metal rod to ensure uniform heat and mass transfer. The reactor was first submerged into a heating-up fluidized sand bath set at 30 °C above the target temperature. The reactor was heated until the target temperature was reached (approximately within 120 s), after which it was transferred into the second fluidized sand bath set at the target reaction temperature. After the reaction time was completed, the reactor was quenched by submerging into cold water bath. After cooling, the whole slurry was mixed with 100 ml of distilled water and vacuum-filtered into a solid and a liquid fraction. The solid fraction was further washed in three washes (each wash with 100 ml) to raise the pH up to 5 prior to enzymatic hydrolysis, and is subsequently referred to as Water Insoluble Solid (WIS) fraction. One part of filtrate was analysed for monomeric sugars content and the other part was used to determine the total sugars in the pretreated liquor as monomers and oligomers by post-hydrolysis as described elsewhere (Jacobsen and Wyman, 2002). The pretreatment conditions used in this study were selected from the previous study based on low severity (170 °C, 0.45% w/w-acid, for 5 min), high combined sugar yield (180 °C, 0.65% w/w-acid, for 10 min) and high enzymatic hydrolysis yield (190 °C, 0.45% w/w-acid, for 15 min) (Benjamin et al., 2014). All pretreatments were performed on duplicate.

7.2.3. Enzymatic hydrolysis of pretreated bagasse

The WIS fractions of pretreated bagasse samples were subjected to enzymatic hydrolysis to evaluate the effect of the pretreatment on the enzyme accessibility. These experiments were conducted in 24 ml glass tubes. The tubes were loaded with 200 mg (dry weight) of WIS and 10 ml of 0.05 M citrate buffer (pH 4.8) with the enzyme solution. Sodium azide was added at a concentration of 0.02% (w/v) to prevent microbial contamination. Two

commercial enzymes preparations were used: Spezyme CP (Genencor-Danisco, Denmark) with cellulase activity of 65 FPU/ml and Novozym 188 (Novozymes A/S, Denmark) with β -glucosidase activity of 995 IU/ml. Enzyme activities of both undiluted enzymes were determined according to García-Aparicio et al., (2010). Cellulase loading of 15 FPU/g WIS of Spezyme CP supplemented with β -glucosidase of 15 IU/g WIS was applied in all the experiments. Tubes loaded with the mixtures were placed in water bath shaker maintained at 50 °C with shaking at 90 rpm. Samples were withdrawn after 72 h, prepared as described below and analysed for sugars by High Performance Liquid Chromatography (HPLC).

7.2.4. Yeast and culture medium for fermentation of pretreated bagasse

Saccharomyces cerevisiae MH1000 was used in the simultaneous saccharification and fermentation (SSF) experiments of pretreated bagasse, to determine possible ethanol yields from bagasse (van Zyl et al., 2011). The yeast strain was stored at -80 °C in the presence of 15% glycerol in vials prior to use. This stock solution was added in a 250 ml Erlenmeyer flask containing 50 ml culture medium (20 g.l⁻¹ yeast extract, 7.5 g.l⁻¹ (NH₄)₂SO₄, 3.4 g.l⁻¹ KH₂PO₄, 0.8 g.l⁻¹ MgSO₄.7H₂O, 1ml trace element solution, 0.05g.l⁻¹ CaCl₂.H₂O, 0.5g.l⁻¹ and Citric acid) and 20g.l⁻¹ glucose. The medium and the flask were autoclaved at 121 °C for 15 min before the yeast was added. The culture was incubated at 30 °C with agitation speed of 150 rpm for 24 h. After incubation the cells' density (OD) was measured by spectrophotometer at a wavelength of 600 nm. The yeast cells were preconditioned in a 1000 ml Erlenmeyer flask containing 300 ml of solution for 24 h. The solution consisted of sterile medium with final concentrations as described above, 20g.l⁻¹ glucose, undiluted pretreated liquor (60 ml) and deionized water. The pretreated liquor was sterile filtered using a (0.22 μ m Stericup, Millipore, Billerica, MA) before being used. For preconditioning the starting OD incubation was 0.2. The seed cells were harvested after 48 h by centrifugation at 8000 rpm for 5 min (Model Z366, Hermle Labortechnik GmbH, Wehingen, Germany), washed four times with sterile PBS solution (containing 8.01g.l⁻¹, NaCl; 0.2g.l⁻¹, KCl; 1.78g.l⁻¹, Na₂HPO₄.2H₂O; 0.27g.l⁻¹, KH₂PO₄; adjust pH to 7.4 with the addition of 3 M KOH) to remove residues. Finally,

20 ml of PBS solution was added to the appropriate weight of yeast cells and then was used for SSF.

7.2.5. Simultaneous saccharification and Fermentation (SSF) of pretreated bagasse

The batch SSF was carried out in a 250mL Erlenmeyer flask. The SSF started by adding 20 g (dry weight) of pretreated material (washed and unwashed). The material was neither sterilized nor detoxified. The pretreated material was diluted to a final solids loading of 10% (w/w) by adding sterile medium and enzymes. The sterile medium contained nutrients resulting to final concentration as described above. The enzymes used were the mixture of Cellic Ctec2 and Cellic Htec2 at a loading of 0.15 and 0.213 ml/g WIS, respectively. The enzymes were obtained from Novozymes (A/S, Denmark). The pH was adjusted to 5 by adding 3 M KOH before adding the enzymes and yeast cells. After an hour of pre-saccharification the inoculum was added at a ratio of 0.05 g wet cell yeast to 1 g dry pretreated material. The SSF experiments were performed at 35 °C, 150 rpm and sampling was done twice a day and was taken for HPLC analysis.

7.2.6. Chemical composition of bagasse and HPLC analysis

The NREL procedure described by Slitter et al. (Sluiter et al., 2008a, 2008b, 2005) was used for the chemical composition analysis of bagasse samples after being consecutively extracted with water and with 95% ethanol for 48 h in total in a Soxhlet apparatus. For the pretreated material, the same procedure was applied; except that no water or ethanol extraction was carried out because of pretreatment of most of extractives. The acid soluble lignin of the pretreated material was not measured.

Monomeric sugars (glucose, xylose and arabinose), acetic acid and ethanol of all bagasse samples were also determined by HPLC. The samples were analysed on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). The column was set to a temperature of 65 °C with a mobile

phase of 5 mM sulphuric acid and a flow rate of 0.6ml/min. The concentrations were measured with a RI detector (Shodex, RI-101) operated at 45 °C. However, under these conditions, the column does not resolve xylose, galactose and mannose. The presence of mannose and galactose in untreated samples were checked on an Xbridge™ Amide column (4.6 x 250 mm, 3.5 µm particle size) equipped with an Xbridge™ Amide precolumn (Waters) at 30 °C, eluted at a rate of 0.7 ml/min with 0.05% ammoniumhydroxide in water (A) and 0.05% ammoniumhydroxide in 90% acetonitrile (B). Sugars were detected by a Varian 380-LC evaporative light-scattering detector. No mannose and galactose peaks were detected. Therefore, the xylose quantification obtained on an Aminex HPX-87H Column was accurate. The glucan, xylan, arabinan, O-acetyl group content in the bagasse were calculated as (0.95 × cellobiose + 0.9 × glucose), 0.88 × xylose, 0.88 × arabinose and 0.683 × acetic acid, respectively (Sluiter et al., 2008b).

7.2.7. Statistical analysis and ethanol calculation

The statistical analysis of the data was employed using one-way-analysis of variance (ANOVA) to determine whether there were significant differences between the varieties or between the harvests on responses. The hypothesis was accepted or rejected at 95% confidence interval. Ethanol yield per hectare was estimated according to equation 1.

$$E_Y = X \times C_Y \times T_C \times \eta_S \times (1000 / \rho_E) \quad 1$$

Where E_Y , ethanol yield (L.ha⁻¹); X , is sugars content in both juice and bagasse (%cane); C_Y is the cane yield (ton.ha⁻¹); T_C , stoichiometric conversion factor (0.538 for sucrose and 0.5111 for other sugars); η_S , sugar conversion efficiency to ethanol and 1000/0.789 (density of ethanol at 20°C, g.mL⁻¹).

7.3. Results

7.3.1. Variability in cane properties and chemical composition of bagasse

Table 7-1 summarises the agronomic properties and chemical composition of six sugarcane varieties, three of which classical breeding and the other three were from precision breeding

across different harvests. Of the preferred varieties from previous rounds of selection (Benjamin et al., 2013), only variety 114 was regrown in 2012. However, there was no systematic pattern in the selection of samples from different harvests/varieties due to the fact that the selection was determined by cane availability, and not just scientific rigor. In addition, all sugarcane varieties used in this study were sufficiently matured when harvested. The maturity of sugarcane varies between 8 and 15 months depending environmental factors and agricultural practice (Wagih et al., 2004). Data for both classical breeding as well as for precision breeding varieties for 2010 season is not taken into account. This is due to the fact that because of drought season experienced during 2010 season (Figure 7-1) the cane growth was marginal and there was not enough material attained to get the required bagasse sample for further analysis. Similarly, no cane yield data in harvest 2009 for varieties 101, 104 and 114 were available. The varieties in 2009 differed slightly in cane yields but the differences were greater in 2011. All varieties showed lower tonnage in cane yields (30% lower) in 2011 than 2009, except variety 74 where the opposite was observed (15% higher). Suboptimal rainfall in 2010 (Figure 7-1) could attribute to the lower cane yields in 2011. All precision breeding varieties (101, 104 and 114) in 2011 were notable for having lower tonnage per hectare compared to the classical breeding varieties (55, 70 and 74). The precision breeding varieties in 2011 also produced shorter stalks, thinner stalks appeared to be more susceptible to smut. Variety 114 was regrown in 2012. The varieties also differed in the content of sucrose, juice sugar and fibre (Table 7-1). The values ranged 39–157, 80–165 and 93–165 kg per ton wet cane, respectively. The best variety in terms of sucrose, juice sugar and fibre content were different within or across the harvests. Notable, all precision breeding varieties in 2009 presented slightly higher sucrose as well as soluble sugar content than classical breeding varieties. However, the classical breeding varieties were superior to precision breeding varieties in 2011. Severe drought did not impact did not impact sucrose or soluble sugar content across harvest years for the classical breeding varieties. The fibre content was also variable depending on the genotype and harvests. The differences in fibre content across harvests were greater for precision breeding varieties (up to 32%) compared to classical breeding varieties (up to 8%).

The average chemical composition and theoretical ethanol of bagasse from six different varieties of sugarcane, which were harvested in between 2009 and 2012, are presented in Table 7-2. The chemical composition of the varieties harvested in 2009 were extracted from Benjamin et al., (2013). The analysis of variance showed considerable variations in chemical components among the varieties within and among harvest seasons. Varieties 55 and 104 exhibited significantly higher glucan content in 2011 than the content observed in 2009. Likewise, glucan content for variety 114 (2012) was significantly higher than that found in 2011 but was statistically the same with that of 2009. Varieties 70 and 74 showed slightly higher glucan content (3.6% and 3.2%, respectively) in 2011 when compared to year 2009, whereas the content for variety 101 was somewhat lower (3.9%). With regard to xylan, all varieties harvested in 2009 exhibited higher xylan content (5.8–10% more) than the content observed in 2011 or 2012. The differences in xylan content across harvests for varieties 70, 74, 101 and 114 were significant. The same pattern was observed by comparing arabinan content among the varieties and across harvests. Varieties 55, 70 and 101 in 2009 exhibited significantly higher arabinan content than that observed in 2011. In the case of lignin content, most of the varieties in 2011 showed higher lignin content with respect to 2009. This increment was significant for varieties 70 and 101. The results also showed that all precision breeding varieties (101, 104 and 114) in 2011 were characterised by higher ash content (117–238% more) with respect to year 2009. Contrariwise, all classical breeding varieties showed significantly lower extractives content (36–57% less) compared to the content in 2009. No clear pattern in acetyl content across the harvests between classical vs. precision breeding varieties was observed. Furthermore, the measured structural carbohydrates content, which includes, glucan, xylan and arabinan in the fibre, ranged from 61% to 69.3%. Suboptimal rainfall did not seem to impact the total structural carbohydrates content across harvest years, except for variety 114, which was significantly lower in 2011. However, all varieties obtained from precision breeding presented significantly higher structural carbohydrates within and between the harvests than the classical breeding varieties, except for variety 104 (2011). Based on glucan and xylan content, the minimum and the maximum theoretical ethanol yields from the varieties corresponded to 34 and 38.4 g

per 100g of dry material (assuming the theoretical conversion factor of 0.511 ton ethanol/ton C6 or C5 sugars). Once again, most of varieties from precision breeding showed higher potential in ethanol yield within and across harvests than the classical breeding varieties due to high structural carbohydrates.

7.3.2. Comparison of sugar yields after pretreatment and enzymatic hydrolysis of bagasse

The pretreatment was performed at three different conditions selected from the previous optimisation study based on lowest pretreatment severity (170 °C, 0.45% w/w-acid, for 5 min), high combined sugar yield (180 °C, 0.65% w/w-acid, for 10 min), and high enzymatic hydrolysis yield (190 °C, 0.45% w/w-acid, for 15 min) (Benjamin et al., 2014). The enzymatic hydrolysis for all pretreated samples was performed at 15 FPU/ g WIS. Sugar yields for varieties harvested in 2009 were taken from the previous study (Benjamin et al., 2014.).

7.3.2.1. Xylose yields from pretreatment of bagasse

Xylose yield as a sum of monomeric and oligomeric sugar in the hydrolysate form, from dilute acid pretreatment is shown in Table 7-3. Significant differences in xylose yields between the varieties were observed mostly at the pretreatment conditions of 170 °C, 0.45%, 5 min and 190 °C, 0.45%, 15 min. Under these conditions, the differences in xylose yields between varieties were generally more variable than the differences between harvests. Differences between the three pretreatment conditions were also observed, with the condition of 180 °C, 0.65%, 10 min favoured xylose production compared to the others (Figure 7-2). However, differences in xylose yields between harvests were statistically similar at 180 °C, 0.65%, 10 min for all varieties, except for variety 74, which produced lower xylose in 2011. Likewise, the best yielding variety was similar in all harvests (2009 and 2011). In case of xylose recovery, which is defined by xylose yield in the hydrolysate divided by xylose content of the raw material, most of varieties showed statistically similar recoveries at 180

°C, 0.65%, 10 min across the harvests (Figure 7-3A). However, variety 114 in (2012) exhibited significantly higher recovery (6.2–8%) compared to those observed in 2009 or 2011. Likewise, variety 55 in 2011 showed substantially higher xylose recovery (5.7%) than the recovery obtained in 2009.

7.3.2.2. EH Glucose yields from pretreated bagasse

Since all varieties from both the 2009 and 2011 harvests showed similar pattern in glucose EH yields, the harvest of 2011 was used to show the effect of pretreatment on enzymatic hydrolysis yield (Figure 7-2). Glucose yield was significantly improved after the materials were pretreated. However, unlike to xylose yield, the pretreatment conditions of 190 °C, 0.45%, 15 min showed the highest glucose yield in all varieties, except for variety 101 (2009). Results regarding the differences in EH glucose yields of varieties within the harvest and between the harvests showed significant difference (Table 7-1). However, the differences in glucose yields between the varieties were greater than those observed between the harvests. The best yielding variety was different across harvests. Variety 101, consistently released significantly higher glucose (17.2–32.3%) in 2009 than that observed in 2011 in all pretreatment conditions tested (Table 7-3). The differences outlined above for EH glucose yields can be reflected in their glucan conversion efficiency (Figure 7-3B). As for other varieties, although they showed different glucose yields, their glucan conversions were statistically similar, with EH glucose yield determined by chemical composition of the raw material.

7.3.2.3. Combined sugar yields from bagasse pretreatment and hydrolysis

The analysis of variance also revealed significant differences in combined sugar yields (sum of glucose, xylose and arabinose after pretreatment and enzymatic hydrolysis) among the varieties as well as between the harvests (Table 7-3). However, the differences in combined sugar yields were bigger than the differences observed between the harvests. The highest yields between the harvests were also different and they were observed on different

varieties. Variety 101, consistently released significantly lower combined sugar in 2011 compared to the yield in 2009 in all pretreatment conditions tested. For the rest of the varieties, no clear trends were observed. As regard to pretreatment conditions, the combined sugar yield was greater at the pretreatment condition of 180 °C, 0.65%, 10min (Figure 7-2, Table 7-3). With this pretreatment condition, the combined sugar yields of the varieties ranged from 51.9 to 65.6 g per 100g dry biomass. In terms of combined sugar recoveries (defined as combined sugar yield divided by potential sugar in the biomass), only two varieties (101 and 114) showed statistically differences in the recoveries between 2009 and 2011 (Figure 7-3C). Likewise, the recovery variety 114 (2012) was different from that observed in 2009 but was statistically the same to the recovery of 2009.

7.3.3. Comparison of combined ethanol yields from sugarcane varieties

Combined ethanol yield for each variety and harvest of sugarcane was estimated by considering ethanol production from both the sugar juice and bagasse. Conversion efficiency of sugars in the juice was assumed to be 85% (Van-Der-Westhuizen, 2013), and the ethanol yield calculated on the basis of cane yield and juice sugar content (Table 7-1). For the bagasse, ethanol yield was calculated based on bagasse yield per hectare (Table 7-1), xylose and EH glucose obtained at 180 °C, 0.65% for 10 min (Table 7-3) and conversion efficiency. Glucose conversion efficiency was calculated based on the correlation between EH glucose yield and ethanol concentration established in the previous study (Benjamin et al., submitted). Xylose to ethanol conversion efficiency was assumed to be 0.36 g/g (Carrasco et al., 2010).

7.3.3.1. Estimated ethanol concentration of the pretreated bagasse

Figure 7-4 shows the highest ethanol concentrations that could be obtained based on EH glucose yield, if the SSF process is to be performed at the solid loading of 10%(w/w) and enzymes dosage of 0.15 ml of Cellic Ctec2/g pretreated material and 0.213ml of Cellic Htec2/g pretreated material. Differences in ethanol concentrations were observed among the

varieties and between the harvests. Within the harvest, ethanol concentrations of the varieties varied from 28.5 to 34.2 g/l in 2009 and between 29.4 and 31.3g/l in 2011. Results regarding the breeding technology, the precision breeding varieties (101 and 114) harvested in 2009 outperformed the classical breeding (55, 70 and 74). Conversely, the opposite was observed in year 2011, in particular for varieties 55 and 70. The estimated ethanol concentrations were verified by performing SSF experiment on variety 114, harvested in 2012 as discussed in the next section.

7.3.3.2. Measured ethanol concentration of the pretreated bagasse

The SSF experiment was performed on the variety 114 harvested in 2012 to verify the accuracy of the above estimation. Figure 7-5 shows the profiles of glucose, xylose and ethanol concentrations measured during SSF. Xylose concentration remained almost constant but no glucose accumulation was observed for the entire period of SSF. This suggests that xylose was not consumed while glucose was completely consumed by the yeast to produce ethanol. As such, ethanol concentration was increasing during SSF. The highest ethanol concentration measured was 30 g/L, which corresponds to ethanol yield of 74.4% of theoretical. This concentration was comparable to the one obtained through estimation (31.9 g/L).

7.3.3.3. Combined ethanol yields

Figure 7-6 depicts ethanol yield in litres per hectare calculated based on equation 1. Ethanol yields for varieties 101, 104 and 114 in 2009 were not calculated because there was no cane yield data. Combined ethanol yield from varieties varied widely from 11,139 to 14,063 L/ha in 2009, and between 2,016 and 12,868 L/ha in 2011. The differences in ethanol yields between harvests were smaller than differences between varieties. The results also showed that best variety was not maintained during different harvests. The precision breeding varieties (101, 104 and 114) showed significantly lower ethanol yields (2,016-2,163 L/ha) compared to the yields (9,459-12,868 L/ha) observed in classical breeding varieties

(55, 70 and 74) in year 2011. Variety 114 was regrown in 2012 and its yield (9,961 L/ha) was comparable to the yield from classical breeding varieties. The contribution of ethanol from the bagasse to the combined ethanol yields was also a variable depending on variety and harvests. It ranged from 23.9 to 59.1% of combined ethanol yield, with the highest contribution being observed on the variety 114 (2011) that presented the lowest sugar juice content (Table 7-3). 88.2 - 90.8% of ethanol from the bagasse was delivered by glucose conversion.

7.4. Discussion

7.4.1. Effect of genotype and harvest on combined ethanol yield

The present study provides the first comparison of sugarcane varieties from different harvests in terms of agronomic properties, chemical composition and processability of bagasse, and calculated ethanol yield from both the sugar juice and bagasse. A key result from this study was that harvest year affect bagasse quality (chemical composition and processability) (Table 7-2 and Table 7-3) and ethanol yield (Figure 7-6) much as variety. This implies that selection of sugarcane varieties for ethanol production, both in terms of sugar juice and bagasse conversion will be determined by genetic variations more so than environment. The exception to this observation was seen in cane yield, sugar juice and fibre content for precision breeding varieties, the crops failed one year (Table 7-1).

The differences in biomass yield and chemical composition between varieties and between harvests have been reported on other energy crops including sweet sorghum (Zhao et al., 2009) and winter triticale (Kučerová, 2007). These studies have demonstrated that factors such as harvest year and maturity and genotype have significant effects on biomass production and carbohydrates and ethanol yields. However, both of these studies did not evaluate biomass conversion using a pretreatment and enzymatic hydrolysis assay.. In this study, the results of sugar yields (xylose, EH glucose and combined sugar) from the bagasse showed that yields for some of the varieties did not change significantly across harvests

(Table 7-3, Figure 7-3). This is important information during crop selection as it eliminates the necessity re-adjusting processing conditions with each new harvest year.

7.4.2. Impact of drought on sugarcane properties and bagasse convertibility

Water plays an important role during early growth and development of sugarcane (Zhao, 2010). Therefore, water deficiency could retard the performance of sugarcane in terms of cane yield and the dry matter content (Ribeiro et al., 2013). As an example Basnayake et al., (2012) found that the sugarcane genotypes grown in a water stressed environment had up to 52% lower biomass yield and 56% lower dry matter content.. The results from the current study demonstrate that the cane yield of a subsequent year could be reduced up to 30% if the ratoons pass through severe drought (Table 7-1, Figure 7-1). Rainfall from January to September (2010) was far below the average in most of coastal areas where these varieties were growth. This also lowered the seasonal average of cane yield from 63.9 to 57.3 ton/ha as reported elsewhere (Singels et al., 2011).

In terms of bagasse processability, lower sugar yields from most of precision breeding varieties were obtained in 2011 (Table 7-3). This could be due to the higher in lignin and ash contents (Table 7-2). For example variety 101 contained higher lignin (7.6%) and higher ash (237.5%) contents in 2011 than in 2009. This increase lowered the EH glucose yield (32.3%) and therefore, combined sugar yield (27.4%) than the yields obtained in 2009. Conversely, the yields from the majority of classical varieties between the two harvest years were not significantly affected. In this regard, Lewis et al. (Lewis et al., 2010) also reported that corn stover had higher heritability than maize grain regardless of differences in seasons.

7.4.3. Importance of drought resistance varieties for sustainable biorefinery

Varieties selection for improved resistance to drought would lead to increase sugarcane yield and sustainable ethanol production. Classical breeding varieties (55, 70 and 74) presented higher cane yield, juice sugar and fibre contents (Table 7-1) and therefore, combined ethanol yield (Figure 7-6). In particular variety 74 was particularly drought resistant

because its yields (cane yield, juice sugar and fibre content) were not affected by the severe drought (Table 7-1). Unfortunately, this variety also had the highest lignin content than the rest (Table 7-2). And likely for this reason, variety 74 was more recalcitrant than the rest (Table 7-3; Figure 7-4). The resilience of this variety toward severe drought could be due to high lignin content (Jung et al., 2012). Lignin content helps to determine the physical properties of plant cell wall as previously been reported on rice straw and spring wheat (Tripathi et al., 2003). High lignin content in the plant could enhance drought resistance because of low water loss (Qin et al., 2012). In this context, a variety that is resilient toward environmental stress and diseases is of great interest during crop development and selection for bioenergy. This might also insure constant yielding and the most important is the improvement of water use efficiency, which is a limiting factor during sugarcane growth (Fargione et al., 2008).

Regardless of the reasons observed above, precision breeding varieties presented lower lignin content than classical breeding varieties for both harvest years (Table 7-2). This result suggests the application of precision breeding technology in this specific case leads to significantly lower lignin content which is crucial a factor in determining the convertibility of the bagasse. However, the assessment of combined ethanol yields per hectare of the varieties demonstrated a major concern for utilization of precision breeding varieties due to the uncertainty of their performance in the field in a water stressed year (Figure 7-1 and Figure 7-6). However, since they have shown a significant potential of good propensity for low lignin content and high carbohydrates content, they may benefit from a classical breeding program to select for better production traits.

7.5. Conclusion

Sugarcane varieties differs significantly in agronomic properties (cane yield, contents of soluble sugar in juice and insoluble structural carbohydrates), sugar released from pretreatment-hydrolysis and calculated ethanol yields from juice and bagasse. The differences among varieties were greater than between harvest years. This observation

implies that selection of sugarcane varieties for ethanol production could be largely determined by genetic differences among the varieties when there is no severe drought. Severe drought also negatively influenced the performance of all the varieties in terms of cane yield, except for the variety containing the highest lignin. Therefore, it was beneficial to identify the genes that promote drought tolerance to provide a fundamental resource for variety improvement.

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Table 7-1: Cane yields and other agronomic properties of different varieties of sugarcane harvested from 2009 to 2012.

Variety	Year of harvest	Maturity (months)	Cane (wet ton/ha)	Sucrose (kg/wet ton cane)	Juice sugar* (kg/wet ton cane)	Fiber (kg/wet ton cane)
55	2009	12	124.1	126	144	146
70	2009	12	126.5	147	164	122
74	2009	12	98.1	136	155	165
101	2009	12	-Na-	150	165	150
104	2009	12	-Na-	146	153	137
114	2009	12	-Na-	153	160	150
55	2011	8	86.8	129	154	148
70	2011	8	89.5	157	162	132
74	2011	8	112.9	138	142	157
101	2011	8	21.1	85	93	109
104	2011	8	36.3	63	86	93
114	2011	8	35.8	39	80	146
114	2012	13	97.3	120	127	138

*Juice sugar is a sum of sucrose, glucose and fructose

-Na- not available

Table 7-2: Chemical compositions and theoretical ethanol yields of bagasse from different varieties of sugarcane harvested from 2009–2012 (% dry weight)

Varieties	Harvest year	Glucan	Xylan	Arabinan	Lignin	Acetyl	Extractives	Ash	TSC ¹	TEY ²
55	2009	35.1±0.4 ^a	24.6±0.5 ^c	2.5±0.2 ^a	19.6±0.6 ^a	3.2±0.1 ^a	9.9±0.3 ^a	1.6±0.1 ^b	62.1±0.7 ^b	34.1±0.5 ^b
70	2009	36.1±0.3 ^{cd}	24.3±0.7 ^c	2.2±0.1 ^b	20.4±0.5 ^a	2.8±0.1 ^b	7.5±0.1 ^b	1.8±0.1 ^a	62.6±0.2 ^b	34.6±0.2 ^b
74	2009	36.9±0.6 ^{bc}	24.0±0.2 ^c	1.5±0.1 ^c	19.7±0.5 ^b	2.9±0.1 ^b	7.5±0.3 ^b	2.0±0.0 ^a	62.9±0.7 ^b	34.8±0.4 ^b
101	2009	40.7±1.0 ^a	26.3±0.6 ^{ab}	2.2±0.1 ^b	14.4±0.3 ^c	3.2±0.2 ^a	7.4±0.9 ^b	0.8±0.1 ^c	69.3±0.9 ^a	38.4±0.5 ^a
104	2009	34.1±1.0 ^d	25.5±0.3 ^b	2.7±0.1 ^a	16.4±0.3 ^b	3.2±0.1 ^a	7.3±0.2 ^b	0.9±0.1 ^c	62.3±0.6 ^b	34.2±0.4 ^b
114	2009	38.3±1.6 ^b	27.2±0.7 ^{a*}	2.5±0.1 ^a	16.1±0.3 ^b	3.3±0.2 ^{a*}	6.1±0.2 ^{c*}	0.9±0.1 ^{c*}	67.9±0.8 ^{a*}	37.5±0.6 ^{a*}
55	2011	38.3±0.5 ^{ab*}	23.3±0.4 ^{cd}	2.0±0.1 ^{ab*}	20.3±0.4 ^b	3.3±0.1 ^{a*}	4.3±0.9 ^{b*}	1.5±0.1 ^d	63.4±0.1 ^b	35.2±0.1 ^{bc}
70	2011	37.4±0.7 ^{ab}	22.5±0.1 ^{de*}	1.7±0.2 ^b	20.1±0.5 ^b	3.0±0.2 ^b	4.8±1.1 ^b	1.9±0.2 ^c	61.6±0.9 ^{bc}	34.2±0.5 ^c
74	2011	38.1±1.0 ^{ab}	21.6±0.6 ^{e*}	1.4±0.2 ^c	22.3±0.2 ^{a*}	2.8±0.1 ^b	4.3±0.1 ^{b*}	1.2±0.1 ^{d*}	61.0±1.0 ^c	34.0±0.4 ^c
101	2011	39.1±0.7 ^a	24.5±0.4 ^{bc*}	2.2±0.3 ^{ab}	15.5±0.2 ^{d*}	3.1±0.2 ^{b*}	7.1±1.7 ^a	2.7±0.1 ^{a*}	65.9±1.7 ^a	36.5±0.9 ^a
104	2011	36.8±0.6 ^{b*}	23.8±0.7 ^b	1.8±0.2 ^{b*}	16.4±0.2 ^c	3.0±0.1 ^b	8.9±0.6 ^{a*}	2.8±0.1 ^{a*}	62.4±0.1 ^{bc}	34.8±0.1 ^c
114	2011	37.3±0.8 ^{ab*}	25.6±0.4 ^a	2.4±0.5 ^a	16.8±0.2 ^c	3.4±0.1 ^{a*}	7.2±0.7 ^a	2.5±0.2 ^{b*}	65.6±0.4 ^a	36.1±0.5 ^{ab}
114	2012	40.1±0.3 [*]	24.1±0.2	2.3±0.3	16.5±0.2	2.8±0.5 [*]	8.2±0.1 [*]	1.6±0.1 [*]	66.5±0.3	36.8±0.1

¹TSC refers to the total structural carbohydrates (glucan, xylan and arabinan)

²TEY is the theoretical ethanol yield based on glucan and xylan content in the bagasse

^{abcde}Means values in the same column and within same harvest year differed significantly at $p<0.05$.

*Means significant differences between the harvests at $p<0.05$.

Table 7-3: The xylose yield after pretreatment, glucose yield after enzymatic hydrolysis and combined sugar yield (g/100g RM) of bagasse from six sugarcane varieties harvested between 2009 and 2012.

Varieties	Harvest year	170°C, 0.45%, 5 min			180°C, 0.65%, 10 min			190°C, 0.45%, 15 min		
		Xyl.Pre	Gluc.EH	CSY	Xyl.Pre	Gluc.EH	CSY	Xyl.Pre	Xyl.EH	CSY
55	2009	6.9±1.2 ^c	21.1±0.8 ^b	32.8±2.2 ^c	19.2±0.4 ^a	33.6±0.8 ^c	58.4±1.2 ^{bc}	14.7±1.2 ^a	35.2±1.5 ^b	55.3±2.3 ^a
70	2009	12.1±0.5 ^b	20.4±0.2 ^b	34.6±1.3 ^c	18.5±1.2 ^a	29.5±0.8 ^d	54.3±2.0 ^{cd}	10.3±1.8 ^b	32.3±1.0 ^c	47.4±3.5 ^b
74	2009	8.3±1.7 ^c	16.7±0.1 ^c	33.0±0.8 ^c	18.9±0.8 ^a	27.2±0.6 ^e	52.2±0.9 ^d	13.0±2.0 ^{ab}	29.9±2.8 ^c	49.5±0.7 ^b
101	2009	14.1±0.3 ^a	31.6±0.5 ^a	51.4±0.3 ^a	20.2±0.7 ^a	39.2±0.5 ^a	65.6±0.9 ^a	13.6±0.2 ^a	39.0±0.8 ^A	58.5±0.5 ^a
104	2009	13.7±0.7 ^{ab}	22.8±0.7 ^b	42.2±1.4 ^b	18.9±0.7 ^a	31.8±0.9 ^{cd}	56.6±0.8 ^c	16.3±2.3 ^a	31.7±1.2 ^c	50.8±2.4 ^b
114	2009	11.6±1.7 ^{b*}	20.9±0.6 ^b	38.4±1.6 ^b	19.3±0.8 ^a	35.5±0.7 ^b	61.1±1.0 ^b	15.8±1.2 ^a	35.9±0.8 ^b	57.5±0.4 ^{ab}
55	2011	12.5±0.4 ^{b*}	23.6±0.6 ^{a*}	41.3±0.5 ^{a*}	19.7±0.4 ^a	31.3±0.8 ^{b*}	57.8±0.8 ^a	16.4±0.4 ^a	34.6±0.3 ^a	56.6±1.0 ^a
70	2011	15.5±1.6 ^{a*}	22.3±0.4 ^{a*}	43.4±0.8 ^{a*}	18.6±0.3 ^a	33.0±0.5 ^{a*}	57.2±0.1 ^a	13.3±0.2 ^{b*}	34.3±1.6 ^a	53.7±2.2 ^{b*}
74	2011	14.8±0.3 ^{ab*}	18.0±0.5 ^{b*}	37.1±0.9 ^{c*}	16.7±0.3 ^{b*}	29.0±0.2 ^{c*}	51.9±0.4 ^b	12.7±1.4 ^b	30.9±0.9 ^a	47.5±0.5 ^c
101	2011	10.2±0.1 ^{bc*}	21.4±1.1 ^{a*}	37.3±0.9 ^{c*}	19.7±1.1 ^a	30.3±0.7 ^{bc*}	56.7±0.3 ^{a*}	15.2±0.5 ^{a*}	32.3±0.3 ^{ab}	53.5±1.1 ^{b*}
104	2011	12.1±0.9 ^b	23.0±0.5 ^a	40.7±0.9 ^b	18.6±0.3 ^a	31.2±1.0 ^{B^C}	56.0±1.5 ^a	14.6±0.0 ^a	32.9±1.1 ^{a*}	53.6±1.2 ^b
114	2011	9.5±0.7 ^{c*}	20.5±1.2 ^a	36.2±1.1 ^c	18.7±1.2 ^a	30.5±1.4 ^{bc*}	54.1±0.7 ^{a*}	14.5±0.5 ^b	33.7±0.7 ^{ab}	54.1±1.6 ^{b*}
114	2012	14.9±0.6 [*]	23.1±1.0	44.8±1.5 [*]	19.3±1.0	34.4±0.6	60.5±1.2	15.3±0.3	37.9±0.3 [*]	59.4±0.1

Xyl.Pre, Gluc.EH and CSY refers to xylose yield after pretreatment, glucose yield after enzymatic hydrolysis and combined sugar yield (sum of glucose, xylose and arabinose after pretreatment and enzymatic hydrolysis), respectively.

^{abcde} Same superscript letters in the same column and within same harvest year signify no significant different at $p < 0.05$

*Means significant differences between the harvests at $p < 0.05$.

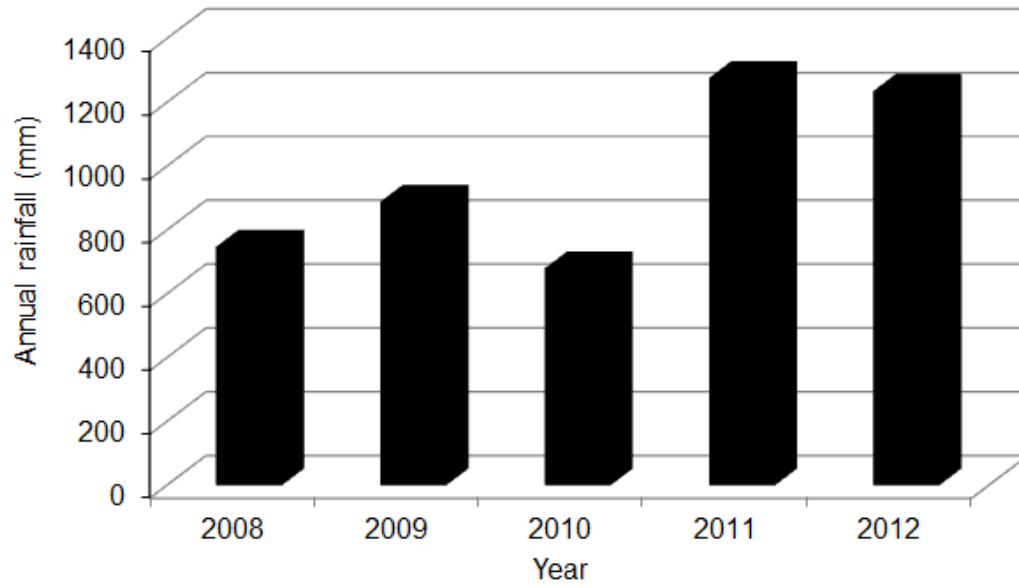


Figure 7-1: Average annual rainfall recorded at Mount Edgecombe via SASRI weather web, where the field experiments were conducted.

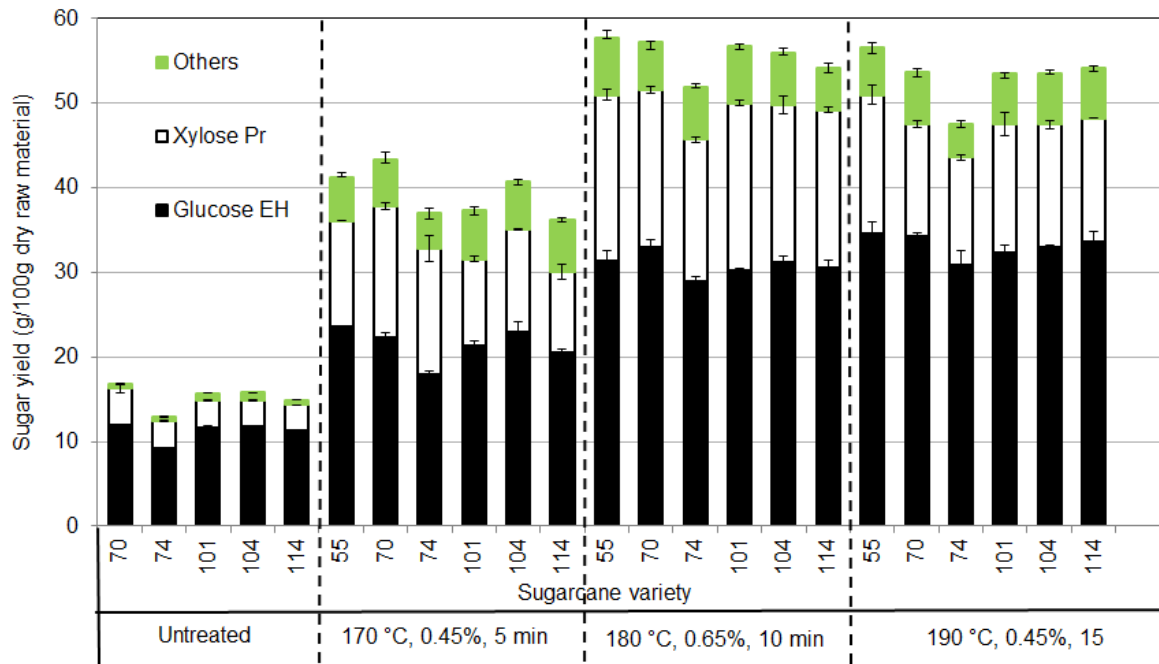


Figure 7-2: Total sugar yields of untreated and pretreated bagasse from different varieties of sugarcane harvested in 2011. With an exception to untreated samples, Xylose Pr. is xylose yield after pretreatment, Glucose EH is glucose yield after enzymatic hydrolysis and others stand for the sum of glucose, arabinose (after pretreatment) and xylose after enzymatic hydrolysis.

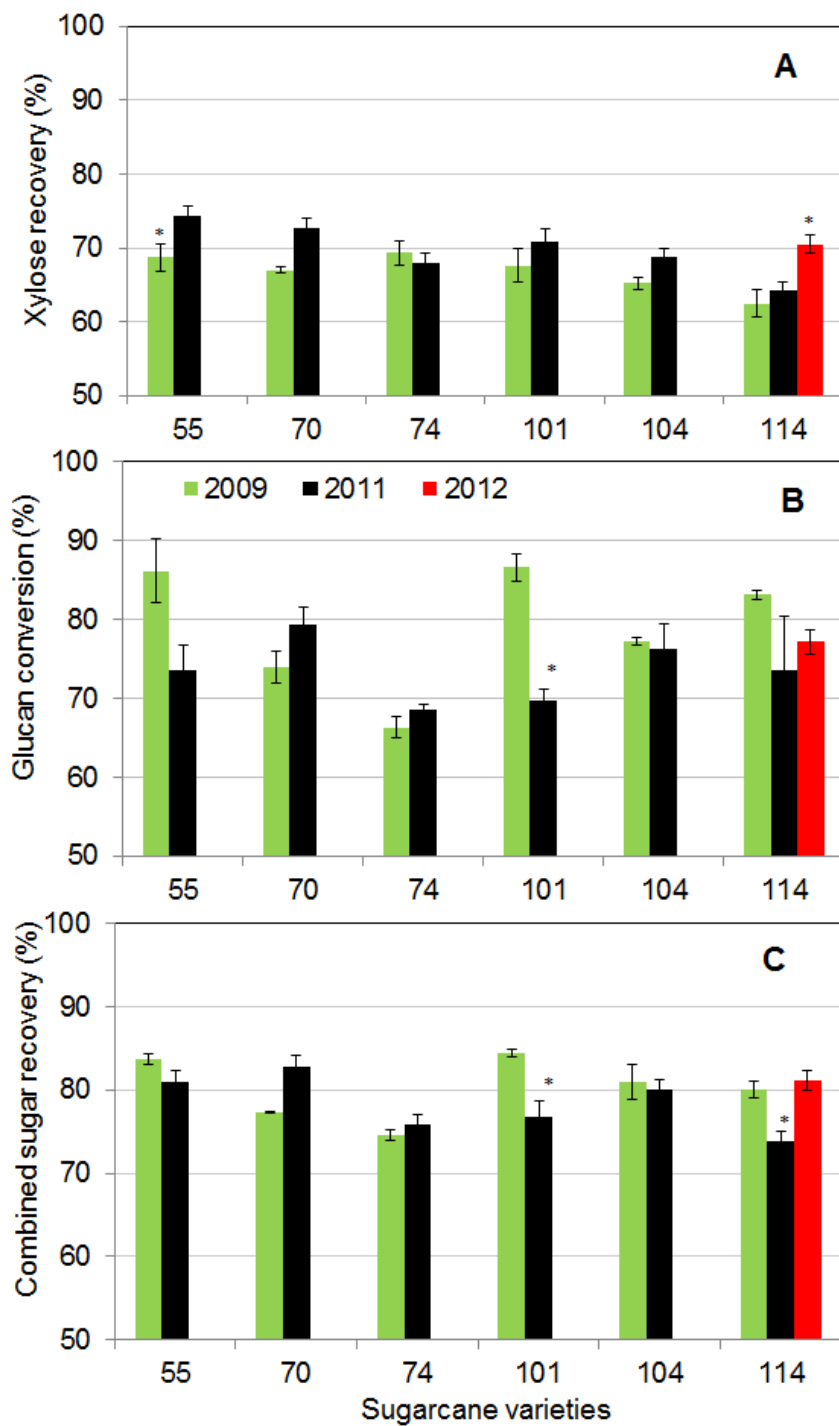


Figure 7-3: Sugar recovery after dilute acid pretreatment at 180°C, 0.65% acid, 10 min and enzymatic hydrolysis of bagasse from different varieties of sugarcane harvested between 2009 and 2012. (A) Xylose recovery in the hydrolysate liquor (B) glucose after enzymatic hydrolysis (C) total sugar recovery. All are expressed as theoretical values in the raw material. *Means significant differences between the harvests at $p < 0.05$.

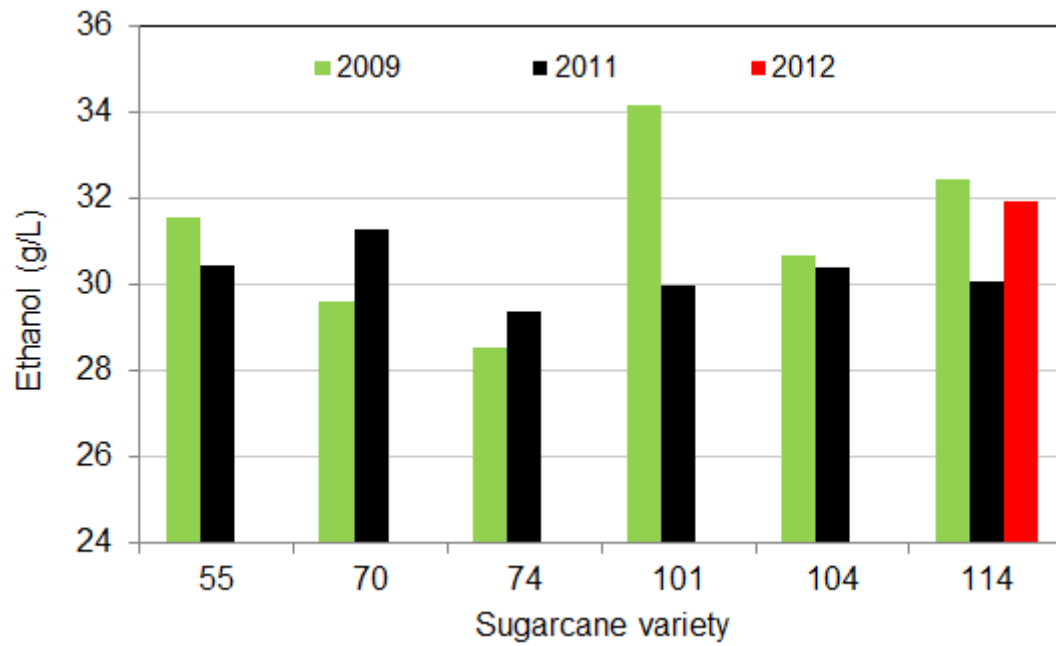


Figure 7-4: Predicted ethanol concentrations based on EH glucose yields obtained at 180°C, 0.65 (%w/w) for 10 min from different bagasse samples of different harvests.

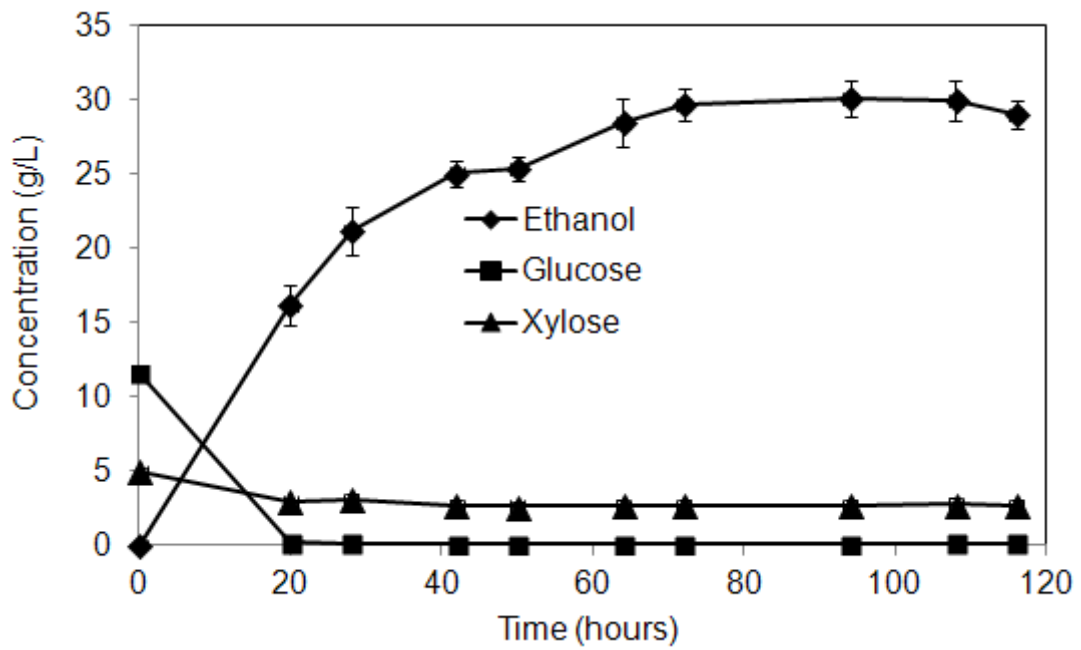


Figure 7-5: Ethanol concentration during SSF of unwashed-pressed pretreated material for variety 114 harvested in 2012. Sample was pretreated at 180°C, 0.65 (%w/w) for 10 min.

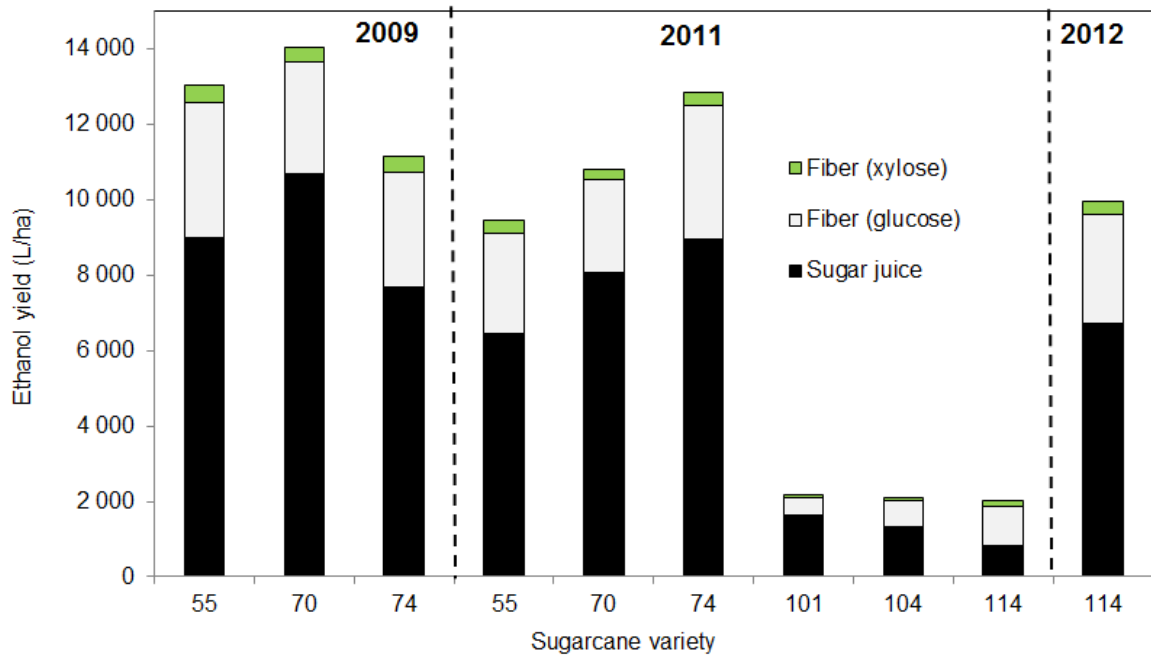


Figure 7-6: Combined ethanol yields of different varieties of sugarcane harvested between 2009 and 2012. Ethanol from the fiber was estimated by using the experimental data obtained after the substrates were pretreated at 180°C, 0.65% acid, 10 min and enzymatic hydrolysis at 15 FPU/g WIS.

Chapter 8

8. Summary of main findings

The present study presents the first data where the impact of sugarcane varieties on the combined ethanol yield from both juice and bagasse was evaluated. The main focus of this thesis was to study differences on the processability of bagasse through pretreatment, enzymatic hydrolysis and fermentation, which were combined with agronomic yields to select preferred varieties for such combined ethanol production. These data guided the selection of varieties with improved properties to increase ethanol yield from the bagasse without compromising juice ethanol per hectare. One hundred and fifteen (115) varieties from classical breeding and precision breeding technologies developed by the breeding program at the South African Sugarcane Research Institute (SASRI) were evaluated. Of these samples, 100 varieties were originated by classical breeding and 15 varieties through precision breeding (genetic engineering). The most important findings obtained in the present study are summarised below.

8.1. Varieties screening

The bagasse samples from all varieties (115) were characterised in terms of chemical composition (Chapter 4). Those varieties with higher content of carbohydrates (cellulose, hemicelluloses), reduced lignin and ash contents are desirable in order to obtain high fermentable sugar yield with reduced process requirements. Additionally, highly branched hemicelluloses (i.e. xylan backbone with arabinose substitution) are associated with reduced recalcitrance.

The results showed considerable variations among the varieties (see Figure 4-1, page 100). The differences were up 11 g/100 g RM for structural carbohydrates content and up to 8.7 g/100 g RM for lignin content (see Table 4-2, page 96). Likewise, the degree of substitution of hemicelluloses between varieties differed significantly. The ratio of xylose to

arabinose ranged from 7.2 to 18.9. In fact, the majority of the precision breeding varieties showed higher structural carbohydrates content (increase of 5.2% on average), lower lignin (11.4% less) and lower ash (50% less) contents compared to bagasse originated from classical breeding. Similarly, xylan in bagasse from precision breeding varieties appeared to be more branched (ratio of xylose to arabinose, 7.2–11.8) than xylan of bagasse from classical breeding varieties (8–18.9).

These results show the capability of precision breeding to modify (improve) the quality of bagasse, to obtain a feedstock more suitable for pretreatment-hydrolysis-fermentation.

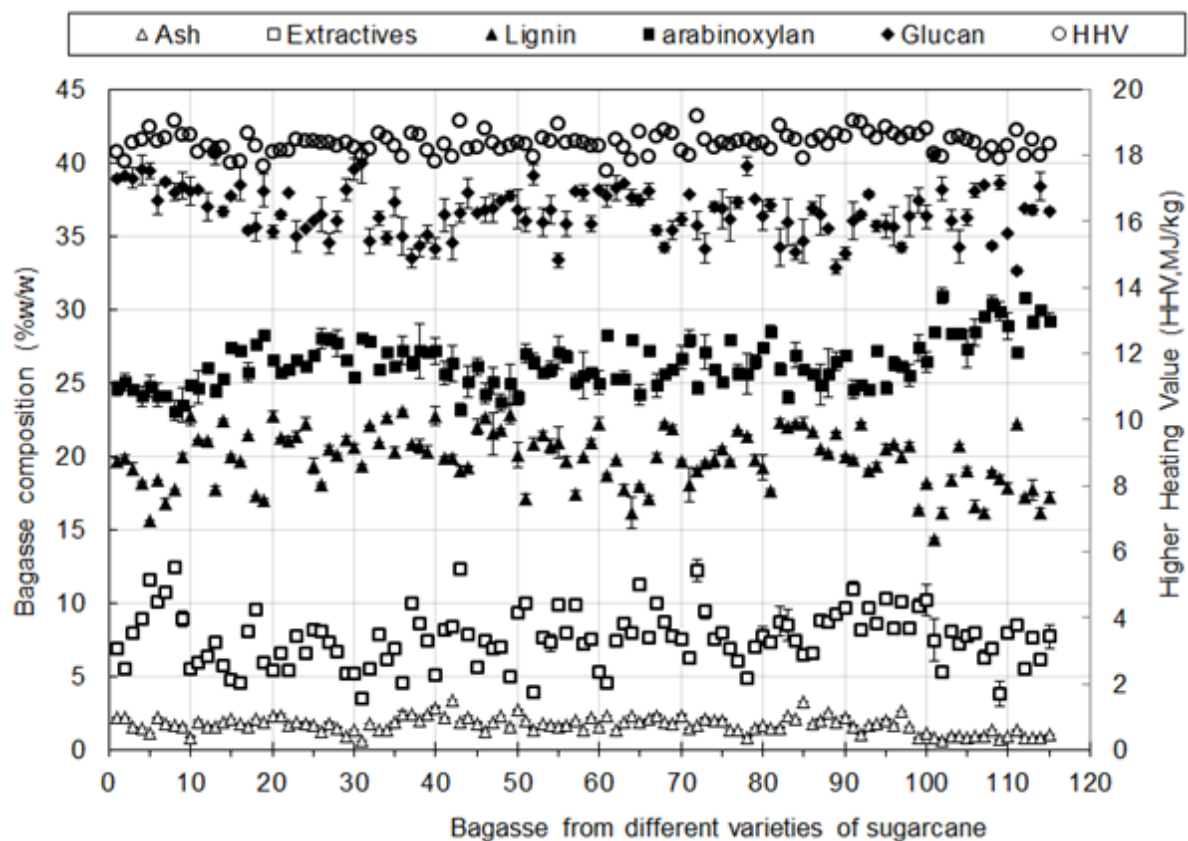


Figure 4-1: Average chemical composition and higher heating values of bagasse from 115 varieties of sugarcane. The error bars represent the variation of four replicates.

Sugarcane bagasse samples were treated with optimum dilute acid pretreatment based on literature prior to enzymatic hydrolysis. The total sugar released from the combined pretreatment-hydrolysis process (“combined sugar”) was used for selection of preferred varieties. The results showed significant differences in combined sugar yield (CSY) between

the varieties (27.3 to 55.2 g/100g dry raw material (RM), which corresponded to 35.8 and 71.1% of theoretical, respectively) (see Figure 4-2, page 101). Precision breeding varieties showed higher CSY (39.9–55.2 g/100 g RM, average 48 g/100 g RM) than classical breeding varieties (27.3–53.3 g/100 g RM, average 42.4 g/100 g RM) (see Table 4-3, page 97). As expected, the CSY was positively correlated with carbohydrates, but negatively correlated with lignin, ash and the ratio of xylose to arabinose (see Table 4-4, page 95).

The application of mild and severe pretreatment conditions showed that the differences in CSY between varieties were greater at less severe conditions (23.5 g/100g RM), compared to more severe conditions (16.6 g/100 g RM) (see Figure 4-6, page 105). Furthermore, the enzymatic hydrolysis at low dosage of 1.5 FPU/g WIS showed that glucose recoveries from the raw material was higher from varieties with improved properties (34–44%) than those obtained from the varieties with non-desired characteristics (19–24%) (see Figure 4-5, page 104). These results demonstrate that mild pretreatment conditions and low enzymes dosage are highly effective in identification of bagasse with improved processability.

When selecting the best varieties for integrated ethanol production, high cane yield combined with high juice sugar together with fibre of high processability are recommended. However, the selection of the varieties also took into account the yield of lignocellulose (cane) per hectare, and not just the properties of the materials present in the cane. The varieties with low lignin content in the bagasse (14-17%) had combined properties of high juice sugar and bagasse of high processability. However, these varieties had a low cane yield (65–98 wet ton/hectare) compared to the 115–126 wet ton/hectare observed in the varieties with intermediate lignin content (18–20%). Still the cane productivity observed on varieties with low lignin content was higher than the average industrial sugarcane yield from South Africa of 62.6 wet ton per hectare. These results show that it is possible to select varieties with reduced lignin without compromising juice and yield/hectare.

Cane yield, juice sugar and bagasse content were used to calculate potential ethanol yield per hectare. The maximum energy from the bagasse was also calculated considering

the chemical composition of the bagasse and the bagasse yield per hectare. Potential cane yield and the maximum energy yield showed a positive correlation (see Figure 4-7, page 106). This shows that the contribution of bagasse, in particular that based on CSY to the total ethanol yield is crucial for a more sustainable and effective use of land.

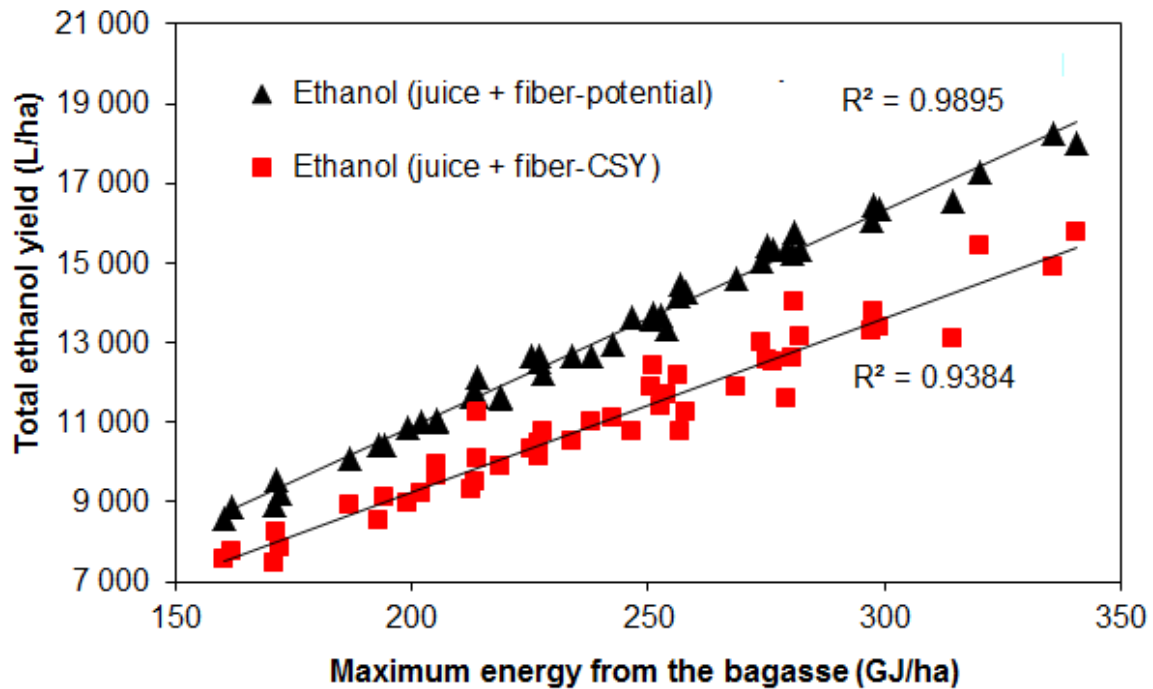


Figure 4-7: Correlation between total potential ethanol yield per unit hectare and the maximum energy from the bagasse per unit hectare for 48 varieties of sugarcane.

8.2. Pretreatment optimisation

Pretreatment optimisation was carried out in small tubular reactors (gram scale) and in a one litre reactor (bench scale): The purpose of the investigation at gram scale was to demonstrate the impact of cultivar selection on increasing fermentable sugar yield, which was determined by optimisation of pretreatment conditions for preferred varieties. On the other hand, the bench scale reactor was used to repeat the optimisation of pretreatment conditions for preferred varieties, taking into account results from grams scale, both to consider the impact of pretreatment scale of optimum conditions and to generate the required amounts of pretreated material for subsequent fermentation studies. A CCD was

applied to evaluate the influence of temperature, acid concentration and residence time on sugar yield at both gram scale and bench scale. Pretreatment optimisation was performed according to variety due to differences on pretreatment requirements caused by differences in chemical composition between varieties.

8.2.1. Pretreatment optimisation at gram scale

Six varieties (i.e. three from each breeding technology) were selected as preferred varieties and were investigated to determine the maximum combined sugar yield after pretreatment-hydrolysis (Chapter 5). The classical breeding varieties were selected based on the cane yield and processability, while the selection of precision varieties was based on juice sugar and processability. For additional comparison, industrial bagasse was included at this stage. The varieties showed considerable differences in sugar yields after pretreatment and enzymatic hydrolysis. Up to 22.7% differences in CSY between preferred varieties were observed at the optimal pretreatment conditions (see Figure 5-7, page 143). The results also showed an improvement in CSY of up to 34.1% by selecting the best performing variety compared to the yield obtained from industrial bagasse.

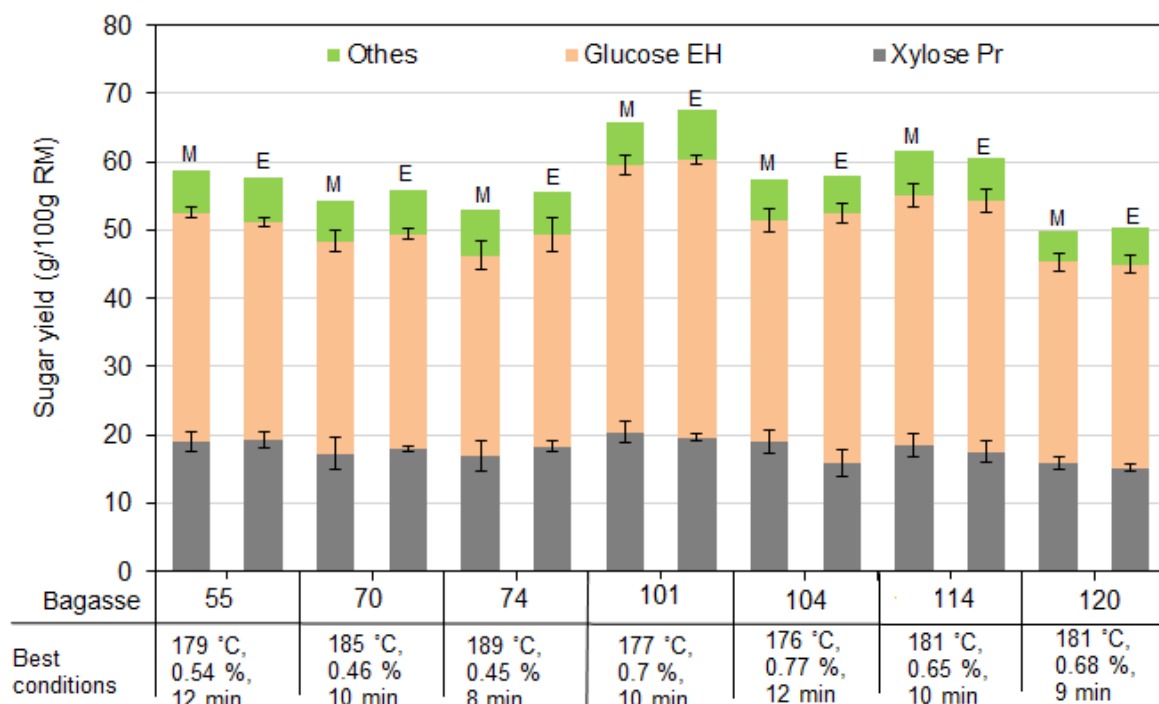


Figure 5-7: The predicted condition for the model optimisation, predicted values (M) and the validation experimental values (E) of combined sugar yields from different sugarcane bagasse samples. Xylose Pr. is xylose yield after pretreatment and Glucose EH is glucose yield after enzymatic hydrolysis. Others stand for the sum of glucose, arabinose (after pretreatment) and xylose after enzymatic hydrolysis. 55, 70 and 74 are classical breeding varieties; 101, 104 and 114 are precision breeding varieties; and 120 is the industrial bagasse.

When evaluating the varieties in terms of pretreatment requirements, the results demonstrated that mild pretreatment conditions such as 170 °C, 0.45%-sulphuric acid for 5 min (part of the CCD) was sufficient to produce highly digestible solid of the best performing variety (see Table 5-4, page 134). As such, its glucose yield (31.2 g/100 g RM) was statistically comparable to the maximum glucose yield from the industrial bagasse (33.7 g/100 g RM) at optimum conditions (196 °C, 0.658% for 10 min). This suggests that high bioconversion efficiency could be obtained by applying less severe conditions when the varieties with preferred characteristics are selected optimally.

8.2.2. Pretreatment optimisation at bench scale

Only classical breeding varieties were investigated for pretreatment optimisation at the bench scale (Chapter 6). Precision breeding varieties were not evaluated due to the lack of materials caused by prolonged drought observed in 2010 (discussed in section 8.4). The maximum combined sugar yield greater than 90% of theoretical was obtained by the best performing varieties, whereas that from industrial bagasse was not more than 75% of theoretical – both at optimum pretreatment conditions.

It was further demonstrated that although the maximum CSY from the substrates differed, the selected varieties and industrial bagasse shared a common set of pretreatment conditions where 95% of the maximum yield could be obtained. This area ranged from 184

to 200 °C for temperature and varying residence time to provide a severity factor between 3.51 and 3.96 (see Figure 6-4, page 184).

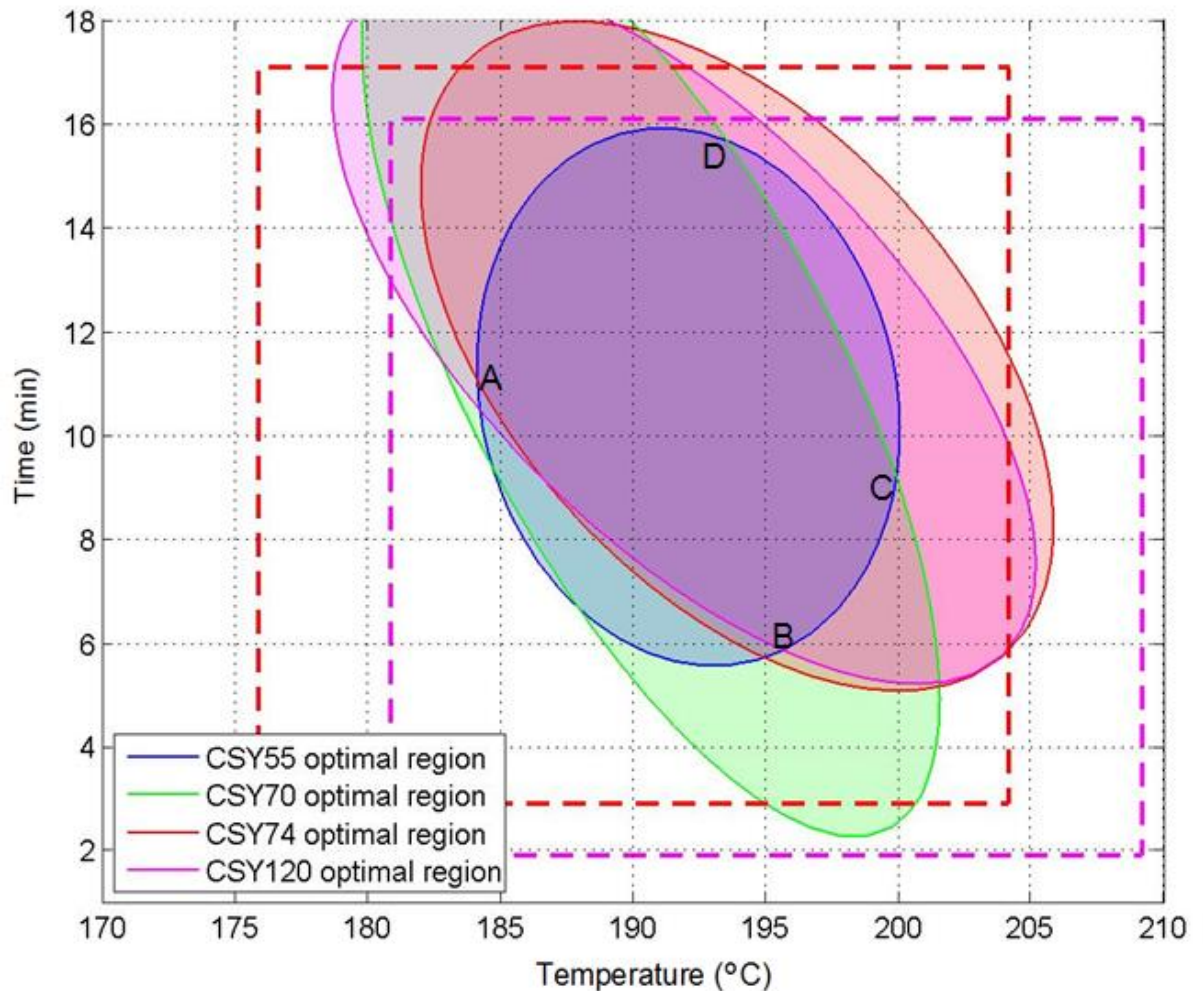


Figure 6-4: Contour plots representing the pretreatment conditions (temperature and reaction time) that provide 95% of the maximum combined sugar yield from bagasse (equations 11 to 14) of different sugarcane varieties. The dotted lines represent the input range of the independent variables for the cultivars: red for varieties 55, 70 and 74; pink for bagasse 120.

8.2.3. Impact of scaling-up on pretreatment

The pretreatment requirements for maximum combined sugar yields from the varieties were found to vary between the two reactors systems. Parr reactor (bench scale) required more severe pretreatment conditions (see Table 6-3, page 178) than those applied at gram

scale (tubular reactor) (see Figure 5-7, page 143). This could be due to limitations in heat transfer (time required to heat up materials) and mass transfer (less efficient mixing, temperature and concentration variations in materials). In fact, although heating mode was the same for both reactors (sand bath), the large reactor required more time for heating-up. For example it required about 7 min for temperature to reach 190 °C (

Figure 8-1) against ~2 min of tubular reactor. On the other hand, the solid loading for the bench scale was limited only to 10% (w/v) due to mixing problems and heat transfer versus 30% (w/v) used in tubular reactor. However, Parr used slightly bigger particle size (600–1000 µm) than that applied in tubular reactor (425–825µm), which could imply more severe conditions for Parr reactor.

Interestingly, despite the differences of the two reactors systems, the differences in maximum CSY between the best performing variety and industrial bagasse between the two reactors were statistically similar between the two reactors systems (33.1–34.1%).

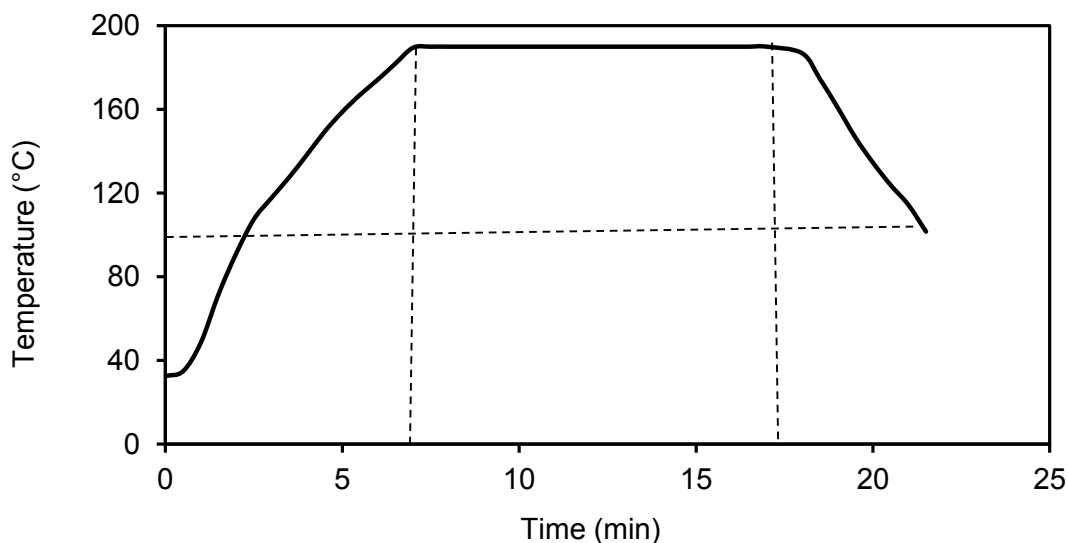


Figure 8-1: Heating-up and cooling down profile of the bench scale Parr reactor

8.3. Process integration and combined ethanol yield

Classical breeding varieties were also evaluated through process integration from pretreatment to fermentation (Chapter 6). The bagasse samples were pretreated at preferred conditions obtained from bench scale optimisation. The pressed, pretreated material was

fermented through Simultaneous Saccharification and Fermentation (SSF) process. The results showed that the preferred varieties had significantly higher ethanol yield (84.5–85.6% of theoretical) than industrial bagasse (74.8% of theoretical) (see Table 8-1). Alternatively, ethanol concentration higher than 48 g/l could be obtained from the best performing varieties, while that from industrial bagasse was less than the benchmark concentration of 40 g/l required for distillation (see Table 8-1). Furthermore, industrial bagasse required twice as much enzyme dosage to obtain comparable ethanol concentration to that was achieved by best performing varieties. The ethanol concentration was inversely correlated with lignin content and the ratio of xylose to arabinose, but it showed positive correlation with glucose yield from pretreatment-hydrolysis (see Figure 6-7 Figure 7, page 187).

Table 8-1: Summary of highest ethanol concentration during SSF of bagasse samples (g/L). The values in parenthesis are overall ethanol yields of theoretical based on glucose content in raw material. The data were extracted from **Figure 6-5**, page 185 and **Figure 6-6**, page 186.

SSF-option	55	70	74	120
¹ Batch	27.1 (70.4%)	29.3 (74.8%)	22.8 (59.3%)	23.1 (61.4%)
² Batch	33.0 (84.5%)	33.1 (85.6%)	29.1 (79.9%)	28.1 (74.8%)
³ Fed-Batch	48.6 (74.3%)	51.3 (77%)	37.1 (57.3%)	38.3 (61.4%)

¹Batch SSF was conducted using solid loading of 10%, (w/w) and moderate enzyme dosage

²Batch SSF was conducted using solid loading of 10%, (w/w) and high enzyme dosage

³Fed-batch SSF was conducted using solid loading of 16%, (w/w) and low enzyme dosage

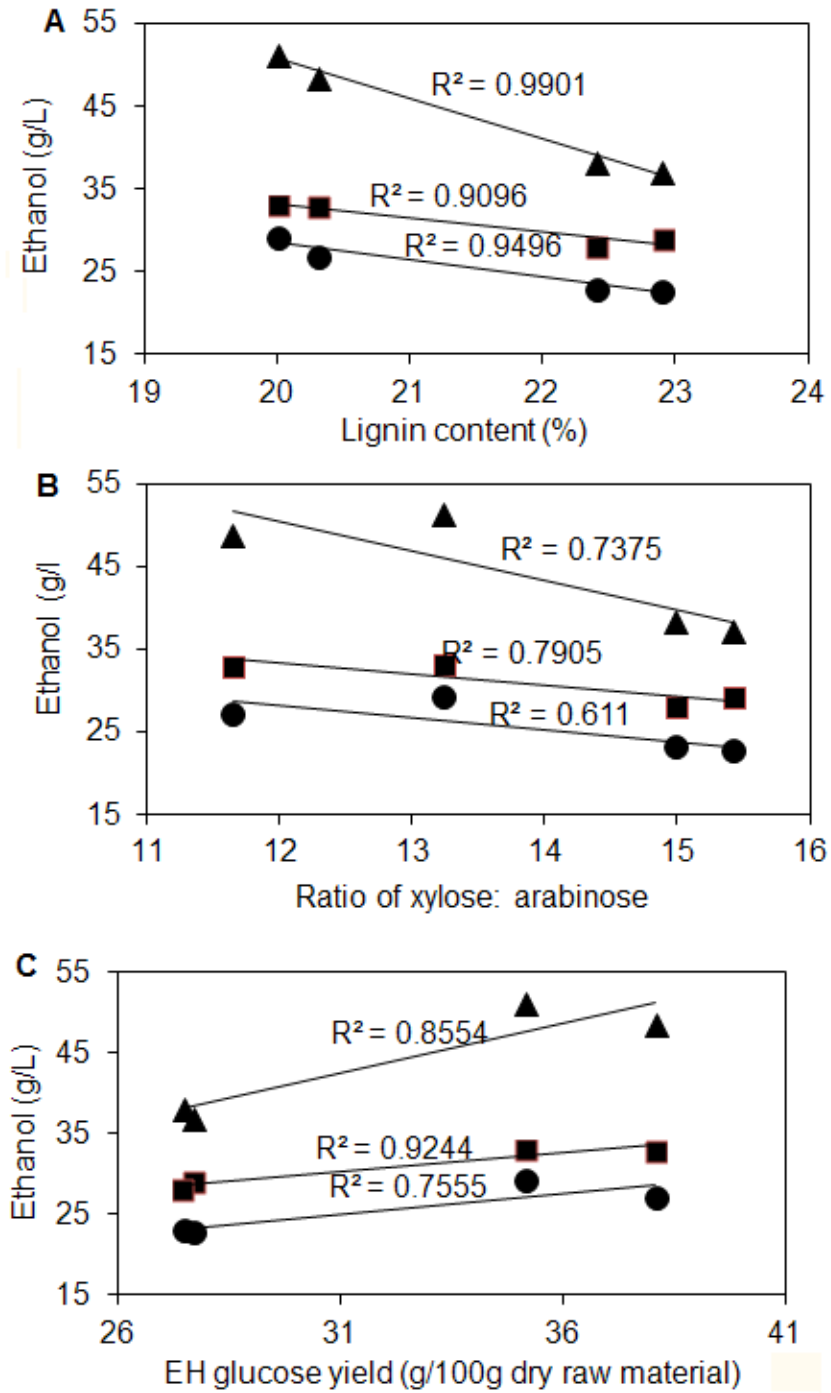


Figure 6-7: Correlations between the highest ethanol concentration during SSF of bagasse samples and (a) lignin content (b) ratio of xylose to arabinose (c) EH glucose yield at the pretreatment condition showed the highest combined sugar yield. Circle (●) and rectangular (■) makers represent ethanol concentration at low and high enzymes loadings for batch process whereas rectangular (▲) maker shows the ethanol concentration during fed-batch process.

As expected, the overall assessment of the varieties showed greater improvement in combined ethanol yields per hectare (71.1–90.7%) for the best performing varieties with respect to industrial sugarcane (see Table 6-5, page 180). The integrated approach shows that ethanol yield could be increased up to 47% when bagasse is used together with sugar juice for ethanol production from sugarcane.

Table 6-5: Cane yield, sucrose content, potential sugars content in juice and bagasse and ethanol yield from different varieties of sugarcane (55, 70, 74 and 120)

	55	70	74	120
Cane yields (wet ton/ha)	105.5 ^a	108 ^a	105.5 ^a	65 ^b
Sugar juice content (kg/ton wet cane)	149 ^a	163 ^a	149 ^a	140 ^b
Bagasse content (kg/ton wet cane)	147 ^a	127 ^a	161 ^a	133 ^b
Ethanol yield (L/ha)				
¹ Juice ethanol	7 731	9 431	8 305	4 967
² Bagasse ethanol (glucose + xylose)	3 605(431)	3 210(368)	3 049(380)	1 660(148)
³ Combined (juice + bagasse)	11 336	12 641	11 354	6 627

^aThe cane yield, sugar juice and bagasse content for varieties 55, 70 and 74 were provided by SASRI, and they represent the average of two harvests (2009 and 2011).

^bThe cane yield, sugar juice and bagasse content for sample 120 were obtained from the literature according to the average values of the industrial sugarcane.

¹Juice ethanol is the ethanol that can be produced by fermentation of juice sugars (sucrose, glucose and fructose).

²Bagasse ethanol is the ethanol that can be produced by fermentation of the xylose obtained after pretreatment and SSF of the pretreated bagasse. Values in parenthesis are ethanol yields from xylose.

³Combined ethanol yield is realistic total ethanol that can be obtained from the sugar juice and bagasse.

8.4. Comparison of the preferred varieties during multiple harvests

The six preferred varieties selected from pretreatment optimisation were compared using different harvests of the same genetic material, taking into account agronomic properties, chemical composition of bagasse, and processability of bagasse and ethanol yield (Chapter 7). The results showed considerable variations in terms of agronomic properties (cane yield, juice sugar and fibre contents). All varieties showed lower cane yield in 2011 than 2009, except for variety 74 (see Table 7-1, page 212). This was due to suboptimal climatic conditions in the 2010 (see Figure 7-1, page 215). Precision breeding

showed lower cane yield and juice sugar content in 2011 than classical breeding varieties. This shows that precision breeding varieties were more susceptible to the impacts of prolonged severe drought compared to classical breeding varieties.

Table 7-1: Cane yields and other agronomic properties of different varieties of sugarcane harvested from 2009 to 2012.

Variety	Year of harvest	Maturity (months)	Cane (wet ton/ha)	Sucrose (kg/ T WC)	Juice sugar* (kg/ T WC)	Fibre (kg/ T WC)
55	2009	12	124.1	126	144	146
70	2009	12	126.5	147	164	122
74	2009	12	98.1	136	155	165
101	2009	12	-Na-	150	165	150
104	2009	12	-Na-	146	153	137
114	2009	12	-Na-	153	160	150
55	2011	8	86.8	129	154	148
70	2011	8	89.5	157	162	132
74	2011	8	112.9	138	142	157
101	2011	8	21.1	85	93	109
104	2011	8	36.3	63	86	93
114	2011	8	35.8	39	80	146
114	2012	13	97.3	120	127	138

*Juice sugar is a sum of sucrose, glucose and fructose

-Na- not available

T WC means kg/wet ton cane

The chemical composition of the bagasse (for example, structural carbohydrates 61–69.3% dry weight, lignin 14.4–22.3%, ash 0.8–2.8%) and sugar yield after pretreatment and enzymatic hydrolysis (for example, combined sugar yield at 170°C, 0.45%, 5 min was 32.8–51.4 g/100 g/100 g RM) and combined ethanol yield (2016–14063 L/ha) between the varieties and across harvests differed significantly (see Table 7-2, page 213 and Table 7-3, page 214; Figure 7-1, page 220). However, the differences between varieties were greater than the differences across harvests. For example, the differences between varieties with the harvest were up to 7.2, g/100g RM for structural carbohydrates content, 6.8 g/100 g RM for lignin content, 18.6 g/100 g RM for combined sugar yield at 170°C, 0.45%, 5 min and 10,780.7 L/ha for combined ethanol yield while that across harvests were 3.4, 2.6, 14.1

g/100 g RM and 3603.6 L/ha, respectively. This implies that selection of sugarcane varieties for ethanol production, both in terms of sugar juice and bagasse conversion would largely be determined by the genetic properties of the varieties, and that such selection of preferred varieties will remain valid over multiple harvests (years).

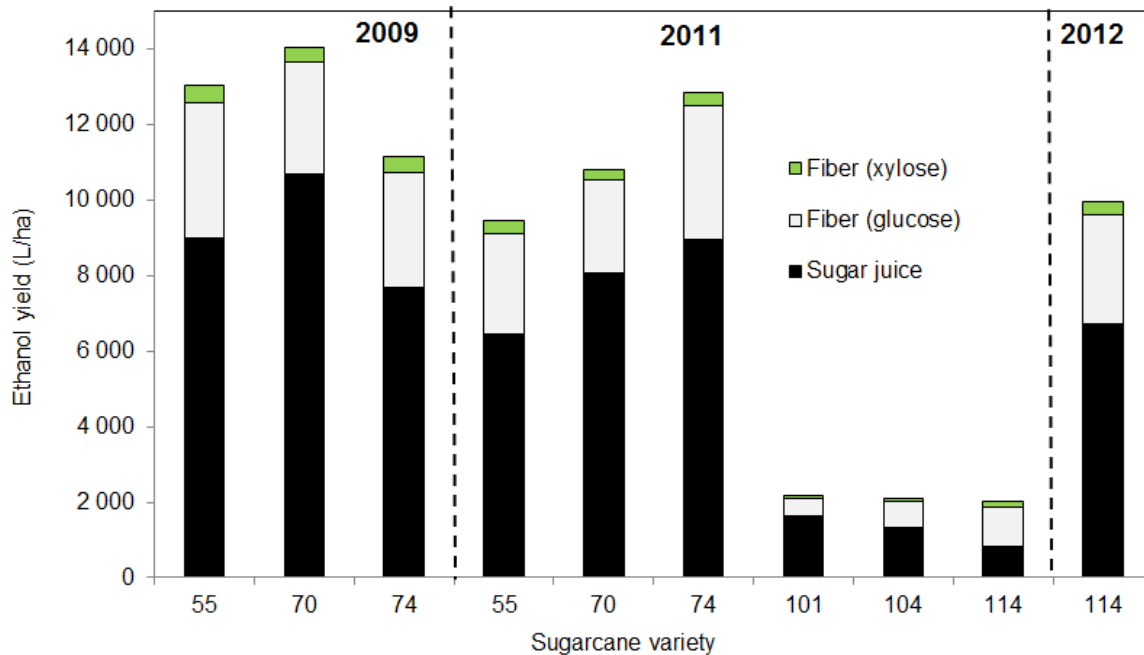


Figure 7-6: Combined ethanol yields of different varieties of sugarcane harvested between 2009 and 2012. Ethanol from the fibre was estimated by using the experimental data obtained after the substrates were pretreated at 180°C, 0.65% acid, 10 min and enzymatic hydrolysis at 15 FPU/g WIS.

Chapter 9

9. Conclusions and recommendations

The present study laid a foundation of research knowledge for development of energycane in South Africa, aiming at crop development for integrated bio-ethanol production from juice and bagasse. Integration of first and second generation technologies is important for the sustainability of biorefinery as it allows optimal use of raw material for ethanol production. Based on the experimental results obtained from the present study, the main conclusions and the direction for the future work are discussed in this chapter

9.1. Conclusions

The present study provides evidence of the impact of cultivar selection and process optimisation in sugar conversion efficiency and ethanol output per feedstock. The integrated approach of the utilisation of juice and bagasse from the preferred varieties significantly increased ethanol yield per hectare. Combined ethanol yield of almost twice as much as that from industrial sugarcane could be obtained by the selection of preferred varieties and optimisation of pretreatment, enzymatic hydrolysis and fermentation. This observation demonstrated that crop development and selection is an important platform for increasing ethanol yield per unit land for modern integrated conversion approach.

9.1.1. Conclusions based on specific objectives

The characterisation of the chemical composition of the bagasse observed considerable variations among varieties (115). Traits of relevance in response to pretreatment (or processability) were structural carbohydrates, lignin and ash contents as they have direct impact on the conversion process of bagasse to ethanol. Another important observation was the differences in structure (i.e. degree of substitution of hemicelluloses) between varieties as it also has direct impact on enzymatic hydrolysis yield as well as ethanol yields. Precision

breeding technology was able to develop traits of interest in the biofuel sector, combining characteristics of higher carbohydrates, lower lignin and lower ash content and a higher degree of xylan substitution, compared to classical breeding. Similarly, precision breeding technology was successful in producing sugarcane with high content of juice sugar per cane, whereas classical breeding was robust in producing varieties of high yielding cane.

Combined sugar yield (CSY) defined as the sum of all pentose and hexose sugar released from the bagasse after pretreatment-hydrolysis between varieties (115) differed significantly. It was observed that the CSY could be doubled by selecting the appropriate variety (27.3–55.2 g/100g raw material (RM)).

Optimisation of pretreatment-hydrolysis significantly improved sugar recovery from the bagasse. Nevertheless, even after pretreatment-hydrolysis optimisation of the 6 preferred varieties there were still differences in maximum CSY (55.1–67.6 g/100 g RM, corresponding to 78.8% and 87.1% of theoretical). Optimisation of pretreatment conditions according to CCD is the best to further improve CSY from the bagasse (9-18%).

Pretreated materials based on optimised conditions were fermented through the simultaneously saccharification and fermentation (SSF) process. Ethanol yield was used to evaluate the fermentability between the selected varieties. Ethanol yield higher than the benchmark concentration for distillation (40 g/l) could be obtained by the best performing varieties. This demonstrates that by selecting preferred varieties one can both (i) increase the sugar yield per ton of bagasse, and (ii) apply less severe pretreatment conditions. Both of these will contribute significantly in reducing the ratio of inhibitors to fermentable sugars, and thus make it much easier to get to 40 g/l (and beyond) without running into the limitations of inhibitor formation. Sugarcane bagasse is better than triticale straw and sorghum bagasse in this regard – it was not possible/easy to get to 40 g/l through cultivar selection and pretreatment optimisation with either of these. Therefore, a combination of crop development, optimal selection of varieties together with process optimisation is an attractive approach for increasing ethanol yield and reducing processing costs.

Nonetheless, although the optimal CSY between samples differed significantly, the preferred varieties and industrial bagasse shared a range of pretreatment conditions providing more than 95% of the maximum fermentable sugar yield. The pretreatment conditions of temperatures ranging from 184 to 200 °C and varying residence time to give the severity factor between 3.51 and 3.96 were identified as the area in common for most of the varieties. This shows that application of pretreatment conditions within that range will probably give the maximum CSY regardless of the variety at bench scale. These conditions should be used in future for the comparison and selection of sugarcane varieties, based on the response of bagasse to pretreatment-hydrolysis.

The agronomic properties and sugar yields as well as ethanol yields from different harvests were investigated. Considerable variations in agronomic properties and sugar yields as well as ethanol yields between the varieties and across harvests were observed. The harvest had a less severe effect on the overall yields than the selection of variety. In spite of their potential for modification of bagasse properties to increase suitability for pretreatment-hydrolysis, the varieties produced by precision technology were more susceptible to climatic changes, in particular drought conditions, compared to classical breeding varieties. Therefore, the final selection of the preferred varieties should consider the field performance in terms of drought tolerance for a constant supply of the raw material in case of drought conditions.

9.1.2. Conclusion specific to methodology

At mild conditions the differences in CSY between varieties were greater but they decreased with increases in pretreatment severity. This observation confirms the notation that at mild pretreatment conditions the conversion efficiency (concisely glucose conversion) of lignocellulose biomass is largely determined by the properties of the feedstock, which is less evident at more severe conditions. At severe conditions the bioconversion efficiency is mostly determined by the severity of pretreatment, rather than by the properties of the

feedstock. Therefore, mild pretreatment condition is preferred in order to do a screening for varieties.

With regards to the investigation of the enzyme requirements, low enzymes dosages were preferred to identify preferred varieties, having bagasse that was more suitable for pretreatment-hydrolysis. Higher glucose recovery and ethanol yield was obtained from the varieties with improved properties, and low enzyme dosage can thus be applied for identification of preferred varieties.

The comparison of pretreatment optimisation at gram and bench scale indicated that more severe pretreatment conditions were required at larger scale. This could be due to differences in geometries between the two systems, together with mass and heat transfer limitations in bench scale as aforementioned in chapter 8. This means that it is more difficult to study the influence of feedstock property on bioconversion in larger scales since more severe conditions will more easily “hide” the differences between bagasse samples in terms of pretreatment response. As such the differences in bioconversion efficiency between the best and poor performing varieties did not decrease with the increasing pretreatment severity, contrary to what was observed at the gram scale.

9.2. Recommendations

Further research can be conducted on the following key areas to compliment the findings of this thesis.

It is observed in this study that the agronomic properties and bagasse quality vary depending on variety and environmental conditions. These characteristics also vary with respect to location [1,2]. Further investigation on the structural differences among the varieties to determine gene/conditions involved in those differences (i.e. the degree of substitution of the hemicelluloses) is recommended.

The precision breeding technology was successful in producing sugarcane with high content of juice sugar and with bagasse of high quality in terms of carbohydrates and

processability. However, the varieties produced by this technology were more susceptible to environmental condition, particularly drought conditions compared with classical breeding varieties. Further studies are recommended to include other genes to increase resistance to abiotic stress.

Pretreatment work could include testing other leading technologies such as sulfur-dioxide impregnated steam explosion, ammonium fibre explosion, liquid hot water and alkaline, in addition to dilute acid to generate an additional database that covers the leading pretreatment technologies. These pretreatments were selected by the biomass refinery Consortium for Applied Fundamentals and Innovation (CAFI) because they consider them to be promising for industrial scale.

The present study has established the common area of pretreatment conditions (temperatures 184–200 °C and varying residence time to provide the severity factor between 3.51 and 3.96) that provides 95% of the maximum combined sugar yield from different varieties. Therefore, this range condition should be used for screening of new bagasse samples in future.

Pretreatment at bench scale required more severe conditions than small scale reactors. Therefore further studies are recommended to see if this happens when up-scaling.

It well known that during dilute acid pretreatment lignin is normally partially depolymerised to phenols and these compounds can inhibit enzymatic hydrolysis and fermentation. Characterisation of soluble lignin (phenols) in the hydrolysate liquor would be useful for understanding the impact of these compounds on both enzymatic hydrolysis and fermentation when pressed or whole slurry are used at high solids loading. However, high ethanol yield was obtained in the present in spite of phenolics supposedly due to the fed-batch approach. The use of whole or pressed slurry is more plausible at industrial scale provided that the fermentative microorganism is robust enough to cope with the harsher conditions. Various lignin components present in pressed or whole slurry can be measured by using HPLC column coupled with mass spectrometer and photodiode array [3].

It is also recommended to study different process configurations such as SHF versus SSF. Various studies that have demonstrated that the enzymes CellicCTEC2 and HTEC2 work better in separate hydrolysis and fermentation when it is conducted at high solids loading due to the formation of gluconic acid [4, 5]. However, gluconic acid formation detected during SSF was minimal under this study probably due to the fed-batch approach.

9.3. References

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Appendix

Appendix A: Results related to Chapter 4

Appendix A-1: Average chemical composition of bagasse samples from different varieties of sugarcane (% dry weight)

Varieties	Ash	Extractives	Lignin	Arabinoxylan	Glucan	TSC*	HHV (MJ/kg)
1	2.2	7.0	19.7	24.6	38.9	63.5	18.1
2	2.2	5.5	19.9	25.0	39.1	64.1	17.8
3	1.6	7.9	19.1	24.6	39.0	63.5	18.4
4	1.5	8.9	18.2	24.1	39.5	63.6	18.5
5	1.2	11.6	15.6	24.7	39.4	64.2	18.8
6	2.2	10.1	18.4	24.2	37.5	61.7	18.4
7	1.8	10.8	16.8	24.2	38.7	62.9	18.5
8	1.6	12.4	17.7	23.6	38.0	61.6	19.0
9	1.6	8.9	19.9	23.8	38.4	62.2	18.6
10	0.8	5.5	22.7	24.9	38.1	63.0	18.6
11	1.9	5.9	21.1	24.7	38.1	62.8	18.1
12	1.6	6.4	21.0	26.0	37.0	63.0	18.3
13	1.6	7.3	17.8	24.5	40.6	65.1	18.1
14	1.9	5.8	22.5	25.3	36.7	62.0	18.2
15	2.1	4.8	20.0	27.4	37.7	65.1	17.8
16	1.8	4.6	19.7	27.2	38.5	65.7	17.8
17	1.6	8.1	21.5	25.8	35.4	61.2	18.7
18	2.1	9.6	17.3	27.6	35.6	63.2	18.3
19	1.9	6.0	17.0	28.3	38.1	66.4	17.7
20	2.3	5.4	22.8	26.5	35.3	61.8	18.1
21	2.3	6.6	21.2	25.8	36.4	62.2	18.2
22	1.7	5.4	21.0	26.0	38.0	63.9	18.2
23	1.9	7.7	21.3	26.6	35.0	61.5	18.5
24	1.8	6.6	22.3	26.1	35.5	61.6	18.4
25	1.6	8.2	19.3	26.9	36.1	62.9	18.4
26	1.2	8.1	18.0	28.1	36.4	64.5	18.4
27	1.8	7.4	20.6	28.0	34.5	62.5	18.4
28	1.5	6.7	20.1	27.7	36.0	63.7	18.3
29	1.0	5.2	21.1	26.6	38.2	64.7	18.4
30	1.4	5.2	20.6	25.4	39.6	64.9	18.2
31	0.6	3.5	19.3	28.0	40.0	68.0	18.0
32	1.8	5.6	22.1	27.8	34.6	62.5	18.2
33	1.3	7.9	20.9	25.9	36.2	62.2	18.7
34	1.4	6.1	22.6	27.2	34.9	62.0	18.5
35	1.9	6.9	20.3	26.1	37.3	63.4	18.3
36	2.5	4.5	23.1	27.2	35.0	62.2	18.0
37	2.4	10.0	20.8	26.3	33.5	59.8	18.7

38	2.0	8.6	20.7	27.2	34.3	61.5	18.6
39	2.4	7.4	20.3	27.1	35.1	62.2	18.2
40	2.9	5.1	22.8	27.2	34.2	61.4	17.8
41	2.2	8.2	19.9	25.7	36.5	62.1	18.3
42	3.4	8.4	19.9	26.3	34.6	60.9	17.9
43	1.9	12.3	19.0	23.7	36.6	60.3	19.0
44	2.2	7.8	19.3	25.1	37.9	63.1	18.2
45	1.8	5.7	22.0	26.1	36.6	62.7	18.2
46	1.2	7.4	22.7	24.3	36.8	61.1	18.8
47	1.9	6.9	21.5	25.1	36.9	62.0	18.4
48	2.4	7.0	21.8	23.7	37.4	61.1	18.2
49	1.6	5.0	22.8	25.0	37.7	62.7	18.3
50	2.7	9.3	20.1	24.0	36.8	60.8	18.4
51	2.0	10.0	17.1	27.0	36.1	63.1	18.4
52	1.4	3.9	20.8	26.5	39.2	65.7	18.0
53	1.8	7.7	21.4	25.7	35.9	61.6	18.5
54	1.7	7.3	20.7	25.9	36.8	62.7	18.4
55	1.6	9.9	19.6	27.1	35.1	62.2	19.0
56	1.7	7.9	19.7	26.8	35.9	62.7	18.4
57	2.0	9.9	17.4	25.1	38.0	63.1	18.4
58	1.4	7.2	20.0	25.5	37.9	63.5	18.4
59	2.2	7.5	21.0	25.7	35.8	61.6	18.3
60	1.6	5.3	22.2	25.0	38.2	63.2	18.3
61	2.3	4.5	18.7	28.3	37.8	66.1	17.5
62	1.4	7.5	19.8	25.3	38.2	63.5	18.5
63	1.9	8.6	17.7	25.4	38.6	64.0	18.3
64	2.3	8.0	16.2	27.9	37.7	65.6	17.9
65	2.0	11.2	17.9	24.2	37.5	61.7	18.7
66	2.1	7.6	17.1	27.2	38.0	65.3	18.0
67	2.3	10.0	20.0	24.8	35.4	60.2	18.6
68	1.9	8.8	22.2	25.7	34.2	59.9	18.8
69	1.8	7.8	21.9	25.9	35.4	61.3	18.7
70	1.8	7.5	20.4	26.7	36.1	62.9	18.1
71	1.4	6.3	18.1	28.0	37.9	65.8	18.0
72	1.7	12.2	19.1	24.6	35.7	60.4	19.2
73	2.2	9.4	19.6	27.1	34.2	61.2	18.5
74	2.0	7.5	19.7	25.5	36.9	62.4	18.3
75	2.0	8.0	20.5	25.1	36.8	61.9	18.4
76	1.3	6.9	19.7	28.0	36.1	64.1	18.4
77	1.4	6.1	21.8	25.7	37.3	63.0	18.4
78	0.9	4.9	21.4	25.6	39.8	65.4	18.5
79	1.5	7.0	19.8	26.4	37.5	63.9	18.4
80	1.6	7.8	19.3	27.4	36.4	63.8	18.4
81	1.4	7.3	17.6	28.5	37.1	65.6	18.2
82	1.5	8.7	22.3	26.0	34.2	60.2	18.9
83	2.3	8.5	22.0	24.1	35.9	60.0	18.6

84	2.1	7.4	22.2	26.9	33.9	60.8	18.5
85	3.3	6.5	22.2	25.9	34.6	60.5	17.9
86	1.8	6.5	21.7	25.6	36.9	62.5	18.4
87	2.0	8.8	20.5	24.9	36.4	61.4	18.6
88	2.5	8.7	20.2	25.7	35.6	61.2	18.3
89	1.9	9.2	21.6	26.4	32.9	59.3	18.7
90	2.3	9.7	20.0	26.8	33.8	60.7	18.6
91	1.6	11.0	19.8	24.6	36.0	60.6	19.0
92	1.1	8.2	22.2	24.8	36.4	61.3	19.0
93	1.7	9.7	19.0	24.5	37.9	62.4	18.7
94	1.8	8.6	19.3	27.3	35.7	63.0	18.5
95	2.0	10.3	20.5	24.6	35.7	60.3	18.9
96	1.7	8.3	20.8	26.3	35.7	62.0	18.7
97	2.6	10.1	20.0	26.1	34.3	60.4	18.5
98	1.6	8.3	20.8	25.5	36.4	61.9	18.7
99	0.8	9.8	16.3	27.4	37.5	64.9	18.6
100	1.1	10.2	18.2	26.4	36.3	62.7	18.8
101	0.8	7.4	14.4	28.5	40.7	69.2	18.1
102	0.7	5.3	16.2	31.0	38.2	69.2	17.9
103	0.9	8.1	18.4	28.3	36.1	64.4	18.5
104	0.9	7.3	16.4	28.2	34.1	62.3	18.6
105	0.8	7.8	19.1	27.3	36.3	63.6	18.5
106	0.9	8.0	16.6	28.5	38.1	66.5	18.4
107	1.0	6.3	16.1	29.6	38.5	68.1	18.0
108	1.4	6.9	19.0	30.4	34.4	64.8	18.3
109	0.7	3.8	18.5	29.8	38.6	68.5	17.9
110	1.0	7.9	17.9	28.9	35.2	64.1	18.3
111	1.4	8.5	22.2	27.1	32.6	59.8	18.8
112	0.9	5.6	17.2	30.8	36.8	67.7	18.0
113	0.8	7.6	17.7	29.2	36.8	66.0	18.5
114	0.9	6.1	16.1	29.7	38.3	68.0	18.0
115	1.0	7.7	17.2	29.3	36.7	66.0	18.4

*TSC means total structure carbohydrates (sum of glucan and arabinoxylan)

Appendix A-2: Average sugar yield after pretreatment at (180 °C, 0.5% w/w, for 15 min) and enzymatic hydrolysis at 15 FPU/g WIS of bagasse from 115 varieties of sugarcane

Variety	Pretreated Liquor			Enzymatic hydrolysis		Combined sugar
	Arabinose	Glucose	Xylose	Glucose	Xylose	
1	1.3	2.7	15.1	28.9	0.9	48.8
2	1.3	2.1	12.4	16.8	1.1	33.7
3	2.0	3.0	16.4	12.8	2.1	36.4
4	1.4	3.3	15.0	29.1	1.8	50.7
5	2.6	3.0	19.0	28.1	0.6	53.3
6	2.0	2.0	12.7	20.9	1.0	38.6
7	1.6	3.1	15.1	15.0	0.7	35.4

8	1.7	2.9	15.6	27.2	1.1	48.6
9	1.4	3.2	14.9	25.6	1.5	46.6
10	1.0	1.9	17.9	17.8	0.4	39.0
11	1.7	2.3	13.6	7.4	2.4	27.5
12	1.0	2.0	16.4	23.0	1.4	43.8
13	1.5	2.4	15.2	24.3	1.1	44.5
14	1.5	2.4	13.2	20.2	0.2	37.4
15	1.0	3.5	18.1	25.3	1.2	49.0
16	1.4	2.4	13.2	28.8	2.9	48.7
17	1.6	2.9	14.0	27.9	0.2	46.6
18	1.5	2.0	16.7	25.4	0.4	46.1
19	1.5	2.2	16.8	30.0	1.5	51.9
20	1.5	3.1	14.5	22.9	1.7	43.6
21	1.1	2.0	14.5	25.3	0.1	42.9
22	1.1	2.8	13.2	20.5	0.5	38.1
23	1.2	2.6	12.5	28.0	1.1	45.2
24	1.2	2.4	11.5	19.6	2.0	36.7
25	1.2	2.0	12.0	25.4	1.7	42.3
26	1.1	2.1	13.0	21.8	0.8	38.8
27	1.2	3.1	14.6	16.0	1.2	36.2
28	1.7	3.5	17.6	24.1	1.5	48.4
29	1.5	3.9	17.6	20.8	1.2	45.0
30	1.0	3.0	14.6	26.0	2.2	46.8
31	0.9	2.6	11.4	11.7	0.7	27.3
32	1.0	2.1	10.6	17.6	0.9	32.1
33	1.0	2.2	10.6	31.3	1.0	46.0
34	0.9	2.3	13.9	31.5	1.4	50.0
35	1.4	2.6	13.6	26.3	0.8	44.8
36	1.7	3.0	15.7	18.2	0.5	39.1
37	2.1	3.4	19.2	13.1	0.7	38.5
38	1.7	1.9	14.8	18.4	0.4	37.2
39	1.2	2.1	12.0	27.3	0.4	43.0
40	1.3	1.2	15.1	22.4	0.4	40.2
41	1.8	2.2	14.0	26.2	0.8	44.9
42	1.7	3.1	17.3	18.3	0.4	40.9
43	0.8	1.9	8.8	25.4	0.4	37.3
44	1.4	2.5	13.1	28.5	0.2	45.7
45	1.0	2.1	14.3	25.2	0.5	43.1
46	0.9	1.6	13.6	26.4	0.6	43.2
47	1.0	1.9	11.2	29.3	0.6	44.1
48	1.2	2.3	14.6	19.1	0.3	37.6
49	0.6	1.1	9.9	21.5	0.4	33.5
50	1.1	1.9	12.8	18.7	0.7	35.0
51	2.0	2.3	14.1	32.9	1.6	52.9
52	1.1	3.0	14.2	17.8	1.9	38.0
53	1.4	4.0	17.7	14.4	0.6	38.1

54	1.6	2.3	14.1	22.3	1.9	42.2
55	1.4	3.1	15.2	27.6	1.6	49.0
56	1.1	2.2	11.5	18.2	0.4	33.4
57	1.7	3.1	15.6	21.2	1.4	43.1
58	1.2	2.7	12.9	23.9	1.2	42.0
59	1.1	3.2	13.8	30.1	0.9	49.1
60	1.1	3.3	13.9	21.0	0.9	40.2
61	1.3	4.4	20.4	19.1	0.3	45.5
62	1.3	2.6	13.8	21.4	1.2	40.3
63	2.0	3.1	16.3	29.8	0.7	51.7
64	2.3	2.1	15.7	16.6	1.7	38.4
65	1.5	2.8	15.4	22.8	0.7	43.2
66	1.9	2.7	14.7	23.4	1.8	44.5
67	2.3	2.3	15.6	26.9	1.7	48.8
68	1.4	1.9	13.6	24.4	0.6	41.8
69	1.3	2.9	13.7	26.7	0.5	45.1
70	1.7	2.8	13.9	27.0	0.4	45.8
71	1.9	2.1	16.3	27.1	3.0	50.4
72	1.3	2.6	15.6	19.4	1.2	40.1
73	2.8	2.2	17.1	13.5	2.6	38.2
74	1.5	1.8	12.5	25.5	2.0	43.3
75	1.8	2.1	14.3	22.1	1.7	42.0
76	1.7	2.0	15.0	15.8	0.5	35.0
77	1.4	1.7	14.0	11.4	0.1	28.5
78	1.0	1.8	15.3	19.7	0.4	38.2
79	2.0	2.9	17.0	16.0	0.5	38.4
80	2.2	3.1	17.1	14.1	1.0	37.5
81	1.6	2.1	17.8	15.2	0.3	37.1
82	1.2	2.2	14.3	21.6	0.5	39.7
83	1.5	2.3	13.6	23.0	0.5	40.8
84	1.7	2.2	15.3	22.9	0.5	42.6
85	2.4	2.7	16.1	22.5	0.3	44.0
86	1.5	0.8	17.3	24.3	0.3	44.2
87	1.3	2.7	15.2	24.0	1.4	44.6
88	1.6	2.4	15.6	24.3	2.6	46.5
89	1.6	2.8	16.3	27.0	0.7	48.4
90	1.6	2.0	16.4	21.6	0.1	41.6
91	1.7	2.5	15.3	24.9	0.5	45.0
92	1.2	3.2	15.0	28.5	1.4	49.3
93	1.6	2.3	17.3	25.2	0.7	47.2
94	1.2	2.0	16.2	21.7	3.1	44.1
95	2.1	2.6	16.8	24.1	0.3	45.9
96	1.7	2.5	15.9	25.2	3.8	49.2
97	2.8	2.1	15.6	24.9	2.4	47.8
98	1.9	3.0	15.4	26.0	0.9	47.2
99	1.4	2.8	13.3	26.7	0.5	44.7

100	1.7	3.2	15.4	20.9	1.3	42.4
101	2.0	2.3	16.9	33.5	0.5	55.2
102	1.9	2.3	13.6	29.2	0.5	51.7
103	2.3	2.5	16.9	32.6	0.0	54.4
104	2.8	2.0	15.7	31.0	0.0	51.6
105	2.4	2.2	15.3	23.3	2.1	45.3
106	2.2	2.7	16.3	28.7	2.1	51.8
107	2.2	2.9	15.9	29.0	0.3	50.3
108	2.8	2.0	15.1	25.1	0.3	45.3
109	3.1	1.5	15.1	29.3	1.0	50.0
110	2.4	2.0	16.3	18.9	0.3	39.9
111	2.8	2.7	18.2	20.9	1.4	45.9
112	2.0	3.1	16.5	21.2	1.9	44.8
113	3.0	2.0	15.8	22.7	1.3	44.9
114	2.4	2.4	15.1	26.4	1.7	48.1
115	1.7	3.1	15.1	20.3	0.5	40.7

Appendix A-3: Sugar yield after pretreatment and enzymatic hydrolysis of bagasse from variety 91

Run	Pretreatment conditions			Sugar yield (g/100 g RM)		
	Temp. (°C)	Time (min)	Acid (%w/w)	Xylose	Glucose	Combined sugar
1	140	10	0.96	5.7	10.8	17.6
2	140	15	0.96	7.9	11.2	20.4
3	140	20	0.96	10.5	12.9	25.5
4	150	10	0.96	10.4	14.3	27.0
5	150	15	0.96	13.4	17.5	34.2
6	150	20	0.96	14.2	15.6	33.5
7	160	10	0.96	13.6	14.2	29.9
8	160	15	0.96	16.6	18.8	38.5
9	160	20	0.96	16.2	18.3	36.6
10	190	10	0.07	10.5	26.0	39.6
11	190	15	0.07	11.6	30.6	45.7
12	190	20	0.07	11.0	29.0	44.0
13	200	10	0.07	9.4	24.5	36.4
14	200	15	0.07	8.9	28.4	40.5
15	200	20	0.07	8.8	26.3	37.5
16	210	10	0.07	10.0	27.3	40.7
17	210	15	0.07	4.9	27.5	36.5
18	210	20	0.07	2.5	26.3	33.8
19	190	10	0	12.5	17.6	31.1
20	190	15	0	12.5	22.5	37.1
21	190	20	0	13.9	27.2	42.1
22	200	10	0	10.7	32.9	44.6
23	200	15	0	9.2	22.4	34.7

24	200	20	0	13.9	27.9	43.9
25	210	10	0	7.7	30.0	41.1
26	210	15	0	9.2	27.1	39.5
27	210	20	0	6.4	29.8	39.5

Appendix A-4: Average xylose yield after pretreatment of bagasse from 34 varieties of sugarcane.

Variety	150 °C, 0.96% (w/w), 15 min	160 °C, 0.96% (w/w), 15 min	180 °C, 0.5% (w/w), 15 min	190 °C, 0.07% (w/w), 15 min	200 °C, no-acid, 15 min
1	15.1	15.5	15.1	9.8	12.2
4	14.4	11.3	15.0	9.2	12.9
5	15.3	16.9	16.0	8.2	12.8
6	16.3	17.6	12.7	15.6	10.3
8	13.0	18.2	15.6	11.2	13.9
12	17.7	18.7	16.4	10.1	13.7
13	13.5	17.0	15.2	10.6	10.8
15	14.6	16.3	18.1	13.8	12.3
16	13.7	14.0	13.2	8.7	11.0
20	12.7	14.8	14.5	7.0	12.3
28	11.1	18.3	14.3	15.0	13.6
30	6.7	17.0	14.6	16.0	11.5
34	15.4	17.4	13.9	10.8	12.8
54	6.9	17.9	14.1	9.9	12.0
55	13.5	17.0	15.2	8.3	11.1
57	13.3	17.1	15.6	9.8	12.0
58	10.7	17.5	13.4	9.6	12.6
63	7.5	14.8	14.3	11.0	9.7
70	13.7	15.2	13.9	14.4	10.5
71	14.0	15.7	16.3	13.2	11.1
74	10.8	15.4	10.5	11.4	8.5
87	13.7	16.6	15.2	14.1	8.4
88	8.3	17.7	15.6	9.4	9.7
89	8.4	17.0	16.3	14.3	9.9
94	7.6	12.8	16.2	10.3	8.3
97	11.8	18.2	15.6	8.4	11.2
101	8.6	17.5	11.6	10.0	8.1
102	8.0	16.1	16.9	15.7	7.4
103	8.3	16.0	13.7	14.7	7.3
104	6.4	15.1	15.3	7.9	5.9
105	16.2	18.1	16.3	14.9	6.3
106	17.2	16.1	15.9	14.2	11.8
109	16.8	18.6	15.1	16.5	6.7
114	16.0	19.6	15.1	15.7	7.3

Appendix A-5: Average glucose yield after enzymatic hydrolysis of the pretreated solids of bagasse from 34 varieties of sugarcane when the hydrolysis was carried out 15 FPU/g WIS.

Variety	150 °C, 0.96% (w/w), 15 min	160 °C, 0.96% (w/w), 15 min	180 °C, 0.5% (w/w), 15 min	190 °C, 0.07% (w/w), 15 min	200 °C, no-acid, 15 min
1	25.5	22.4	28.9	29.1	24.5
4	22.4	20.4	29.1	26.9	27.4
5	25.3	19.8	28.1	29.9	24.8
6	19.1	18.8	20.9	20.6	25.6
8	24.9	24.0	27.2	27.0	29.6
12	19.8	22.4	23.0	24.1	29.6
13	25.6	20.7	24.3	26.4	27.5
15	18.9	18.6	24.3	19.3	26.5
16	23.1	24.0	28.8	24.8	29.9
20	15.3	19.8	22.9	20.4	19.5
28	15.7	18.4	25.5	15.9	21.6
30	19.7	23.1	25.0	27.2	26.6
34	18.3	21.7	31.5	25.0	29.1
54	18.5	23.3	22.3	24.5	23.8
55	15.0	18.4	27.6	27.1	28.7
57	15.2	18.8	21.2	22.6	18.8
58	16.1	20.2	23.9	19.5	30.6
63	25.6	26.0	29.8	22.4	30.4
70	20.4	24.5	27.0	17.6	25.3
71	22.5	24.7	27.1	17.4	28.4
74	17.5	23.9	25.5	17.9	26.8
87	16.2	20.6	24.0	24.5	24.6
88	18.0	21.1	24.3	26.3	27.8
89	16.6	18.2	27.0	20.6	25.0
94	23.5	26.3	21.7	18.9	28.3
97	18.8	22.2	24.9	24.1	23.5
101	26.9	29.9	32.5	29.6	34.6
102	25.2	29.5	32.6	23.6	27.2
103	27.2	25.0	30.0	26.0	30.5
104	23.4	18.2	22.3	18.7	31.8
105	24.6	23.6	28.7	27.0	30.0
106	25.8	25.0	29.0	29.8	33.8
109	21.7	23.2	29.3	33.9	31.9
114	27.8	27.6	25.4	24.5	32.3

Appendix A-6: Average glucose yield after enzymatic hydrolysis of the pretreated solids of bagasse from 34 varieties of sugarcane when the hydrolysis was carried out 1.5 FPU/g WIS.

Variety	150 °C, 0.96% (w/w), 15 min	160 °C, 0.96% (w/w), 15 min	180 °C, 0.5% (w/w), 15 min	190 °C, 0.07% (w/w), 15 min	200 °C, no-acid, 15 min
1	10.1	11.1	10.2	11.9	11.7
4	12.3	11.7	11.7	13.1	14.6
5	12.8	13.0	12.2	12.6	13.2
6	8.6	10.2	8.9	9.9	10.0
8	10.5	11.6	9.5	13.8	13.4
12	8.7	9.9	10.2	11.2	11.7
13	12.2	13.1	12.4	12.9	10.9
15	8.6	11.7	8.7	10.6	11.0
16	10.6	11.8	12.6	12.4	10.3
20	7.5	8.5	7.3	9.1	9.0
28	7.4	7.6	9.7	9.7	9.5
30	7.8	11.1	10.0	9.9	12.4
34	8.9	10.0	10.4	11.2	9.8
54	8.9	10.7	10.7	11.4	11.5
55	7.8	10.2	8.6	10.5	10.1
57	7.5	8.6	8.8	8.8	9.2
58	7.0	8.0	10.0	9.6	10.9
63	12.9	14.7	13.9	13.9	14.3
70	10.5	11.7	11.7	12.0	12.2
71	9.8	9.4	11.6	12.5	8.3
74	8.4	10.0	12.0	12.4	13.5
87	7.4	9.1	9.8	8.7	9.4
88	8.3	10.8	12.2	10.4	12.5
89	7.9	7.8	10.4	8.7	11.5
94	8.9	10.5	11.7	9.9	12.8
97	7.6	10.4	11.6	10.3	11.8
101	15.2	16.5	18.3	13.0	15.8
102	8.2	10.1	11.1	9.0	14.6
103	15.0	18.6	17.7	17.9	17.0
104	13.2	13.2	15.3	14.8	14.9
105	14.5	13.5	15.9	13.3	14.3
106	8.5	7.9	9.8	9.9	13.1
109	9.0	10.7	10.4	12.3	9.7
114	13.8	15.6	14.5	14.8	16.4

Appendix A-7: Average combined sugar yield after and enzymatic hydrolysis of bagasse from 34 varieties of sugarcane when the hydrolysis was carried out 15 FPU/g WIS.

Variety	150 °C, 0.96% (w/w), 15 min	160 °C, 0.96% (w/w), 15 min	180 °C, 0.5% (w/w), 15 min	190 °C, 0.07% (w/w), 15 min	200 °C, no-acid, 15 min
1	46.1	43.6	48.8	44.9	44.2
4	43.0	37.0	50.7	42.9	45.7
5	48.3	45.8	53.3	43.5	46.4
6	41.6	43.3	38.6	42.1	41.4
8	43.7	49.2	48.6	45.8	50.0
12	42.8	47.3	44.8	40.4	48.3
13	45.7	47.7	48.5	43.5	44.7
15	38.6	41.0	49.0	43.5	44.4
16	42.7	45.5	47.1	40.3	47.2
20	32.3	39.6	41.6	34.2	36.7
28	29.8	44.7	45.9	35.9	41.4
30	30.0	48.1	46.8	49.9	46.1
34	39.1	46.4	51.0	42.5	47.7
54	28.8	48.0	42.7	40.2	43.5
55	33.4	43.1	49.0	40.3	44.2
57	30.8	44.1	43.1	38.0	39.8
58	28.1	46.4	42.0	35.4	47.6
63	37.7	49.1	51.7	39.5	46.0
70	39.8	46.3	45.2	38.3	40.6
71	42.3	46.8	50.4	38.1	45.3
74	33.1	45.7	41.3	34.0	39.5
87	34.7	42.6	44.6	41.6	39.5
88	30.6	45.8	46.5	40.3	42.3
89	28.5	39.8	48.4	41.5	39.3
94	36.2	42.7	44.1	36.3	40.6
97	36.1	47.1	45.8	38.1	42.4
101	42.5	54.7	55.2	44.2	47.4
102	38.2	54.1	54.4	44.9	47.2
103	40.3	49.3	51.6	48.1	41.8
104	34.5	39.8	45.3	31.1	41.8
105	48.3	50.7	51.8	48.2	40.2
106	49.0	45.3	50.3	49.5	50.2
109	42.8	50.4	50.0	53.9	45.1
114	51.6	53.7	48.1	49.5	45.2

Appendix B: Results related to Chapter 5.

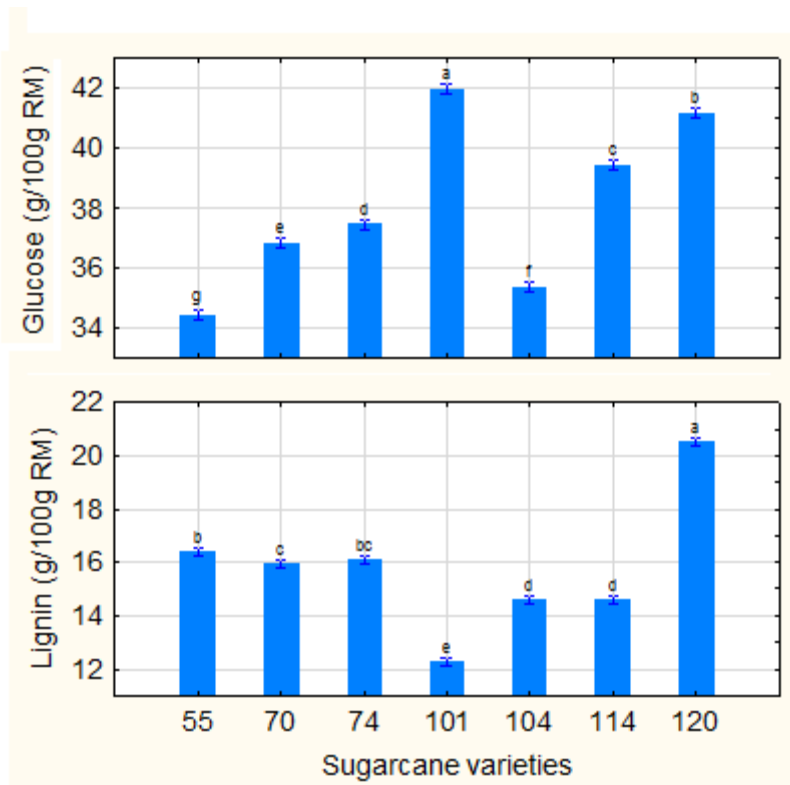
Appendix B-1: Preliminary experiments to select a range conditions that could be applied during central composite design (CCD) of the preferred varieties.

Standard Design					
Pretreatment conditions			g/100g raw bagasse		
Temp, °C	Acid, % (w/w)	Time, min	Xylose	Glucose	Combined sugar
165	0.15	7	2.8	15.2	24
195	0.15	7	11.4	21.9	38.3
165	0.65	7	12.2	21.4	39
195	0.65	7	14	29.9	47.8
165	0.15	21	5.6	20.2	30.9
195	0.15	21	7.8	27	38.2
165	0.65	21	19.4	24.3	49.5
195	0.65	21	8.9	21.5	37.2
180	0.4	14	16.1	30.2	51.8
180	0.4	14	15.9	30.6	52.3
Proposed Centre point by the model					
160	0.96	15	17.5	29.9	54.7
Steepest ascent					
165	0.65	7	12.7	29.1	47.3
170	0.65	8	16.9	27.2	49.5
175	0.65	9	18.2	33.8	57.5
180	0.65	10	19.1	34.7	59.7
185	0.65	11	18.2	34.3	57.6
Centre point selected for CCD					
180	0.65	10	19.1	34.7	59.7

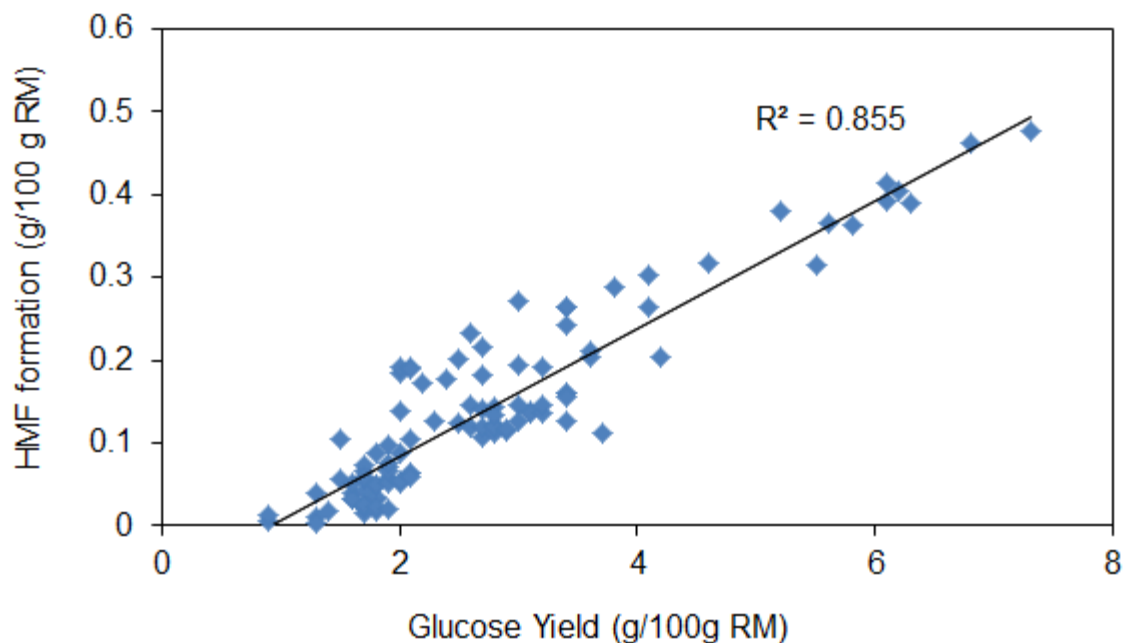
Appendix B-2: Recovery of WIS and sugar yield in the pretreated liquor (glucose and arabinose) after dilute acid pretreatment of different sugarcane bagasse samples (55, 70, 74, 101, 104, 114 and 120)

Run	Variables ^a			Pretreated liquor (g/100g RM)																							
				WIS recovery (%)							Glucose							Arabinose									
	X ₁	X ₂	X ₃	55	70	74	101	104	114	120	55	70	74	101	104	114	120	55	70	74	101	104	114	120			
1	170	0.45	5	68.0	61.1	70.5	70.3	65.6	70.1	77.6	1.4	1.6	1.7	1.8	1.7	1.7	0.9	0.9	1.7	0.9	1.8	1.6	1.6	0.7			
2	190	0.45	5	59.9	57.0	60.7	62.6	58.8	61.5	68.7	1.9	2.0	3.5	2.4	2.1	2.7	2.0	1.3	1.9	1.9	1.9	1.8	2.4	0.8			
3	170	0.85	5	64.2	58.1	65.1	65.4	62.8	64.8	74.4	1.6	2.2	2.5	2.5	1.9	2.0	1.3	1.5	1.7	1.6	1.9	2.1	1.9	1.0			
4	190	0.85	5	57.4	53.7	58.9	60.5	56.6	58.8	67.4	2.8	2.4	2.9	3.6	3.0	4.2	1.9	1.7	1.6	1.1	1.8	2.1	2.0	1.0			
5	170	0.45	15	61.2	69.5	63.4	63.3	63.3	64.0	73.4	2.1	2.1	2.4	2.3	1.7	1.7	1.7	1.8	1.5	1.4	2.0	1.9	1.7	1.1			
6	190	0.45	15	57.6	55.6	57.0	58.8	57.6	58.1	66.3	3.5	3.1	4.1	3.4	2.7	3.8	2.0	1.5	1.2	1.5	1.7	1.9	1.8	0.7			
7	170	0.85	15	58.7	62.3	60.6	60.4	59.5	59.7	69.0	2.0	2.7	2.8	2.9	2.3	2.7	1.3	1.6	1.7	1.8	2.4	1.9	2.2	1.0			
8	190	0.85	15	51.9	58.0	53.4	55.3	53.7	54.4	63.3	6.5	6.3	6.8	6.2	5.6	7.3	2.4	1.2	1.4	1.4	1.7	1.7	1.7	0.7			
9	163.2	0.65	10	63.3	71.0	67.9	67.3	63.2	66.8	72.8	2.6	1.8	2.6	2.8	2.2	2.2	0.9	1.7	1.3	1.2	1.9	2.1	2.1	0.8			
10	196.8	0.65	10	50.1	57.4	53.1	56.1	54.0	54.4	62.6	6.1	4.6	6.1	5.2	5.8	5.5	3.2	1.6	1.4	1.5	1.7	1.9	1.4	0.7			
11	180	0.31	10	59.8	60.0	63.1	64.2	62.9	63.1	70.5	1.7	1.9	2.1	2.2	1.7	1.9	1.9	1.3	1.9	1.4	1.8	2.1	2.1	1.0			
12	180	0.99	10	54.7	60.4	59.0	59.1	56.9	58.8	66.8	3.6	3.7	3.4	3.5	3.5	3.2	1.8	1.5	1.1	1.7	1.8	1.8	1.9	1.0			
13	180	0.65	1.6	69.2	59.6	70.4	69.7	62.5	69.4	76.5	1.5	1.6	1.7	1.8	1.8	1.8	1.3	1.2	1.6	0.8	1.6	1.6	1.9	0.8			
14	180	0.65	18.4	57.3	57.4	59.0	58.1	56.9	58.7	67.4	3.4	4.1	3.9	3.6	2.6	3.0	1.5	1.4	0.9	1.1	1.6	1.7	1.8	0.8			
15	180	0.65	10	59.2	60.2	55.7	62.5	58.8	61.1	68.2	3.1	2.0	3.4	2.5	2.4	3.4	1.7	1.7	3.2	1.9	2.1	1.9	2.2	1.1			
16	180	0.65	10	58.9	59.8	59.2	62.0	58.7	60.2	67.9	3.4	2.5	3.9	2.8	2.3	2.6	1.6	1.8	2.4	1.9	2.1	1.9	2.2	1.0			
17	180	0.65	10	58.2	60.5	60.2	61.9	58.9	59.8	68.3	3.4	2.2	3.9	2.8	3.5	3.4	2.2	1.7	3.7	2.1	2.0	2.2	2.1	1.1			
18	180	0.65	10	58.4	60.2	59.3	62.8	58.7	60.5	67.5	3.2	2.1	3.6	2.8	2.6	2.8	1.9	1.6	3.5	1.9	2.0	2.1	2.1	1.5			
19	180	0.65	10	59.5	58.8	60.8	62.6	58.9	60.8	68.4	3.1	2.0	3.5	2.8	2.7	2.6	1.8	1.7	3.2	1.8	2.0	2.1	2.1	1.2			
20	180	0.65	10	59.4	60.3	60.0	61.8	58.7	61.0	68.0	3.2	2.1	3.5	3.5	3.0	3.0	1.9	1.9	3.3	1.9	2.2	2.0	2.0	1.2			

^aPretreatment conditions applied in the study, runs 1 to 8, 9 to 14 and 15 to 20 represents factorial points, star points and centre points, respectively, X₁, temperature (°C); X₂, acid concentration (%w/w); X₃, reaction time (min).



Appendix B-3: Comparison of chemical composition (glucose and acid insoluble lignin) in the WIS of different sugarcane bagasse samples (55, 70, 74, 101, 104, 114 and 120) after the dilute sulphuric acid pretreatment. The yields are based mean values of all factorial points. The columns with similar insulated letters do not differ between each other at a significance level of 0.05.



Appendix B-4: Relationship between glucose yield in the pretreated liquor and HMF formation after pretreatment.

Appendix B-5: Analysis of Variance of the proposed model

Source	55					70				
	<i>SS</i> ^a	<i>DF</i> ^b	<i>SM</i> ^c	<i>F</i>	<i>p</i>	<i>SS</i> ^a	<i>DF</i> ^b	<i>SM</i> ^c	<i>F</i>	<i>p</i>
Model	1026.19	8	128.27	56.06	< 0.0001	658.87	8	82.36	14.03	< 0.0001
Residual	25.17	11	2.29			64.59	11	5.87		
Lack of Fit	18.19	6	3.03	2.17	0.2058	44.65	6	7.44	1.87	0.26
Pure Error	6.97	5	1.39			19.94	5	3.99		
Cor Total	1051.35	19				723.46	19			
			74			101				
Model	689.88	8	86.24	52.21	< 0.0001	413.80	9	45.98	17.44	< 0.0001
Residual	18.17	11	1.65			26.37	10	2.64		
Lack of Fit	14.41	6	2.40	3.19	0.1119	21.90	5	4.38	4.90	0.05
Pure Error	3.76	5	0.75			4.47	5	0.89		
Cor Total	708.05	19				440.16	19			
			104			114				
Model	349.92	7	49.99	36.37	< 0.0001	784.52	8	98.07	36.20	< 0.0001
Residual	16.49	12	1.37			29.80	11	2.71		
Lack of Fit	13.49	7	1.93	3.21	0.1087	24.68	6	4.11	4.02	0.07
Pure Error	3.00	5	0.60			5.12	5	1.02		
Cor Total	366.41	19				814.32	19			
			120							
Model	427.45	9	47.49	38.82	< 0.0001					
Residual	12.23	10	1.22							
Lack of Fit	9.05	5	1.81	2.85	0.1378					
Pure Error	3.18	5	0.64							
Cor Total	439.68	19								

^a *SS* sum of square

^b *DF* degree of freedom

^c *SM* mean square

Appendix C: Results related to Chapter 6.

Appendix C-1: ANOVA test p -values for the comparison of chemical compositions of bagasse samples

p-values							
Glucan	Xylan	Arabinan	Acid soluble	Acid insoluble	Acetyl	Extractives	Ash
0.3213	0.0094*	0.2435	0.4087	0.0035*	0.0836	0.1234	0.1609

*Means significant differences between the harvests at $p < 0.05$.

Appendix C-2: Inhibitors formation after dilute acid pretreatment of sugarcane bagasse samples when the acid loading was kept at 0.5% (w/w)

Run	Pretreatment conditions			Furfural				HMF				Acetic Acid				Formic acid			
	Temp (°C)	Time (min)	logR'0	55	70	74	120	55	70	74	120	55	70	74	120	55	70	74	120
1	180(185)	5(4)	0.94(0.91)	0.1	0.2	0.1	0.3	0.1	0.1	0.1	0.0	0.5	0.5	0.5	0.8	0.0	0.0	0.0	0.1
2	200(205)	5(4)	1.59(1.58)	0.8	1.2	1.1	1.9	0.3	0.4	0.4	0.1	1.5	1.9	1.9	2.3	0.1	0.1	0.1	0.1
3	180(185)	15(14)	1.46(1.56)	0.5	0.4	0.5	0.6	0.3	0.2	0.2	0.0	1.4	1.0	1.4	1.1	0.1	0.0	0.1	0.1
4	200(205)	15(14)	2.06(2.16)	2.6	3.1	2.6	4.6	0.7	0.8	0.6	0.2	3.2	3.0	3.0	3.8	0.1	0.2	0.1	0.2
5	176(181)	10(9)	1.10(1.20)	0.1	0.2	0.1	0.5	0.1	0.2	0.1	0.0	0.6	0.7	0.5	1.2	0.0	0.0	0.0	0.1
6	204(209)	10(9)	1.98(2.06)	2.8	2.9	2.3	3.0	0.7	0.8	0.5	0.1	3.1	3.2	2.8	2.9	0.2	0.2	0.1	0.1
7	190(195)	3(2)	1.07(0.95)	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.0	0.8	0.7	0.8	0.6	0.1	0.1	0.0	0.1
8	190(195)	17(16)	1.82(1.92)	1.6	1.4	1.3	2.6	0.5	0.5	0.4	0.1	2.3	2.1	2.4	2.7	0.1	0.1	0.1	0.1
9	190(195)	10(9)	1.57(1.63)	0.9	1.1	0.8	1.3	0.4	0.5	0.3	0.1	1.7	1.9	1.8	1.9	0.1	0.1	0.1	0.1
10	190(195)	10(9)	1.56(1.65)	1.0	1.2	0.7	1.2	0.4	0.5	0.3	0.1	1.8	1.8	1.8	1.9	0.1	0.1	0.1	0.1
11	190(195)	10(9)	1.58(1.64)	0.9	1.2	0.8	1.2	0.4	0.5	0.3	0.1	1.7	1.8	1.8	1.9	0.1	0.1	0.1	0.1

The conditions in parenthesis were used for bagasse 120 only

Appendix C-3: Analysis of Variance of the proposed model

Source	Xylose-Variety 55					Glucose-Variety 55				
	SS ^a	DF ^b	SM ^c	F	p	SS ^a	DF ^b	SM ^c	F	p
Model	659.17	5	131.83	7.28	0.024	200.24	4	50.06	81.30	< 0.0001
Residual	90.51	5	18.10			3.69	6	0.62		
Lack of Fit	85.03	3	28.34	10.34	0.0894	3.51	4	0.88	9.44	0.0981
Pure Error	5.48	2	2.74			0.19	2	0.09		
Cor Total	749.68	10				203.93	10			
	CSY -Variety 55					Xylose-Variety 70				
Model	194.77	4	48.69	36.41	0.0002	868.13	5	173.63	10.71	0.0105
Residual	8.02	6	1.34			81.03	5	16.21		
Lack of Fit	7.12	4	1.78	3.94	0.2126	61.01	3	20.34	2.03	0.3467
Pure Error	0.90	2	0.45			20.02	2	10.01		
Cor Total	202.79	10				949.15	10			
	Glucose-Variety 70					CSY -Variety70				
Model	145.59	4	36.40	69.53	< 0.0001	280.85	5	56.17	110.53	< 0.0001
Residual	3.14	6	0.52			2.54	5	0.51		
Lack of Fit	3.09	4	0.77	33.71	0.029	1.32	3	0.44	0.72	0.6273
Pure Error	0.05	2	0.02			1.23	2	0.61		
Cor Total	148.73	10				283.39	10			
	Xylose-Variety 74					Glucose-Variety 74				
Model	216.54	5	43.31	19.65	0.0027	139.4421	2	69.72104	29.72645	0.0002
Residual	11.02	5	2.20			18.76337	8	2.345421		
Lack of Fit	10.26	3	3.42	9.06	0.101	18.70337	6	3.117228	103.9076	0.0096
Pure Error	0.76	2	0.38			0.06	2	0.03		
Cor Total	227.56	10				158.2055	10			
	CSY -Variety74					Xylose-Variety 120				
Model	594.36	5	118.87	7.18	0.0248	1842.02	5	368.40	29.84	0.001
Residual	82.83	5	16.57			61.73	5	12.35		
Lack of Fit	63.50	3	21.17	2.19	0.3288	51.05	3	17.02	3.19	0.248
Pure Error	19.33	2	9.67			10.68	2	5.34		
Cor Total	677.18	10				1903.75	10			
	Glucose-Variety 120					CSY -Variety 120				
Model	113.16	3	37.72	80.04	< 0.0001	94.19014	5	18.83803	11.86129	0.0084
Residual	3.30	7	0.47			7.940967	5	1.588193		
Lack of Fit	3.22	5	0.64	17.40	0.0552	5.812758	3	1.937586	1.82086	0.3737
Pure Error	0.07	2	0.04			2.128209	2	1.064105		
Cor Total	116.46	10				102.1311	10			

Appendix C-4: Correlation between cellulose digestibility and ratio of xylose to arabinose and lignin content

Run	1	2	3	4	5	6	7	8	9
Ratio of xylose to arabinose	0.962*	0.767	0.543	0.904*	0.830	0.974*	0.744	0.935*	0.949*
Lignin content	-0.974*	-0.979*	-0.879	-0.997*	-0.876	-0.906*	-0.966*	-0.986*	-0.979*