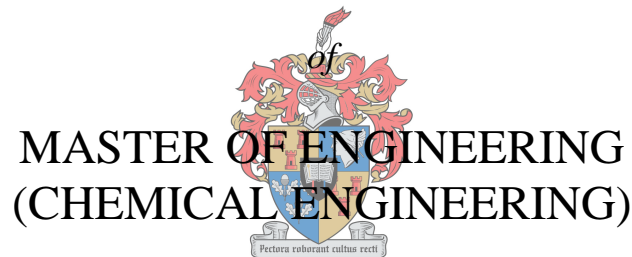


# Lactic acid production from sugarcane bagasse and harvesting residues

*by*

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Thesis presented in partial fulfillment  
of the requirements for the Degree



UNIVERSITEIT  
iYUNIVESITHI  
STELLENBOSCH  
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in the Faculty of Engineering  
at Stellenbosch University

1918 · 2018

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed, and conclusions arrived at, are those of the author and are not necessarily attributed to the NRF.

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March 2018

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## Abstract

Sugarcane bagasse and harvesting residues collected from different sugars mills across South Africa were evaluated for potential use in a biorefinery for ethanol, lactic acid and electricity co-production after pretreatment using dilute sulphuric acid as catalyst. On a dry mass basis, sugarcane bagasse consisted of 38% glucan, 15% arabinoxylan, 27% lignin, 7% extractives, 9% acetyl groups and 3% ash. By comparison, harvesting residues consisted of 33% glucan, 17.5% arabinoxylan, 20% lignin, 16% extractives, 5 % acetyl groups and 9% ash.

Following pre-screening experiments to appraise the differences between the responses of the two feedstocks, a central composite, rotatable design was used to optimise the xylose from hemicellulose, glucose from cellulose and combined sugar yield after pre-treatment and enzymatic hydrolysis, where temperature, sulphuric acid concentration and residence time were the independent variables.

Based on optimised regression at a 95% confidence level, all three factors had a significant effect on the pre-treatment of sugarcane bagasse, whereas only temperature and sulphuric acid concentration were significant during the pre-treatment of harvesting residues. Based on model predictions, optimal conditions resulted in the production of 24.5 g xylose, 32.4 g glucose and 63 g combined per 100 g DM for sugarcane bagasse and 17.4 g xylose, 42.9 g glucose and 66.7 g combined sugar per 100 g DM for harvesting residues.

Steam pre-treatment was used to produce sufficient quantities of hemicellulose-rich hydrolysate for lactic acid production during fermentation using six different lactic acid bacteria obtained from various research groups and culture collections. These strains were selected based on the ability to (i) ferment xylose, arabinose and glucose simultaneously; (ii) operate at moderately to high temperatures, and (iii) were tolerant to inhibitor compounds produced during pre-treatment.

The innate tolerance of each strain to inhibitory compounds found in hemicellulose hydrolysates were tested under anaerobic and micro-aerobic conditions. The latter was included to determine if the low oxygen tensions in shake flask cultures negatively affected fermentation of five-carbon sugars, usually assimilated via the pentose phosphate pathway where the absence of oxygen could lead to redox imbalances. Higher lactic acid concentrations were generally observed under anaerobic conditions in a fermentation broth supplemented with 75% (v/v) hemicellulose hydrolysate where *Bacillus coagulans* P38 produced 4.18 and 20.42 g/L lactic acid from the hydrolysates of sugarcane bagasse and harvesting residues, respectively. By comparison, *Bacillus coagulans* MXL-9 produced 5.58 g/L and 16.97 g/L lactic acid from the hydrolysates of bagasse and harvesting residues, respectively, and *Lactococcus lactis* IO-1 produced 8.68 g/L and 17.44 g/L lactic acid from the respective substrates. These results accentuated the importance of bacterial strain selection when

using complex and relatively toxic substrates for production of lactic acid as an economically important platform chemical.

## Uittreksel

Die potensiaal van suikerrietbagasse en suikerriet oesreste, wat van verskillende Suid Afrikaanse suikermeule verkry is, en met verdunde suur katalise behandel is, is vir bioraffinadery-gebaseerde etanol-, melksuur- en elektrisiteitproduksie in hierdie studie geassesseer. Op 'n droëmassabasis het suikerrietbagasse 38% glukosaan, 15% arabinoxilaan, 27% lignien, 7% ekstraktiewe, 9% asetiel groep en 3% as bevat. Daarteenoor het suikerriet oesreste 33% glukosaan, 17.5% arabinoxilaan, 20% lignien, 16% ekstraktiewe, 5% asetiel groep en 9% as bevat.

Na aanvanklike keuringseksperimente om die verskille tussen response vanaf die twee voerstowwe te assesser, is 'n roteerbare, sentrale saamgestelde ontwerp gebruik om die xilose vanaf hemisellulose, glukose vanaf sellulose en die gekombineerde suikeropbrengs ná verdunde suurbehandeling en ensimatiese hidrolise te optimeer, waar temperatuur, swaelsuurkonsentrasie en tydskuur die onafhanklike veranderlikes was.

Op grond van 'n geoptimeerde regressiemodel met 'n 95% vertrouwe vlak het al drie faktore 'n statisties beduidende effek op die behandeling van suikerrietbagasse gehad, terwyl slegs temperatuur en swaelsuurkonsentrasie 'n beduidende effek op die behandeling van die oesreste gehad het. Modelvoorspellings het tot die optimale produksie van 24.5 g xilose, 32.4 g glukose en 63 g gekombineerde suiker per 100 g DM vir suikerrietbagasse, en 17.4 g xilose, 42.9 g glukose en 66.7 g gekombineerde suiker per 100 g DM vir oesreste gelei.

Stoombehandeling is gebruik om voldoende hoeveelhede, hemisellulose-ryke hidrolisaat vir melksuurfermentasie eksperimente te produseer, waar ses verskillende melksuurbakterieë vanaf verskillende navorsingsgroepe en kultuurversamelings gebruik is. Dié stamme is op grond van hul vermoëns om (i) xilose, arabinose en glukose gelyktydig te fermenteer, (ii) by matig tot hoë temperature te funksioneer en (iii) bestand te wees teen hoë inhibitorikonsentrasies wat tydens stoombehandeling geproduseer word, geselekteer.

Die natuurlike weerstand van elk van die bakteriële stamme teen inhibitoriese verbindings wat in hemisellulose hidrolisate aangetref word, is onder anoksiese asook aërobiese toestande getoets. Laasgenoemde is ingesluit ten einde vas te stel of lae suurstofspannings in skudfleskulture die fermentasie van vyf-koolstofsuike negatief beïnvloed, aangesien dié suikers gewoonlik via die pentose-fosfaat weg geassimileer word, en waar lae suurstof tot redoks wanbalanse kan lei. Oor die algemeen was melksuurkonsentrasies onder anoksiese toestande heelwat hoër in fermentasiesop wat met 75% (v/v) hidrolisaat aangevul was. *Bacillus coagulans* P38 het onderskeidelik 4.18 en 20.42 g/L melksuur vanaf die hidrolisate van bagasse en suikerriet oesreste geproduseer terwyl *Bacillus coagulans* MXL-9 onderskeidelik 5.58 g/L en 16.97 g/L melksuur van die twee substrate geproduseer

het. Daarteenoor het *Lactococcus lactis* IO-1 onderskeidelik 8.68 g/L en 17.44 g/L melksuur geproduseer. Die resultate het die belang van bakteriële stamseleksie beklemtoon wanneer relatief toksiese substrate vir die produksie van 'n ekonomies-belangrike platformverbinding soos melksuur gebruik word.

## **Acknowledgements**

Firstly, I would like to give all the glory to God, for the strength, perseverance and abundant blessings, that was graciously provided to me in my times of need. Psalm 121

I would like to thank my supervisor, Prof Johann Görgens, for his patience, leadership and invaluable support during the past 4 years. Your exceptional work ethics and driven nature, have assisted me not only in my studies but also in my working environment as an engineer. Thank you for taking a chance on me as a student and allowing me the opportunity to complete my Masters.

My sincerest gratitude to my sponsors, the National Research Foundation of South Africa and the Counsel for Science and Industrial Research, for the financial support whilst completing my Masters.

To my co-supervisor, Dr Eugene van Rensburg, I will always be grateful for the unwavering moral support that you provided, even when things seemed dire. Your assistance, advice and insights were most valuable. Thank you for always believing in me.

Many thanks to Mrs Levine Simmers and Mr Jaco van Rooyen for your friendly smiles and assistance with the analytical processing of all the hundreds of samples submitted.

To my parents, thank you for never giving up on me. Thank you for the constant support and prayers over the years and always encouraging me to achieve my goals. To my best friends and partners in crime, Maryke and Niel, thank you for always being a phone call away. You were the pillars against whom I could lean when I could no longer stand on my own. You provided me with constant motivation and encouragement. Your prayers, unconditional love and friendship carried me through some of my hardest days.

To all my friends at Process Engineering, Anné, Lia, Sonja, Martin, Bianca and Logan. Thank you for your friendship, encouragement and contribution to my coffee addiction. You made the journey so much more fun.

## List of abbreviations

AH	Acid hydrolysis
CCR	Carbon catabolite repression
CSY	Combined sugar yield
DA	Dilute acid
DM	Dry material
EH	Enzymatic hydrolysis
EMP	Embden-Meyerhof Pathway
LA	Lactic acid
LAB	Lactic acid bacteria
NREL	National Renewable Energy Laboratory
PK	Phosphoketolase
PP	Pentose phosphate
PT	Pretreatment
SB	Sugarcane bagasse
ST	Sugarcane harvesting residues
WIS	Water Insoluble Solids



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## Chapter 1: Introduction

### 1.1 Background

The increase in global energy use and depletion of fossil fuel resources over the last few decades has sparked the search for alternative renewable energy and fuel resources. Global warming due to the increase in greenhouse gas (GHG) emissions and environmental issues have become a major concerns, and steps need to be taken to minimise these impacts (Cherubini, 2010).

The bioconversion of renewable lignocellulosic biomass to biofuels and value-added products has generated interest in several industries. This led to the development of the biorefinery concept, which is based on the maximal use of organic carbon molecules through exploitation of the whole plant to substitute hydrocarbons from fossil-based oil and gas (Kamm and Kamm, 2004). Ideally, the processing of biomass in a biorefinery would attempt to render the term “waste” as obsolete (FitzPatrick *et al.*, 2010). Biorefineries based on renewable feedstocks can be regarded as a direct outcome of the prominent growth and demand for bioenergy, biofuels and biochemicals (Cherubini, 2010).

Lignocellulosic biomass has gained increased interest as an alternative source for production of platform chemicals and renewable energy. Key advantages of this feedstock include its abundance and non-competition with staple food (Octave and Thomas, 2009). The disadvantages of using lignocellulose include limitations of maximum production rates and the limited supply of biomass to meet the demands for energy and fuel (FitzPatrick *et al.*, 2010).

Due to the recalcitrant nature of sugarcane bagasse and harvesting residues, an effective pretreatment is required, which results in a mixture of fermentable sugars (glucose, xylose and arabinose), sugar degradation products (5-hydroxymethylfurfural and furfural) and inhibitory by-products (formic and acetic acid). Pretreatment is not only energy intensive, but the most expensive part of the process, and thus the most efficient process, be it dilute acid or steam, should be used (Behera *et al.*, 2014). Downstream processing is greatly impacted by the pretreatment method. As the type and intensity of the pretreatment process determines how much of the cellulose and hemicellulose can be recovered (Yang and Wyman, 2008). Three proposed optima; namely hemicellulose yield, combined sugar yield (CSY) and glucose yield, have been identified, to describe the overall performance of the combined pre-treatment and enzymatic hydrolysis process. Each optimum provides benefits in terms of lactic acid, ethanol and/or electricity production. In the end, it comes down to an economic optimisation in which various scenarios are evaluated. With the primary focus on lactic acid production, hemicellulose yield is of greatest interest, while other aspects, such as maximum glucose

yield for ethanol production are kept in mind. It is thus important to do comprehensive research to generate the necessary data for each scenario, and use an integrated pretreatment-hydrolysis process, to prove what the advantages would be in producing various biorefinery products at each optimum. The experimental data collected in the thesis will be utilised in subsequent projects for process modelling to assess the economic and environmental impacts of various process scenarios.

Lactic acid can be applied in a variety of industries, such as pharmaceutical, in which lactic acid is a building block compound for production of poly-lactic acid, an environmentally friendly and biodegradable poly-plastic (Ye *et al.*, 2013). Depending on the severity of the pretreatment, by-products pose a significant challenge due to their inhibitory effect on the fermenting organism (Hofvendahl and Hahn-Hägerdal, 2000). Each organism is affected differently by these compounds and thus the sub-lethal toxicity hydrolysate concentration for each organism should be established.

Glucose fermentation for lactic acid production has been extensively investigated and defined in literature (Taniguchi *et al.*, 2004; Ouyang *et al.*, 2013; Xu and Xu, 2014). However, there remains a distinct paucity in the literature where hemicellulose-derived sugars, especially xylose, are converted to lactic acid using bacteria, as few strains can (i) ferment xylose, arabinose and glucose simultaneously; (ii) operate at moderate to high temperatures, and (iii) are tolerant to inhibitor compounds produced from pretreatment conditions.

The present study forms a sub-component of a broader project, namely “Utilising agricultural residue from sugarcane harvesting to produce bio-energy and chemicals in a biorefinery” at the Department of Process Engineering at Stellenbosch University, in collaboration with the Bioenergy and Energy Planning Research Group at EPFL in Switzerland. This study covers part of the experimental work to describe processes included in one scenario of a proposed biorefinery, in which sugarcane bagasse and harvesting residues are used as feedstock for the co-production of lactic acid, ethanol (not evaluated) and electricity (not evaluated). More specifically, this thesis deals with optimisation of dilute acid pretreatment of the selected lignocellulose materials, together with lactic acid production from hemicellulose hydrolysates obtained by steam-explosion pretreatment of sugarcane bagasse and harvesting residues.

The intention of the biorefinery is to co-produce lactic acid and ethanol, whereby lactic acid is produced from hemicellulose hydrolysate and ethanol from cellulose rich solids. Hence the pretreatment must deliver a hemicellulose-rich hydrolysate for lactic acid production and a highly digestible solid for subsequent ethanol production. The experimental data obtained from the three proposed optima would assist in further modelling work (done elsewhere) to determine which is economically and environmentally preferred.

## 1.2 Thesis Outline

The thesis outline can be summarised as follows:

**Chapter 1: Introduction.** This chapter contains background information and context to the study. The aims and objectives for this research are given within the layout of the thesis.

**Chapter 2: Literature review.** The purpose of this review is to provide background on lactic acid production from the hemicellulose hydrolysates of sugarcane bagasse and harvesting residues within a biorefinery context, where other products, notably ethanol, can also be produced. This section focuses on the steps required to use lignocellulosic biomass, such as sugarcane bagasse and harvesting residues, as feedstock for lactic acid co-production with ethanol, by means of pretreatment and fermentation. It introduces lignocellulose material, its chemical composition and structure, and its role in a biorefinery. Conventional pretreatment processes (dilute acid and steam) used for pretreatment of lignocellulosic materials to obtain a fermentable hemicellulose hydrolysate are discussed. Lastly, the production of lactic acid and lactic acid producing microbial organisms are evaluated.

**Chapter 3: Dilute acid pretreatment of sugarcane bagasse and harvesting residues for maximum hemicellulose, glucose recovery and combined sugar recovery.** This research chapter contains the experimental work pertaining to the dilute acid pretreatment screening and optimisation of hemicellulose, glucose and combined sugar yield derived from sugarcane bagasse and harvesting residues.

**Chapter 4: Lactic acid production from hemicellulose hydrolysate generated from sugarcane bagasse and harvesting residue.** This research chapter contains all experimental work dealing with fermentation development to produce lactic acid from hemicellulose-rich hydrolysate derived from steam-explosion pretreated sugarcane bagasse and harvesting residues.

**Chapter 5: Conclusions.** The last chapter summarises the main findings and conclusions of this study.



### 1.3 Aims and Objectives

The main aims and objectives of this study are summarised as follows:

1. Evaluate if sugarcane harvesting residues could serve as a potential feedstock for a biorefinery alongside sugarcane bagasse.
  - a. Determine the compositional differences between sugarcane bagasse and harvesting residues
  - b. Using dilute acid pretreatment establish the pretreatment ranges using conditions identified from literature
  - c. Using dilute acid pretreatment, optimise pretreatment conditions to evaluate whether a hemicellulose-rich hydrolysate (with low inhibitor concentrations) for lactic acid production, and a highly digestible solid residue, for subsequent ethanol production could be obtained (not evaluated).
2. Identify lactic acid bacteria for lactic acid production in a biorefinery from hemicellulose hydrolysate.
  - a. Under micro-aerobic conditions, evaluate xylose fermenting capability of lactic acid bacteria.

Determine the sub-lethality and tolerance of lactic acid bacteria to produce lactic acid under micro-aerobic vs anaerobic fermentation conditions. The effect of oxygen was tested as strict anaerobic conditions at industrial scale are costly and difficult to achieve.

## Chapter 2: Literature review

### 2.1 What is a biorefinery?

The development and implementation of biorefinery processes is of utmost importance to meet the vision towards a sustainable bio-economy. The present biorefinery concept is aimed at utilisation of non-food lignocellulosic biomass to produce biofuels and value-added products. Replacement of petroleum-derived chemicals with those from biomass will play a key role in sustaining the growth of the chemical industry (Menon and Rao, 2012).

The recent imbalance in oil market and hike in fuel costs have initiated a global challenge for biofuel production from lignocelluloses. First generation biofuel derived mainly from food crops creates many problems ranging from net energy losses to greenhouse gas emission to increased food prices (Menon and Rao, 2012). Cost-effective conversion of lignocellulosic biomass is still, to date, a challenging proposition. The main idea behind a biorefinery is to extract more value from lignocellulosic biomass by co-producing chemicals (e.g. lactic acid, succinic acid and furfural) with fuels (e.g. ethanol and butanol) and electricity. Producing only ethanol and electricity from lignocellulose does not provide economically attractive outcomes, and therefore it is necessary to determine if this can be improved by co-production of chemicals such as lactic acid. Sugarcane bagasse and harvesting residue are ideal and abundantly available resources, which can be utilised as feedstock for producing a number of bulk chemicals such as lactic acid and ethanol (Adsul, Varma and Gokhale, 2007).

### 2.2 Lignocellulosic biomass as feedstock for biorefinery

Lignocellulosic biomass is a complex biological material considered to be the most abundant plant biomass (Claassen *et al.*, 1999). 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 (Wyman, 1999; Sanchez and Cardona, 2008). Each constituent (that is, hemicellulose, cellulose and lignin) in plant biomass can be functionalised to produce non-food and food fractions and intermediate agro-industrial products (Octave and Thomas, 2009). Thus, a complete set of specific technologies must be developed to efficiently convert each fraction into value-added products. These fractions can be used directly as desired bio-chemicals or can be converted by chemical, enzymatic, and/or microbial approaches (Menon and Rao, 2012). Conversion of these by-products to high-value co-products will offset the cost of biofuel, improve the production economies of a lignocellulose biorefinery, minimise waste discharge, and reduce the dependence of petroleum-based products (Menon and Rao, 2012). A biorefinery would also offer new economic opportunities

for agriculture and chemical industries through the production of a tremendous variety of chemicals, transportation fuels and energy (FitzPatrick *et al.*, 2010). Conceptually, a biorefinery would apply hybrid technologies from different fields including polymer chemistry, bioengineering and agriculture (Ohara, 2003) to produce these various products.

Bio-ethanol is one of the many products that can be produced from lignocellulosic biomass, and its potential application in the transport sector, make it the most sought-after product that can be derived from biomass (Bailey, 1996). Second generation bio-ethanol is generally produced from the cellulosic component in lignocellulose. However, cellulose is recalcitrant to enzymatic attack and needs to be pretreated before it can be enzymatically hydrolysed and then fermented into ethanol (Chandra *et al.*, 2007; Zhu *et al.*, 2008). Glucose, the hydrolysis product of cellulose, is readily fermentable and, can be converted into ethanol effectively (Diedericks, 2013). Alternatively, ethanol can also be derived from xylose, the main hydrolysis product of hemicellulose. However, the inability of organisms such as *Saccharomyces cerevisiae* to achieve high ethanol yields (Slininger *et al.*, 1985) have redirected research into the use of genetically modified organisms (Eliasson *et al.*, 2000; Hahn-Hägerdal *et al.*, 2001; Erdei *et al.*, 2013).

Corn starch and sugars from sugarcane and beets are currently being used directly for biofuels such as ethanol. Brazil has been using sugarcane as raw material for large scale bio-ethanol production for more than 30 years (Goldemberg, 2007). Bio-ethanol from lignocellulosic biomass such as sugarcane bagasse has been studied for more than two decades, but its production is not economically feasible at industrial scale (Clomburg and Gonzalez, 2010; Rodríguez-Moyá and Gonzalez, 2010). Ethanol is mainly produced from sucrose (Brazil), molasses (India) and corn starch (USA). It is produced, in the rest of the world, from variety of sugar rich crops and also from biomass derived sugar syrups (Adsul *et al.*, 2011).

### 2.2.1 Lignocellulosic biomass as feedstock

Lignocellulosic biomass is the most abundant source of unutilised biomass, and is mainly composed of cellulose, hemicellulose and lignin. It can also contain other minor components such as soluble sugars, extractives, minerals, ash and oil (Wyman, 1999). Several factors affect the actual chemical composition and structure of lignocellulose materials such as variety, environmental conditions, geographic location, tissue, harvest period, agricultural practice, breeding technology, harvest season and maturity (Wang and Sun, 2010; Kim *et al.*, 2011; Larsen, Bruun and Lindedam, 2012). Plant biomass in general consists of 40 % - 50 % cellulose, 25 % - 30 % hemicellulose and 15 % - 20 % lignin and other extractable components (Knauf and Moniruzzaman, 2004). Table 2-1 shows the variation in chemical composition of different lignocellulosic biomass. The effective utilisation of

all three these components would play a significant role in the economic viability of a biorefinery (Menon and Rao, 2012). 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 (Figure 2-1) (Mosier *et al.*, 2005).

Table 2-1: Chemical composition (% (w/w) of different lignocellulosic biomass

Substrate	Cellulose	Hemicellulose	Lignin	Reference
Sugarcane bagasse	43	31	11	DOE (US Department of Energy), (2006)
Sugarcane tops & leaves	30	19	26	Sindhu <i>et al.</i> , (2014)
Corn stover	38	26	19	DOE (US Department of Energy), (2006); Li, Kim and Nghiem, (2010)
Corn cobs	34	32	6	nee' Nigam, Gupta and Anthwal, (2009)
Switch grass	37	29	19	DOE (US Department of Energy), (2006)



Figure 2-1: Organization of lignocellulosic biomass (redrawn from Mosier *et al.* (2005)

### 2.3 Potential of sugarcane bagasse and harvest residues

The constant demand for non-food and feed-based sources resulted in the utilisation of sustainable and cheaper resources for their bioconversion into value-added products of commercial interest through basic routes of microbial bio-conversion (Hatti-Kaul *et al.*, 2007a). With this objective, a variety of products were derived from renewable resources. Due to advancement in the agricultural industries, millions of tons of wastes and by-products are generated each year that have potential as low-cost sources of energy and material (Pandey *et al.*, 2000; Hatti-Kaul *et al.*, 2007b; Somerville *et al.*, 2010; Chandel and Singh, 2011).

Compared to the world's major regions, Sub-Saharan Africa has the greatest bioenergy potential because of large areas of suitable cropland, vast unused pasture land and low crop productivity under

agriculture (Smeets, Faaij and Lewandowski, 2004). The Cane Resources Network for Southern Africa (CARENSA) focused on sugarcane because it is currently the world's most significant energy crop, with much experience exploiting this crop in the Southern African Development Community (SADEC) region (Johnson *et al.*, 2007). In 2012, sugarcane was amongst the top ten food and agricultural commodities produced worldwide (Figure 2-2A) and in South Africa (Figure 2-2B) (FAOSTAT, 2014). In Figure 2-2, it is apparent that sugarcane production is nearly double the closest competing commodity produced, thus strengthening the argument to use it as feedstock.

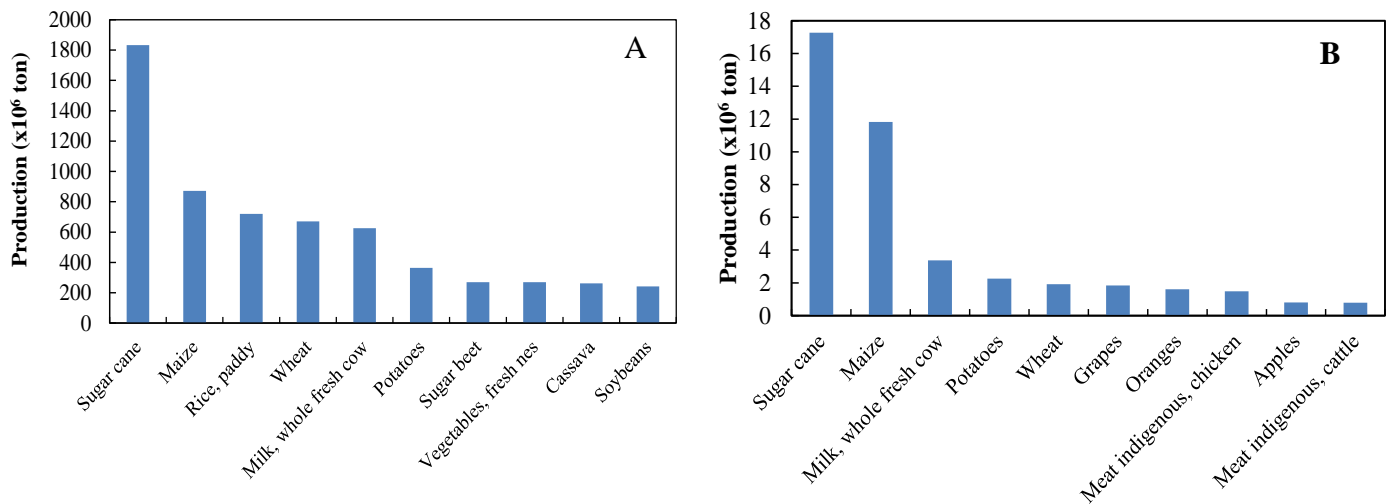


Figure 2-2: Top ten food and agricultural commodities produced (A) worldwide and in (B) South Africa (FAOSTAT, 2014)

With improvements in sugarcane harvesting and co-generation technology, sugarcane bagasse and tops and leaves came to the forefront as important sources of bioenergy (Alonso-Pippo *et al.*, 2009). Sugarcane bagasse is a fibrous residue of sugarcane stalks left over after the crushing and extraction of the juice (Pandey *et al.*, 2000). It is one of the largest agro-industrial by-products as inefficient sugar mills burn most of the bagasse in boilers to for heating energy (Pandey *et al.*, 2000). Generally, 280 kg of bagasse (wet basis) is generated from 1 ton of sugarcane (Soccol *et al.*, 2010). Furthermore, approximately one-third of the energy available from sugarcane is contained in the tops and leaves (referred to as harvesting residues), which are generally burnt prior to harvesting or are not recovered from the field (Smithers, 2014). As a significant quantity of post-harvesting residues is also generated (250 kg dry weight per ton of sugarcane) (Singh *et al.*, 2008), incorporating the sugarcane leaves and tops in the harvesting process, could result in a significant increase in the value that sugarcane can provide for multi-stream exploitation (Bocci, Di Carlo and Marcelo, 2009). The use of sugarcane harvesting residues has the added benefit of not competing as a food source and has similar energy content as bagasse per unit weight, but is frequently burnt off to facilitate harvesting of the stalks

(Alonso-Pippo *et al.*, 2009). Therefore, the bioconversion of bagasse and harvesting residues into value-added products may have sustainable economic and strategic benefits (Chandel *et al.*, 2012) for sugar mills.

## 2.4 Pretreatment methods

Pretreatment is the process of disrupting the naturally recalcitrant structure of lignocellulosic biomass to enable enzymatic hydrolysis of cellulose and hemicellulose to generate fermentable sugars (Yang and Wyman, 2009). The pretreatment step is regarded as the bottleneck, as well as the most expensive part of bioconversion processes due to high energy demand. Depending on pretreatment conditions, significant amounts of sugar degradation products are released that are inhibitory to enzymatic hydrolysis and/or microbial conversions to ethanol or lactic acid (Adsul *et al.*, 2011). On the other hand, effective pretreatment is indispensable to render the feedstock material amenable to enzymatic digestion and/or fermentation.

### 2.4.1 Dilute acid

Pretreatment of lignocellulose to obtain fermentable sugars is an essential step for lignocellulose conversion by microbial fermentation. Acid pretreatment involves the use of concentrated or diluted strong acids to disrupt the rigid, crystalline structure of the lignocellulosic material (Menon and Rao, 2012). In general, dilute acid pretreatment is conducted with acid concentrations ranging from 1 % - 5 % and performed at temperatures of about 160 °C (Sun and Cheng, 2002). Dilute sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) is most commonly used, especially for the commercial utilisation of a wide variety of biomass types. Dilute sulphuric acid has traditionally been used to manufacture furfural (Zeitsch, 2000) by hydrolysing the hemicellulose to simple sugars, such as xylose, followed by dehydration of xylose to produce furfural. Due to its ability to remove hemicellulose, acid pretreatment has been used as part of overall processes in fractionating the components of lignocellulosic biomass (Zhang *et al.*, 2007). Depending on the conditions of the pretreatment, the hydrolysis of the sugars could take from a few minutes to hours (Menon and Rao, 2012). Generally, the pretreatment should promote high product yields in a subsequent enzymatic hydrolysis and/or fermentation operation with minimum cost (Menon and Rao, 2012). It has been demonstrated that the dilute acid pre-hydrolysis can achieve high reactions rates in short time and significantly improve hemicellulose extraction and cellulose hydrolysis (Xiang, Kim and Lee, 2003). However, pretreatment operating conditions must be tailored to the specific chemical and structural composition of the various sources of biomass (Menon and Rao, 2012).

#### 2.4.2 Steam explosion

Steam explosion is typically initiated at a temperature range of 160 °C - 260 °C for several seconds to a few minutes before the material is exposed to atmospheric pressure (Menon and Rao, 2012). The biomass/steam mixture is held for a period to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression (Menon and Rao, 2012).

The steam pretreatment process has been a proven technique for the pretreatment of different biomass feedstocks. It can generate high sugar recovery while utilising a low capital investment and low environmental impacts concerning the chemicals and conditions being implemented and has a higher potential for optimisation and efficiency (Focher, Marzett and Crescenzi, 1991). The difference between 'steam pretreatment' and 'steam explosion' pretreatment is the quick depressurization and cooling down of the biomass at the end of the steam explosion pretreatment, which causes the water in the biomass to 'explode' (Menon and Rao, 2012). During steam pretreatment parts of the hemicellulose hydrolyse and form acids, which could catalyse the further hydrolysis of the hemicellulose. This process, in which the *in situ* formed acids catalyse the process itself, is called 'auto-cleave' steam pretreatment (Menon and Rao, 2012). The role of the acids, is probably not to catalyse the solubilisation of the hemicellulose, but to catalyse the hydrolysis of the soluble hemicellulose oligomers (Hendriks and Zeeman, 2009)

It should, however, be emphasised that, although conventional steam explosion processes are conducted in the absence of an added catalyst, acid catalysts such as sulphurous acid (derived by mixing sulphur dioxide and water), may also be added, similar to the dilute acid process, to limit hemicellulose degradation (Mackie *et al.*, 1985). The main feature that distinguishes dilute acid from steam explosion is their mechanism of heating. Steam explosion process makes use of direct steam injection, whereas heat is transferred to and from the dilute acid process through conduction (Diedericks, 2013).

#### 2.4.3 By-product formation and detoxification

One of the drawbacks of pretreatment is the formation of compounds that can inhibit enzymatic hydrolysis and fermentation. Examples of these inhibitors include aliphatic acids (acetic acid, formic acid and levulinic acid), furan derivatives (furfural and 5-hydroxymethylfurfural) and phenolic compounds (Benjamin, 2014). Furfural and 5-hydroxymethylfurfural (HMF) are formed through chemical decomposition of pentose and hexose sugars, respectively (Neureiter *et al.*, 2002). Furfural and HMF can further breakdown to formic acid as well as levulinic acid (Taherzadeh and Karimi, 2007).

Commonly a detoxification process must be performed to decrease the inhibitors concentration in the hydrolysate generated by pretreatment. This process can be performed in several ways such as evaporation (Larsson *et al.*, 1999), overliming with calcium hydroxide (Larsson *et al.*, 1999) and use of enzymes with phenoloxidase or laccase (Martín *et al.*, 2002), metabolic processes by the yeast (Larsson *et al.*, 1999) and extensive washing (Olofsson, Bertilsson and Lidén, 2008). 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 (Larsson *et al.*, 1999). Overliming lowers the concentration of various by-products, but also results in some sugar loss, whereas phenoloxidase and laccase enzymes remove phenolic compounds (Martín *et al.*, 2002).

## **2.5 Metabolism of C<sub>5</sub> and C<sub>6</sub> sugars and the production of lactic acid**

### **2.5.1 Overview on C<sub>5</sub> and C<sub>6</sub> metabolic pathways**

The metabolism of eukaryotes and bacteria is a complex process that includes the synthesis (anabolism) and breakdown (catabolism) of complex substrates and their intermediates for cell growth and survival (Dobbins, 2010). For this thesis metabolism will mainly refer to catabolism and the breakdown of the simple sugars hexoses (C<sub>6</sub>) and pentoses (C<sub>5</sub>) by bacteria. In bacteria, metabolic breakdown of hexoses initially involves either the Embden-Meyerhof Pathway (EMP) or Entner-Doudoroff Pathway (EDP) (Dobbins, 2010). The EMP is by far the most common pathway used in the first step of hexose metabolism; where-as the EDP is mainly used by certain soil bacteria. Both pathways produce pyruvate as the final product, although the EDP only produces one molecule pyruvate while the EMP produces two (Dobbins, 2010). Alternatively, bacteria can also metabolise pentoses through the Pentose Phosphate Pathway (PP) to produce fructose-6-phosphate or glyceraldehyde-3-phosphate – the precursor of pyruvate. Important to note, the EMP, EDP and PP pathway are all metabolically active under either aerobic or anaerobic conditions (Dobbins, 2010).

During the final stage of metabolism, pyruvate produced from either the EMP, EDP or PPP will either undergo aerobic or anaerobic respiration, or fermentation. In aerobic respiration oxygen is the final electron acceptor where pyruvate is metabolised in the tricarboxylic acid cycle to CO<sub>2</sub>, H<sub>2</sub>O and adenosine triphosphate (ATP) (Dobbins, 2010). This process mostly leads to cellular growth. Alternatively, in the absence of oxygen (anaerobic respiration) electrons can be donated to a variety of other electron acceptors with numerous ecological and practical consequences (e.g. denitrification of NO<sub>3</sub><sup>-</sup>) (Dobbins, 2010). However, these two processes are of little importance to the industrial setting, whereas the process of fermentation is industrially of great importance with the commercialization of products such as ethanol, butanol, butyric, formic, acetic and lactic acid, to name but a few. This thesis however only focuses on lactic acid production.



### 2.5.2 Lactic acid production

Over the last decade the demand for lactic acid (LA) increased substantially due to its favourable properties. As a natural organic acid, lactic acid is widely used in food, pharmaceutical, cosmetic and industrial applications (Abdel-Rahman, Tashiro and Sonomoto, 2011). Biopolymer poly-lactic acid (PLA), which is a promising biodegradable, biocompatible, and environmentally friendly alternative to plastics derived from petrochemicals, is also produced from lactic acid. The use of PLA in surgical sutures, orthopaedic implants, drug delivery systems, and disposable consumer products (Adnan and Tan, 2007), would significantly alleviate waste disposal problems.

Lactic acid is produced commercially either by chemical synthesis or by microbial fermentation. Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation, whereas the remainder is produced synthetically by the hydrolysis of lactonitrile. The chemical synthesis of lactic acid always results in a racemic mixture of lactic acid. Fermentative production of lactic acid offers the advantages in both utilisation of renewable carbohydrates and production of optically pure L- or D-lactic acid, depending on the strain selected (Adsul *et al.*, 2011).

### 2.5.3 Lactic acid bacteria

The efficiency of lactic acid fermentation processes mainly depends on the lactic acid organism, fermentation substrate, and operational modes. Lactic acid can be produced from renewable materials by various microbial species, including bacteria, fungi, yeast, microalgae, and cyanobacteria. Selection of the strain is of foremost importance, particularly in terms of high optical purity of lactic acid and high production capacity. Pure sugars and food crops have been partially replaced by non-food carbohydrates in the fermentation industry in recent years. The use of various low-cost raw materials has been extensively investigated (Budhavaram and Fan, 2009; Laopaiboon *et al.*, 2010; Mazumdar, Clomburg and Gonzalez, 2010; Abdel-Rahman, Tashiro and Sonomoto, 2011; Talukder, Das and Wu, 2012). Another method that reduces the cost of lactic acid production is to improve the production, productivity and yield of lactic acid fermentation. Although batch fermentation is the most widely used in lactic acid production, it suffers from low productivity due to long fermentation times and low cell concentrations. In addition, substrate and product inhibition are also considered major bottlenecks of this fermentation manner. To overcome such problems, fed-batch fermentation, repeated fermentation, and continuous fermentation have been investigated (Abdel-Rahman, Tashiro and Sonomoto, 2013).

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive microorganisms that exist within plants, meat, and dairy products and can produce lactic acid as an anaerobic product of glycolysis with high yield and high productivity. The optimal growth conditions vary depending on

the organisms, since these bacteria can grow in the pH range of 3.5–10.0 and temperature of 5–45 °C (Abdel-Rahman, Tashiro and Sonomoto, 2013). LAB are efficient producers of lactate from the currently used substrates glucose and sucrose, but they are not well capable of converting C<sub>5</sub>-sugars, and require high amounts of complex nitrogen sources, which add significantly to the costs. Most LAB are hetero-fermentative and produce by-products such as ethanol and acetic acid (Abdel-Rahman, Tashiro and Sonomoto, 2013), adding to purification costs and decrease final product yield.

Lactic acid production has also been reported by some *Bacillus* species, including *Bacillus coagulans*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus* sp. (Abdel-Rahman, Tashiro and Sonomoto, 2013). In comparison to LAB, *Bacillus* spp. have several potential improvements to lactic acid production that may help for the reduction of costs in lactic acid fermentation as follows: (i) *Bacillus* ssp. can grow and produce lactic acid by using mineral salt medium with few nitrogen sources instead of expensive media (Wang *et al.*, 2011); and (ii) *Bacillus* ssp. can produce lactic acid in thermal fermentation ( $\geq 50$  °C). These characteristics should give *Bacillus* spp. several advantages over other bacteria, as costs associated with the coolant water after medium sterilization would decrease. And use of *Bacillus* spp. would enable open fermentation using non-sterilized media at higher temperatures than 40 °C (Qin *et al.*, 2009; Zhao *et al.*, 2010).

Table 2-2 below contains an overview of lactic acid producing organisms from literature and compared based on fermentation conditions, medium, substrate type, lactic acid concentration, productivity and yield (g lactic acid produced per g substrate consumed). The organisms evaluated, were selected based on their ability to ferment C<sub>5</sub> and C<sub>6</sub> sugars present in a hydrolysate that had undergone pretreatment. Inhibitor tolerance is a major factor in the selection of the organism, as detoxification will lead to increase in production cost.

Most of the *Bacillus coagulans* strains could ferment in a lignocellulosic hydrolysate. Detoxification was also not required due to the inhibitor tolerance exhibited by most of the strains. *B. coagulans* NL01 simultaneously fermented xylose, glucose and arabinose within first 24 h. It was inhibited by acetic acid (> 15 g/L) and levulinic acid (> 1 g/L). Furfural and HMF did not have a significant negative effect on the strain (Ouyang *et al.*, 2012). *B. coagulans* MXL-9 was tolerant to the presence of furfural (2.5 g/L) and HMF (2.5 g/L) (Bischoff *et al.*, 2010). *B. coagulans* JI12 did not experience glucose repression (i.e. no or little xylose is fermented until all the glucose present is consumed) that is observed in other strains. It was also able to metabolise furfural at concentrations lower than 1.5 g/L to furoic acid and was tolerant to furfural concentration up to 4 g/L and acetic acid concentration up to 20 g/L (Ye *et al.*, 2014).

Table 2-2: Overview of L-lactic acid producing organisms

Organism	T °C	pH	pH Control	Medium additions (L) <sup>1</sup>	Fermentation method <sup>2</sup>	Substrate type <sup>3</sup>	[Substrate] (g/L) <sup>4</sup>	Lactic acid (g/L)	Time (h)	Productivity (g/L/h)	Yield (g/g)	Reference
Extremophiles used for L-lactic acid production												
<i>B. coagulans</i> 36D1 (ATCC PTA-5827)	55	5.0	KOH	2.5 g CSL	SSCF	Sugarcane bagasse hyd. + SF-cellulose	81.3 + 20	36	144	0.6	0.36	Patel <i>et al.</i> , (2005)
<i>B. coagulans</i> 36D1 (ATCC PTA-5827)	50	6.0	KOH	10 g P, 5 g YE	FB	Xylose	100 + 50 + 50	163	216	-	0.87	Ou, Ingram and Shanmugam, (2011)
<i>B. coagulans</i> 17C5 (ATCC PTA-5826)	50	5.0	KOH	5 g CSL	B	Sugarcane bagasse hyd.	60*	55.5	144	0.8	0.89	Patel <i>et al.</i> , (2004)
<i>B. coagulans</i> NL01	50	6.5	CaCO <sub>3</sub>	2.5 g YE	B	Corn stover hyd.	25.45*	18.2	48	-	0.734	Ouyang <i>et al.</i> , (2012)
<i>B. coagulans</i> C106	50	6.0	NaOH	10 YE	B	Xylose	85	83.6	12	7.5	0.98	Ye <i>et al.</i> , (2013)
<i>B. coagulans</i> C106	50	6.0	Ca(OH) <sub>2</sub>	20 g YE	FB	Xylose	120 + 80 + 60	215.7	60	4.0	0.95	Ye <i>et al.</i> , (2013)
<i>B. coagulans</i> MXL-9	50	6.0	NaOH	10 g T, 5 g YE	B	Corn fibre hyd.	50*	40.2	72-106	2.7	0.80	Bischoff <i>et al.</i> , (2010)
<i>B. coagulans</i> JI12 (ATCC PTA-13254)	50	6.0	Ca(OH) <sub>2</sub>	10 g YE	B	Oil palm empty fruit bunch	87.5*	73.9	50.5	1.5	1.09	Ye <i>et al.</i> , (2014)
<i>B. coagulans</i> IPE22	50	5.0-6.0	NA	10 g P, 10 g BE, 5 g YE	SSCF	Wheat straw + cellulose + CSL	26.46* + 20 + 10	38.73	65	-	0.47	Zhang, Chen, Luo, <i>et al.</i> , (2014)
Mesophiles used for L-lactic acid production												
<i>L. pentosus</i> (ATCC 8041)	31	6.0	NaOH	10 g YE, 10 g CSL	B	Corn cob hyd.	46*	24.7	60	0.34	0.53	Moldes <i>et al.</i> , (2006)
<i>L. brevis</i>	30	6.0	Ca(OH) <sub>2</sub>	10 g YE, 10 g P, 6 g BE,	B	Corn cob hyd.	56.9*	39.1	48	0.81	0.7	Guo <i>et al.</i> , (2010)

<i>L. xylosus</i> (ATCC 15577)	30	6.5	NaOH	7 g YE, 3 g P	B	Xylose	31	13	54	-	0.41	Tyree, Clausen and Gaddy, (1990)
<i>L. lactis</i> IO-1	37	6.0	NaOH	5 g polypeptone, 5 g YE,	B	Xylose	51.2	24	38	0.6	0.47	Ishizaki <i>et al.</i> , (1992)
<i>L. lactis</i> IO-1	37	6.0	NaOH	5 g YE	B	Sugarcane bagasse hyd.	32.8*	10.85	64	0.14	0.36	Laopaiboon <i>et al.</i> , (2010)

<sup>1</sup>CSL: corn steep liquor, YE: yeast extract, T: tryptone, P: peptone, BE: beef extract.

<sup>2</sup>SSCF: simultaneous saccharification and co-fermentation, FB: fed-batch, B: batch

<sup>3</sup>hyd: hydrolysate, SF-cellulose: Solka Floc cellulose

<sup>4</sup>\* sugar amount is the total sugar content (glucose, xylose, arabinose, etc.), as reported by the authors in original article.

## 2.6 Gaps in literature

Sugarcane bagasse has been established as a feedstock for use in ethanol and lactic acid generation, however, the harvesting residues have been studied to less of an extent. Most lignocellulosic biomass feedstocks are only optimised for one downstream product to be produced, and not for entire plant utilisation as would be required in a biorefinery. In this study, the focus will be on whether dilute acid pretreatment can be done in a way that maximises both the yield of hemicellulose (and lactic acid produced from it) and the yield of glucose from enzymatic hydrolysis of solids (and the ethanol produced from it). The focus of obtaining the hemicellulose yield will be to generate hydrolysate that is rich in xylose, to be used in lactic acid production. To date, harvesting residues has not been investigated as feedstock for LA production. At large scale, LA is mostly produced from glucose, as most LAB are only capable of fermenting glucose. Hemicellulose hydrolysate is a cheaper alternative as a potential carbon source, but mainly contains xylose. Very few bacteria have been reported that are capable of fermenting xylose as well as glucose and other minor sugars present. Depending on the pretreatment conditions at which the hemicellulose hydrolysate was produced, it would also contain some sugar degradation and inhibitory by-products. These inhibitors could negatively affect the fermentation capabilities of the microorganism used. Hence, the innate tolerance of the cultures will be investigated to determine the sub-lethal hydrolysate toxicity. The hemicellulose hydrolysate will be tested at various volume concentrations and without prior detoxification. At large scale, it is also difficult to achieve strictly anaerobic conditions and hence cultures will be subjected to micro-aerobic and anaerobic conditions. To date, a combination of these factors and the responses, have not been addressed.

## **Chapter 3: Dilute acid pretreatment of sugarcane bagasse and harvesting residues for maximum hemicellulose, glucose recovery and combined sugar recovery**

### **3.1 Abstract**

Sugarcane bagasse (SB) and harvesting residues (i.e. tops and leaves) (ST) were collected from various sugarcane mills across South Africa. These feedstocks were evaluated for potential use in a biorefinery for co-production of ethanol, lactic acid and generation of electricity from residual waste. On a dry mass basis, sugarcane bagasse consisted of 38% glucan, 15% arabinoxylan, 27% lignin, 7% extractives, 9% acetyl groups and 3% ash. In comparison, the harvesting residues consisted of 33% glucan, 17.5% arabinoxylan, 20% lignin, 16% extractives, 5% acetyl groups and 9% ash. A set of pre-screening experiments were performed to evaluate the difference in the feedstock using dilute sulfuric acid. An optimisation study was then executed according to central composite rotatable design evaluating the feedstock response regarding temperature, acid concentration and residence time. Pretreatment with each feedstock was optimised for three distinctly different optima, i.e. maximum hemicellulose, glucose and combined sugar yield, as obtained after pretreatment and enzymatic hydrolysis. At a 95% confidence interval, all three factors had a significant effect in the pretreatment of sugarcane bagasse, whereas, only temperature and acid concentration significantly affected the optimisation of harvesting residues. The three proposed optimal conditions would result in 24.5 g hemicellulose, 32.4 g glucose and 63 g combined sugar yield (/100 g DM) for sugarcane bagasse and 17.4 g hemicellulose, 42.9 g glucose and 66.7 g combined sugar yield (/100 g DM) for harvesting residues.

**Keywords:** Sugarcane bagasse, harvesting residues, hemicellulose, glucose, combined sugar, dilute acid pretreatment

### **3.2 Introduction**

Ever rising fossil fuel cost, depletion of fossil fuel resources (such as coal) and increased awareness in greenhouse gas reduction, has sparked the need to find alternative renewable fuel sources. Lignocellulosic biomass is an abundantly available resource that has been identified as an important feedstock for the production of biofuels and other value-added products, such as lactic acid (Pereira *et al.*, 2015). To compete with fossil fuels from a cost perspective, it is necessary to fully utilise the lignocellulosic biomass and reduce waste generated from the process. This gives rise to the concept of a biorefinery. A biorefinery utilises a wide range of technologies to separate biomass feedstocks

into their precursor components which can be converted to value-added products, biofuels and chemicals, such as ethanol and lactic acid (Cherubini, 2010).

Fermentable sugars are a precursor chemical to be used for platform chemical production such as lactic acid and/or ethanol. Extensive research has been done to optimally obtain the cellulosic sugars generated during pretreatment (Canilha *et al.*, 2011; Benjamin, Cheng and Görgens, 2014; Pereira *et al.*, 2015). However, to successfully utilise the entire sugarcane plant in a biorefinery set-up, it is necessary to also incorporate the hemicellulose sugars released into the hydrolysate to improve overall product yield.

Sugarcane lignocelluloses will be explored here as a feedstock for both lactic acid and ethanol production. Pretreatment (PT) is done to isolate hemicellulose for lactic acid and produce digestible solids for ethanol. Such a process requires simultaneous maximisation of hemicellulose and glucose (from hydrolysis) yields. Other literature reports that it is not possible to maximise both under one set of pretreatment conditions, and that a compromise is always required, that is, maximum hemicellulose yield produces solids with low digestibility, while maximum digestibility is at high severity where a substantial portion of the hemicellulose is degraded. Sugarcane bagasse is a feedstock that is widely used in the production of bio-ethanol, of which US and Brazil are global leaders (*Renewable Fuel Association (RFA)*, 2017). Two of the waste residues generated in the sugar industry are sugarcane bagasse and harvesting residue (i.e. tops, leaves and straw). Sugarcane bagasse is a by-product generated in the process of extracting juice from the sugarcane (Antonio Bizzo *et al.*, 2014). After the sugarcane has been harvested from the plantation, the tops, leaves and straw get left on the field either to be burnt, or used for agricultural purposes as fertiliser (Pereira *et al.*, 2015). For every ton of sugarcane harvested, 140 kg of bagasse and 140 kg of harvesting residues (on a dry basis) is generated (Pippo *et al.*, 2011).

Due to the recalcitrant nature of lignocellulosic biomass, a pretreatment process is required to access the cellulose and hemicellulose to be converted to fermentable sugars. However, the selection of pretreatment process to be used needs to account for sugar-release patterns, solid concentrations and compatibility with overall process and downstream biological application (Yang and Wyman, 2008). Dilute sulphuric acid pretreatment is one of the most widely studied pretreatment methods on a variety of feedstocks (Lloyd and Wyman, 2005; García-Aparicio *et al.*, 2011; Moutta *et al.*, 2012; Uppugundla *et al.*, 2014). Even though extensive research has been done on dilute acid pretreatment on sugarcane bagasse (Neureiter *et al.*, 2002; Canilha *et al.*, 2011; Diedericks, van Rensburg and Görgens, 2013; Benjamin, Cheng and Görgens, 2014), to the author's knowledge, little to no work has been reported in literature for the maximisation of hemicellulose, glucose as well as combined sugar yield from dilute-acid pretreatment of harvesting residue, that include tops, leaves and straw.

In the present work, experimental conditions for dilute acid pretreatment of sugarcane bagasse and harvesting residues were investigated to establish the key differences between the two feedstocks. After which an optimisation study was performed to determine the pretreatment conditions required for maximum hemicellulose, glucose and combined sugar yield for each feedstock. For the design of experiment, a central composite design with response surface methodology was used, incorporating changes of temperature, acid concentration and residence time.

### 3.3 Materials and methods

#### 3.3.1 Feedstock and sample preparation

Sugarcane bagasse and harvesting residues (tops and leaves) were sourced from TSB Sugar (Malelane, Mpumalanga, South Africa) and SASRI (Mount Edgecombe, Kwazulu-Natal, South Africa) to obtain a representative sample of South African SB and ST. The SB and ST originally had a moisture content of ~50 %, where after it was air-dried in a greenhouse to an average moisture content of ~6 %. Following this, the material was separately shredded with a Condux mill (used for SB) or Hammer mill (used for ST) and mixed for even distribution before being stored in a container until needed. SB and ST were further ground and sieved using a centrifugal mill (Retsch ZM 200 basic, Haan, Germany) and vibratory sieve shaker (Retsch AS 200 Basic, Haan, Germany) sequentially, to achieve a particle distribution between 425 – 850  $\mu\text{m}$  suitable for material composition analysis and gram scale pre-treatment. Prior to use, the material was coned and quartered to obtain a representative sample and ensure homogenous mixing. The chemical composition of the raw SB and ST was determined according to the NREL procedure (Sluiter *et al.*, 2011) for biomass analysis (carbohydrates, lignin, ash and extractives).

#### 3.3.2 Experimental setup and operation

The SB and ST were pretreated in tubular reactors, manufactured in house according to specifications of Yang and Wyman (2009) and carried out by using a sand bath heating system described elsewhere (Diedericks, van Rensburg and Görgens, 2013). The tube reactors were submerged into sand bath set at 30 °C above the set point temperature and monitored using a fitted temperature probe. Once the desired reaction temperature was reached, the reactors were immediately transferred to a second sand bath set at the reaction temperature and reaction time was started. Following the required incubation time, the reactors were transferred to a water bath to quench the reaction to room temperature. Finally, the content of the reactors was transferred to a beaker and mixed with 100 mL distilled water. The slurry was vacuum-filtered to separate the wet solids and supernatant. Consequently, the wet solids were washed with 200 mL distilled water to remove excess inhibitors and the pH set to 5. This is referred to as Water Insoluble Solids (WIS). Finally, the WIS was dried in an oven at 30 °C for three



to five days until a constant average mass was observed and enzymatic hydrolysis (Section 3.3.5) could be performed.

### 3.3.3 Dilute sulphuric acid pre-treatment

Dilute sulphuric acid pretreatment was performed in two phases. First, the SB and ST were treated over a wide range of conditions, according to literature (Lloyd and Wyman, 2005; Neureiter *et al.*, 2002; Diedericks *et al.*, 2013; Benjamin *et al.*, 2014), to identify the differences in pretreatment conditions between the two feedstocks. Second, based on the results from phase one, the pretreatment conditions were optimised for each feedstock. Each reactor was loaded with 1.5 g dry material (DM) and compressed with a metal rod to ensure uniform heat and mass transfer during pre-treatment. Five millilitres of dilute sulphuric acid solution was added to each reactor to obtain a solid loading of 30% (w/v) and left to soak overnight at room temperature.

### 3.3.4 Post-hydrolysis of pre-treatment supernatant

The supernatant collected in Section 3.3.2 was analysed according to the NREL procedure (Sluiter *et al.*, 2006) for determining solubilised sugars and by-product formation during pre-treatment. One part of the supernatant was analysed for total monomeric sugars, while the remainder was used to determine the total sugars (oligomers and monomers) through mild acid hydrolysis. The difference between the total monomers before and after acid hydrolysis was indicative of the total oligomer concentration. All post-hydrolysis experiments were completed in duplicate and average results with standard deviation are reported.

### 3.3.5 Enzymatic hydrolysis

To evaluate the effect of pretreatment on the digestibility of the material, SB and ST WIS was enzymatically hydrolysed according to the NREL procedure (Selig, Weiss and Ji, 2008). In short, enzymatic hydrolysis (EH) was performed in 100 mL screw cap Erlenmeyer flasks (30 mL working volume) at a 2% WIS loading (0.6 g dry weight). Each flask contained 0.05 M sodium citrate buffer at a pH 5, supplemented with 0.02% (w/v) sodium azide, to prevent microbial contamination. An industrial enzyme cocktail, Cellic® CTec 2 was kindly provided by Novozymes (Novozymes A/S, Denmark) and added to each flask at an enzyme loading of 0.12 mL/g dry WIS (equivalent to 15FPU/g WIS) (Pengilly *et al.*, 2015). The flasks were incubated at 50 °C for 72 h in an orbital shaker at 150 rpm. Samples were taken at 0 h and 72 h and prepared for sugar analysis. All enzymatic hydrolysis experiments were performed in triplicate and average results with standard deviations are given.

### 3.3.6 Analytical methods

The concentrations of sugar monomers (glucose, xylose and arabinose), cellobiose as well as acetic acid and the by-products formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (5-HMF) were analysed using HPLC. The Aminex HPx-87 column was equipped with a cation-H Micro Guard Cartridge and an AS3000 AutoSampler (all Bio-Rad, Johannesburg, South Africa). The column temperature was operated at 65 °C with 5 mM H<sub>2</sub>SO<sub>4</sub> as a mobile phase at the flowrate of 0.6 mL/min. Sugar concentrations were measured with a RI detector (Shodex, RI-101, Munich, Germany) operated at 45°C. 5-HMF and furfural were analysed on a Phenomenex Luna C18(2) reversed phase column equipped with a Phenomenex Luna C18(2) precolumn (Separations, Johannesburg, South Africa) with column temperature set to 25°C and a flow rate of 0.7 mL/min.

### 3.3.7 Experimental design and statistical analysis

In phase one of the PT experiments, two statistical designs (Table 3-1) were used to evaluate the differences between SB and ST with regards to sugar yields after PT and EH at various PT conditions. The ranges for the independent variables (temperature and time) were selected based on similar studies performed in literature (Neureiter *et al.*, 2002; Lloyd and Wyman, 2005; Diedericks, van Rensburg and Görgens, 2013; Benjamin, Cheng and Görgens, 2014). The purpose of using two sets of experiments was to cover the wide set of conditions. At lower temperatures, longer residence times are required, whereas at higher temperatures, shorter residence times are preferred. The acid concentration was kept constant at 0.5% (w/w) H<sub>2</sub>SO<sub>4</sub>. One-way-analysis of variance (ANOVA) was determined to evaluate the statistical differences.

Table 3-1: Preliminary experimental designs to evaluate difference between sugarcane bagasse and harvesting residues

<b>3<sup>2</sup> Full factorial design</b>			
Temperature (°C)	120	155	190
Time (min)	5	10	15
<b>2<sup>2</sup> Full factorial design with centre point</b>			
Temperature (°C)	120	137.5 (C)	155
Time (min)	20	30 (C)	40

In phase two of the dilute acid PT, a central composite rotatable design (CCRD) with response surface methodology (RSM) was applied to optimise the conditions that would maximise each of the three response variables - hemicellulose (H) and glucose yield after pre-treatment, enzymatic hydrolysis (G) and combined sugar yield (CSY). Temperature (T), acid concentration (c) and time (t) were

specified as the three independent variables. The proposed design comprised of six-star, eight-factorial, and a centre point which tested each of the independent variables at five levels (Table 3-2).

Table 3-2: Range of independent variables for CCRD expressed in terms of natural values

Independent variables	Natural factor levels				
	$-\alpha^a$	-1	0	1	$+\alpha$
T: Temperature (°C)	140	150	165	180	190
c: Acid concentration (% w/w)	0.16	0.3	0.5	0.7	0.84
t: Time (min)	6.6	10	15	20	23.4

<sup>a</sup> $\alpha = 1.682$

A second-order polynomial regression model was used to develop the response surface as fitted to the experimental data. This standard second-order polynomial regression model can be expressed as

$$Y_i = \beta_0 + \sum_{i=1}^3 \beta_i \cdot x_i + \sum_{i=1}^3 \beta_{ii} \cdot x_i^2 + \sum_{i<j}^3 \beta_{ij} \cdot x_i \cdot x_j + \xi \quad (1)$$

where  $\beta$  represents the various regression coefficients which included an intercept ( $\beta_0$ ) and the three different effects namely linear ( $\beta_i$ ), interaction ( $\beta_{ij}$ ) and quadratic ( $\beta_{ii}$ ) effect. The experimental error was expressed as  $\zeta$ . Per ANOVA, the model was adjusted and regression coefficients that were deemed insignificant ( $p > 0.05$ ) were removed. However, to retain the integrity of model hierarchy, some of the non-significant terms were included. ANOVA and CCRD were carried out using STATISTICA (software, version 13).

### 3.4 Results and Discussions

#### 3.4.1 Chemical composition of sugarcane bagasse and harvesting residues

The chemical composition of SB and ST were determined (Table 3-3). Arabinoxylan consists of xylose and arabinose, with xylose the major component. Based on the glucan and arabinoxylan content of each material, the average maximum potential for recovery of monomeric sugars for SB and ST were 60.84 g/100 g DM, and 56.77 g/100 g DM respectively.

Table 3-3: Chemical composition of sugarcane bagasse and harvesting residues (% (w/w), dry basis)

<b>Component</b>	<b>Sugarcane Bagasse</b>	<b>Sugarcane Harvest Residues</b>
Glucan	38.6 ± 1.3	33.2 ± 0.4
Arabinoxylan	15.8 ± 0.5	17.5 ± 1.3
Lignin	27.4 ± 1.4	19.7 ± 3.0
Extractives	6.9 ± 0.3	15.8 ± 0.1
Acetyl groups	8.7 ± 0.3	4.9 ± 0.1
Ash	2.7 ± 0.1	8.9 ± 0.1
<b>Total Mass Closure</b>	<b>88.7 ± 2.8</b>	<b>96.6 ± 4.1</b>

The chemical composition of the SB and ST samples were within composition ranges as reported in literature (Table 3-4). For different samples of SB as reported by other researchers, the glucan content varied between 33.3 to 44.9 g/100 g DM, arabinoxylan content between 15.8 to 24.9 g/100 g DM, and lignin content between 17.8 to 27.4 g/100 g DM. For ST, the content of glucan varied from 29.7 to 37.5 g/100 g DM, arabinoxylan content between 17.5 to 27.5 g/100 g DM, and lignin content from 15.4 to 19.7 g/100 g DM.

The diversity of the compositional data makes it difficult to compare the results as the material composition of lignocellulosic biomass depends on various factors, such as geographical location, variety and breeding, and the analytical methods used to analyse the composition (Canilha *et al.*, 2011; Benjamin, 2014; Szczerbowski *et al.*, 2014). SB is a by-product generated from the sugar processing industry, which introduces additional factors from the original process and adds to variance in composition (Hames *et al.*, 2003). It is also worth noting that ST can be differently defined by various research groups. The current ST being investigated, included the green leaves, dry leaves and the tops of the sugarcane plant. Dry leaves are also known as straw, and sometimes get classified on its own and excluded from the ST group.

Table 3-4: Chemical composition comparison of sugarcane bagasse and harvesting residues

Method	Glucan	Arabinoxylan	Lignin	Acetyl groups	Extractives	Ash	Reference
<b>Bagasse</b>							
NREL	39.6	20.8	22.4	3.2	5	1.3	Benjamin (2014)
NREL	39.1	24.8	18.9	-	6	4	Diedericks (2013)
NREL	33.3	20.9	18.9	4.1	6.8	2.2	Hamann (unpublished)
NREL	43.7	24.9	22.4	3.9	-	2.6	Hamann (unpublished)
NREL	44.9	23.3	19.3	2.6	8.5	1.4	Canilha <i>et al.</i> (2011)
-	36.1	23.6	17.8	-	6.1	2	Martin <i>et al.</i> (2007)
NREL	41.95	21.7	23.61	-	-	-	Gao <i>et al.</i> (2013)
NREL	38.6	15.8	27.4	8.7	6.9	2.7	This work
<b>Harvesting Residues</b>							
NREL	29.74	21.3	15.4	2.8	14.8	7	Hamann (unpublished)
NREL	37.5	27.5	16.7	2.3	11.7	7.1	Mokomele (unpublished)
NREL	33.2	17.5	19.7	4.9	15.8	8.9	This work

The differences in chemical composition as reported for the same feedstock could be attributed to the variability of the feedstock sampling as it was sourced from various location across South Africa, even though care was taken to ensure homogeneity. From a chemical composition point of view, SB and ST have a high glucan content that would be advantageous for ethanol production. The arabinoxylan content of the ST is high enough to be viable for xylose recovery to be used in downstream biological fermentation.

#### 3.4.2 Phase one: Screening of pretreatment conditions to identify suitable operating regimes for SB and ST

From the design of experiment, ANOVA was applied to each of the factorial designs to evaluate the statistical significance of the independent variables (temperature and time) on the response variables (xylose, glucose and CS yield) for each feedstock (data not shown). Two-way linear, quadratic and cross product interactions were considered. Depending on the degrees of significance ( $p < 0.05$ ), effects and interactions were included or removed, however, to retain the integrity of the model hierarchy, some insignificant effects were included.

For ST, the measured xylose yields (/100 g DM) ranged from 0.85 g (5 min, 120 °C) to 9.28 g (10 min, 190 °C). While the total xylose yield ranged from 1.33 g (15 min, 120 °C) to 16.22 g

(40 min, 155 °C) for SB (Figure 3-1). Glucose and arabinose were also present in the pretreated liquor of both feedstock and contributed 4 % to 31% of the total sugar detected (Appendix A-1).

For the xylose yield of SB, temperature (linear and quadratic) and time (linear) were significant ( $p < 0.05$ ) as well as the interaction between the two variables. In comparison, the xylose yield in ST, only temperature (linear) had a significant effect on the model. The maximum xylose yields in the screening were observed either at a moderate temperature with long residence time (155 °C, 40 min) or at a high temperature with a short residence time (190 °C, 5 min). In general, as temperature and time increased, more xylose was released. However, when the pretreatment conditions became too severe, degradation of xylose became significant. The xylose content of SB decreases from 16.22 g to 8.11 g/100 g DM when temperature was increase from 155°C to 190°C.

The effect of the different PT conditions on the WIS was evaluated in terms of glucose yield after EH. The pretreated solids were hydrolysed with a standard enzyme loading of 15 FPU/g WIS. For SB, the measured glucose yields (/100 g DM) ranged from 8.32 g (15 min, 120 °C) to 35.34 g (15 min, 190 °C). The glucose yields for the ST ranged from 11.62 g (5 min, 120 °C) to 23.61 g (15 min, 190 °C) (Figure 3-2).

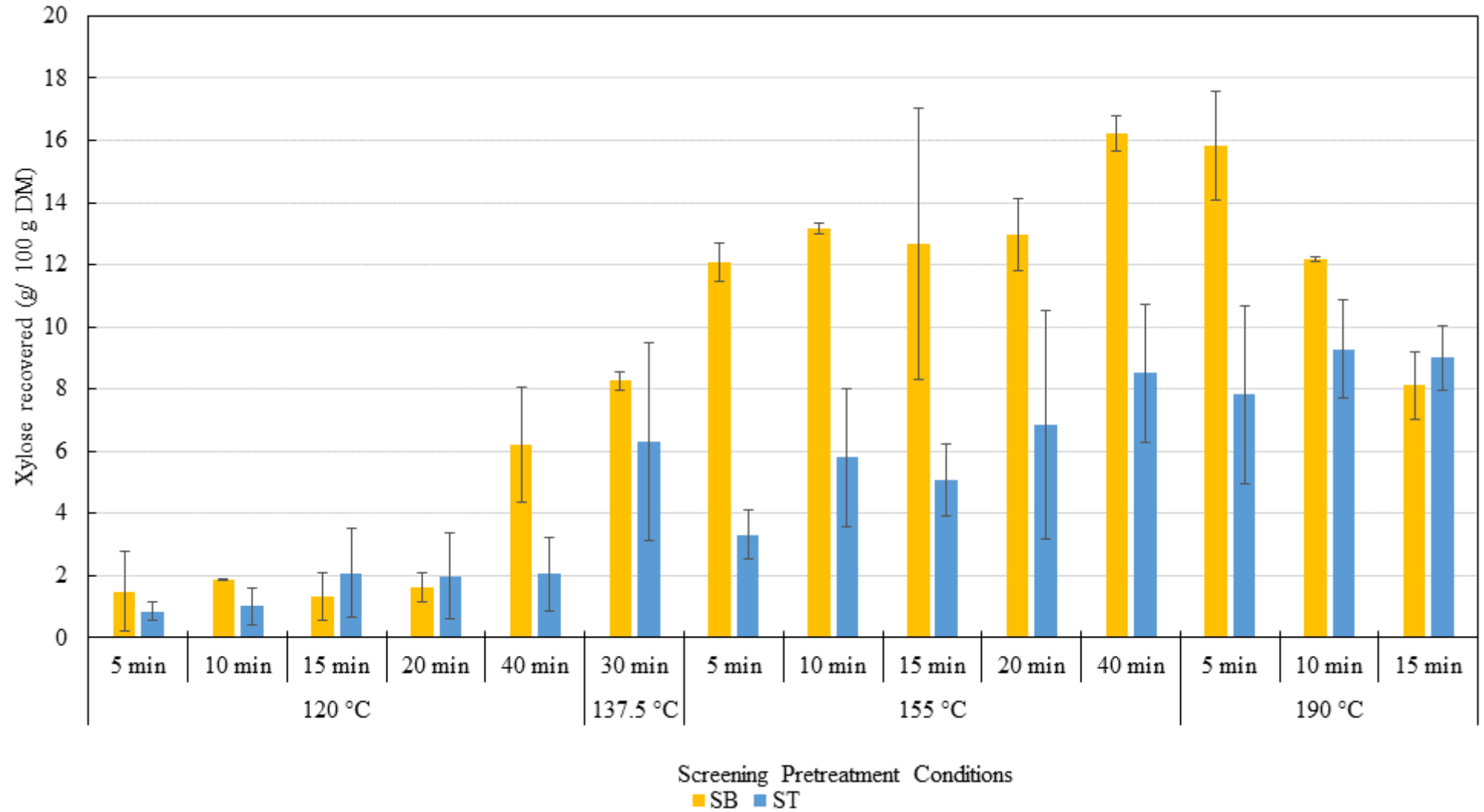


Figure 3-1: Comparison of xylose yield (oligomers and monomers) after pretreatment of sugarcane bagasse and harvest residues at various screening conditions

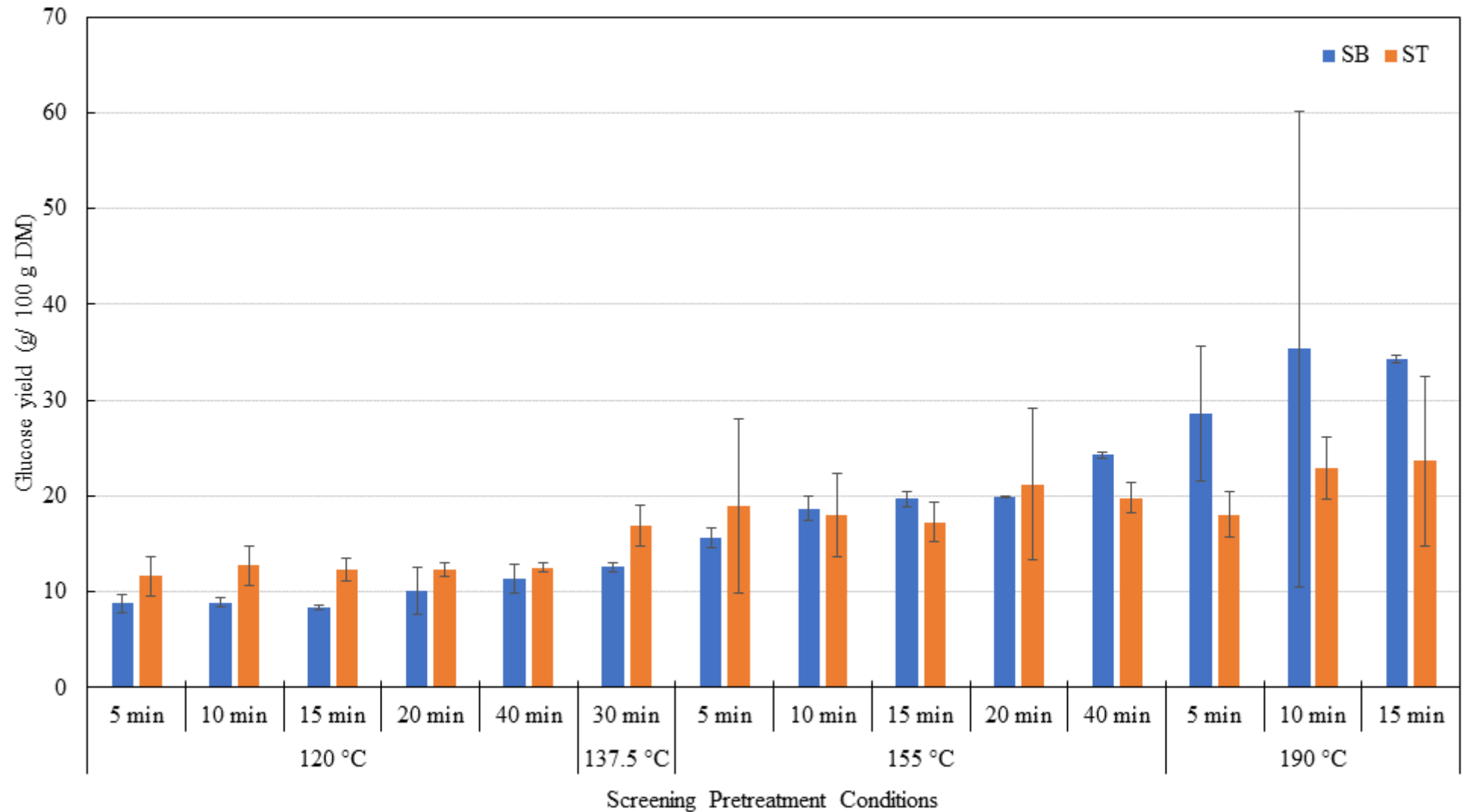


Figure 3-2: Comparison of glucose yield after pretreatment and enzymatic hydrolysis between sugarcane bagasse and harvest residues



From the statistical analysis, the glucose yields in SB were significantly affected by temperature (linear and quadratic) and time (linear and quadratic) and the interaction between the two variables. For the glucose yield in ST, only temperature (linear and quadratic) had a significant effect on the model. However, the linear expression of time was not significant but was included in the model as two of the interactions between temperature and time were significant. The maximum glucose yields for SB and ST in this screening were observed at a high temperature with a moderate residence time (190 °C, 15 min). In general, as temperature and time increased, the digestibility of the material improved as more glucose became accessible to the enzymes to be hydrolysed.

The combined sugar yield (CSY) was calculated by summing all the sugars (glucose, xylose and arabinose) released after PT and EH. The ability to maximise the yields of pentose and hexose sugars, relates to the efficiency of the PT and EH processes. The CSY varied between 14.22 g (120 °C, 15 min) to 52.55 g (190 °C, 15 min) for SB and between 16.75 g (120 °C, 5 min) to 40.78 g (190 °C, 15 min) for ST (Figure 3-3).

The ANOVA results for SB indicated that temperature (linear and quadratic) had a significant effect on the CSY, along with the cross-product interactions between the temperature and time. Time (linear) was however not significant but was included in the model as the interaction were significant. For ST, temperature (linear) and time (linear) were significant and the cross interactions between the variables. The quadratic term of temperature was however not significant but still included. As the temperature increased, the CSY for SB and ST improved, along with an increase in time. A decrease in the CSY was observed at more severe pretreatment conditions, which is attributed to the degradation of the xylose in the liquor fraction.

The presence and significance of quadratic terms in some of the models of the response variables suggest that curvature is present, and that each response variable could be optimised to obtain a maximum response. The surface and contour plots for SB (Figure 3-4) and ST (Figure 3-5) were plotted for each of the response variables. From the surface and contour plots, it is evident that further investigation is needed regarding temperature ranging from 155 – 190 °C and time varying from 10 to 20 min if the three response variables are to be optimised.

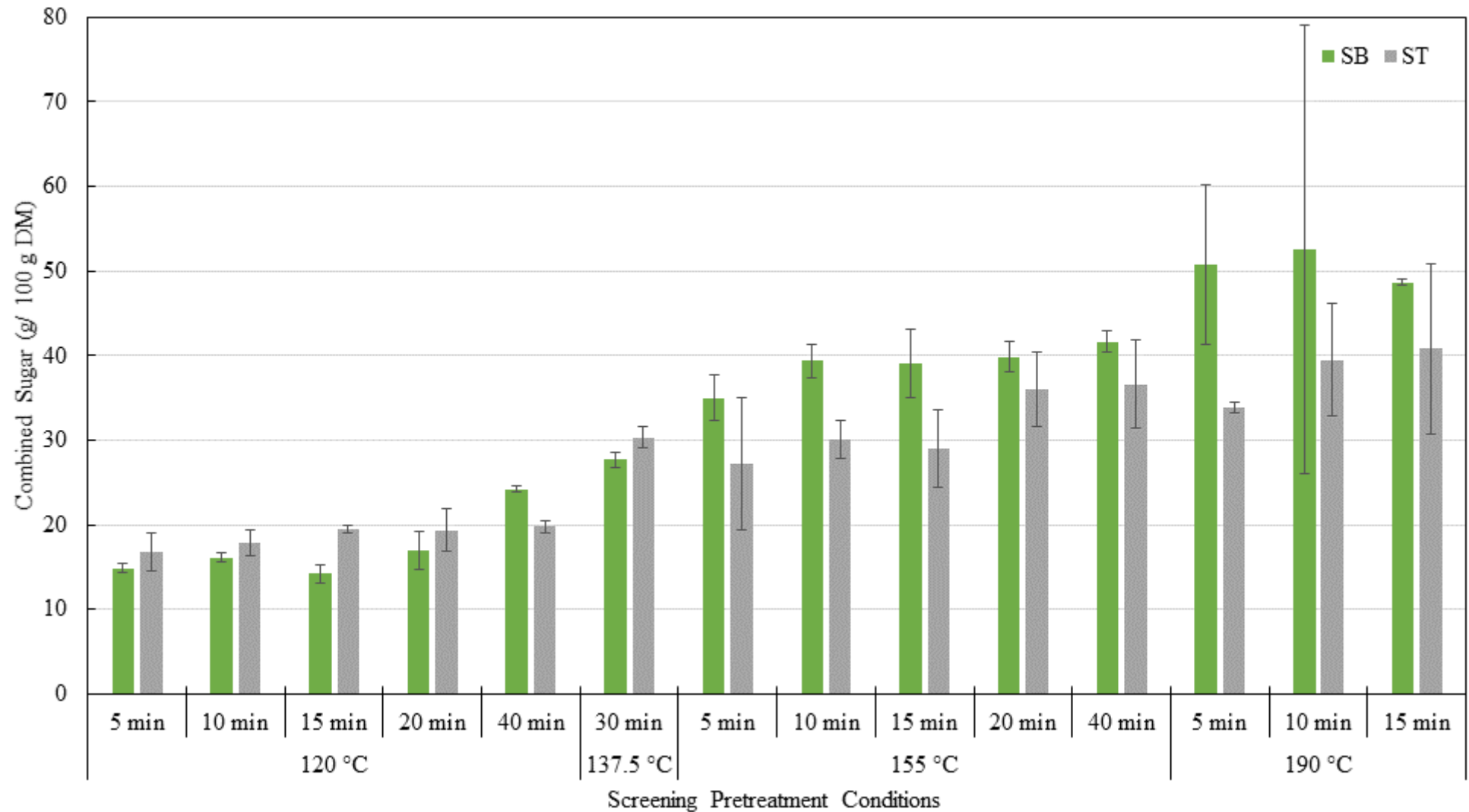


Figure 3-3: Combined sugar yield (glucose, xylose and arabinose) after pretreatment and enzymatic hydrolysis of sugarcane bagasse and harvest residues

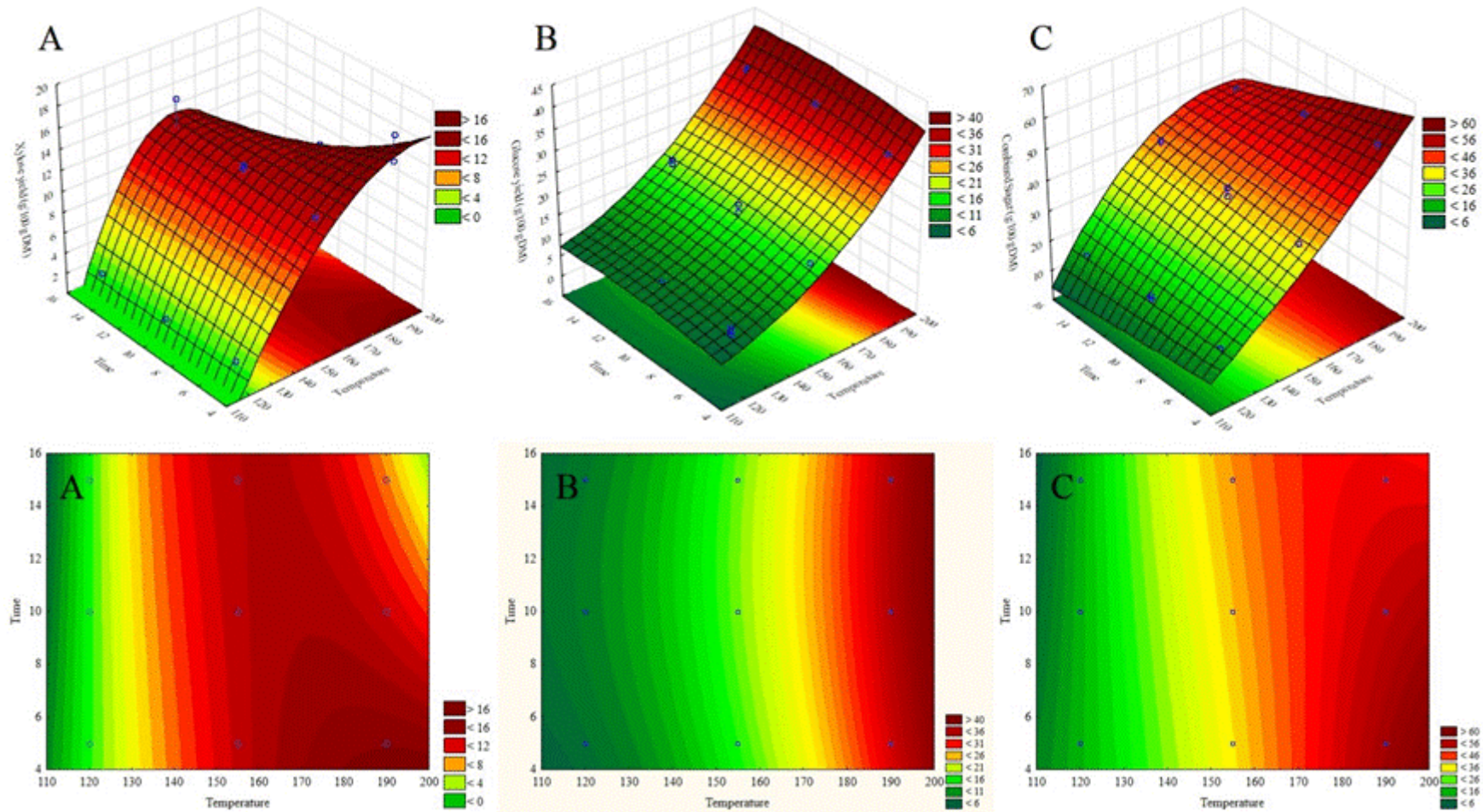


Figure 3-4: The surface and contour plots from the screening experiments of sugarcane bagasse showing the influence of temperature and time on (A) xylose yield, (B) glucose yield and (C) combined sugar yield.

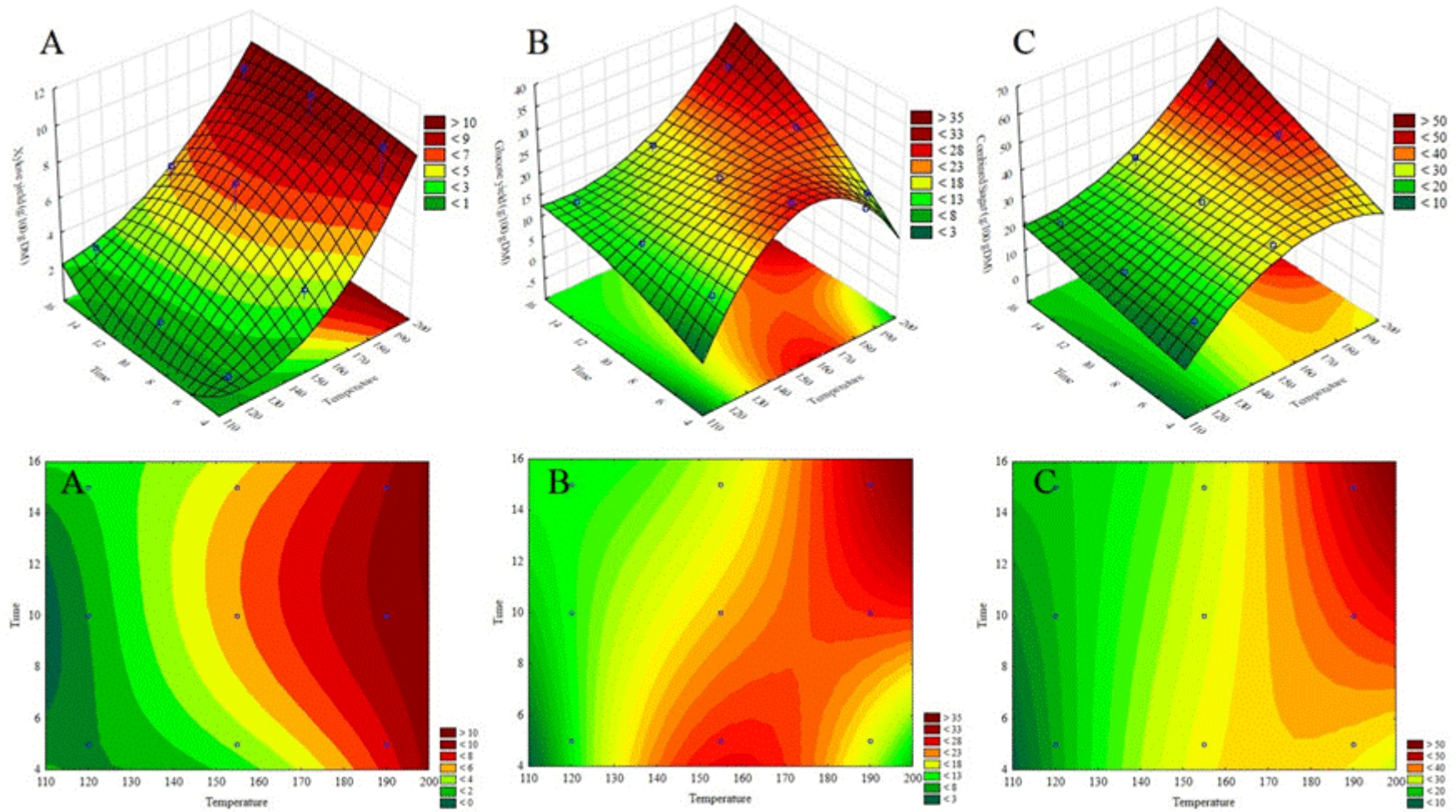


Figure 3-5: The surface and contour plots from the screening experiments of sugarcane harvest residues showing the influence of temperature and time on (A) xylose yield, (B) glucose yield and (C) combined sugar yield.

### 3.4.3 Phase two: Dilute acid pretreatment optimisation of hemicellulose, glucose and combined sugar yield for sugarcane bagasse and harvest residues

#### *Statistical analysis of experimental results*

Based on the findings from Phase One, to optimise the hemicellulose, glucose and combined sugar yield for each feedstock, the dilute acid pretreatment was conducted at different temperatures (140 to 190°C), acid concentrations (0.16 to 0.84% H<sub>2</sub>SO<sub>4</sub>(w/w)) and reaction times (6.6 to 23 min). For each material, a central composite rotatable design was applied, represented by factorial, star and centre points (Table 3-5).

For the response surface methodology (RSM), second-order polynomial equations were fitted to the experimental data of SB and ST (Table 3-6). For SB, the response variables were described by second-order polynomial equations, while for ST, the response variables were best fitted to linear models as only temperature and acid concentration had significant ( $p < 0.05$ ) effects, while time had a marginally significant effect ( $0.05 < p < 0.1$ ).

Table 3-5: Hemicellulose, glucose and combined sugar yield at pretreatment conditions as determined by a central composite rotatable design

No.	Pretreatment Conditions			Sugarcane Bagasse			Sugarcane Harvest Residues		
	Temperature	[Acid]	Time	Hemicellulose yield <sup>a</sup>	Glucose yield <sup>b</sup>	Combined sugar yield <sup>c</sup>	Hemicellulose yield	Glucose yield	Combined sugar yield
	°C	% (w/w)	min	g/100 g DM			g/100 g DM		
<b>Factorial Points</b>									
1	150	0.30	10	11.54 ± 0.01	12.75 ± 1.63	31.82 ± 0.85	2.46 ± 0.02	15.84 ± 0.71	24.81 ± 0.88
2	150	0.30	20	16.73 ± 0.15	13.58 ± 0.96	38.06 ± 0.58	3.73 ± 0.03	20.93 ± 1.25	33.39 ± 1.57
3	150	0.70	10	20.68 ± 4.8	18.21 ± 1.06	45.47 ± 3.26	7.97 ± 0.1	21.30 ± 0.62	38.14 ± 0.73
4	150	0.70	20	27.40 ± 0.73	24.61 ± 2.78	58.48 ± 1.89	12.53 ± 0.38	23.80 ± 1.36	46.09 ± 1.93
5	180	0.30	10	26.17 ± 0.03	32.24 ± 1.86	65.25 ± 0.97	12.24 ± 0.6	29.83 ± 3.7	53.73 ± 5.09
6	180	0.30	20	18.95 ± 0.11	31.27 ± 2.83	56.48 ± 1.68	15.19 ± 0.18	32.85 ± 2.28	58.04 ± 2.08
7	180	0.70	10	21.80 ± 0.04	31.50 ± 3.63	62.59 ± 1.86	18.87 ± 0.67	38.08 ± 1.00	66.62 ± 1.09
8	180	0.70	20	14.80 ± 0.16	29.04 ± 3.53	53.07 ± 1.84	13.34 ± 0.37	39.86 ± 1.03	60.61 ± 2.16
<b>Star Point: Temperature</b>									
9	140	0.50	15	16.35 ± 0.41	14.31 ± 0.37	38.10 ± 0.49	5.92 ± 0.17	13.62 ± 0.44	24.90 ± 0.59
10	190	0.50	15	16.05 ± 0.92	34.08 ± 2.98	58.98 ± 2.08	17.47 ± 1.95	31.27 ± 0.11	54.89 ± 2.43
<b>Star Point: Acid Concentration</b>									
11	165	0.16	15	18.01 ± 0.06	18.19 ± 1.25	44.89 ± 0.82	5.17 ± 0.11	14.49 ± 0.12	26.09 ± 0.28
12	165	0.84	15	20.55 ± 1.93	27.63 ± 4.51	54.06 ± 3.53	19.44 ± 1.00	25.39 ± 0.19	50.81 ± 1.53
<b>Star Point: Time</b>									
13	165	0.50	6.6	23.04 ± 0.23	23.60 ± 0.48	53.89 ± 0.37	6.57 ± 0.29	16.29 ± 0.32	28.92 ± 0.64
14	165	0.50	23.4	22.54 ± 0.48	27.57 ± 0.52	56.73 ± 0.52	14.91 ± 0.98	22.42 ± 0.11	44.17 ± 1.69
<b>Centre Point</b>									
15	165	0.50	15	26.38 ± 0.49	30.95 ± 1.74	64.07 ± 1.13	12.40 ± 0.42	22.07 ± 0.85	41.67 ± 1.16
16	165	0.50	15	25.25 ± 0.79	28.87 ± 0.63	59.15 ± 0.80	11.28 ± 0.29	22.09 ± 2.61	41.46 ± 2.36
17	165	0.50	15	25.03 ± 0.34	29.49 ± 1.31	59.59 ± 0.91	10.16 ± 0.01	18.49 ± 0.03	36.55 ± 0.04

<sup>a</sup> Xylose (mono- and oligosaccharides) and arabinose recovered in the hydrolysate following DA PT. <sup>b</sup> Glucose (mono- and oligosaccharides) recovered following DA PT and EH. <sup>c</sup> Sum of total sugars, xylose, glucose and arabinose, from DA PT and EH.

Table 3-6: Adjusted response surface methodology predictive models for the yields of hemicellulose (H), glucose (G) and combined sugar (CS) for sugarcane bagasse (B) and harvest residues (T)

Response variable g/100 g DM	Adjusted Regression Equation	Coefficient of determination R <sup>2</sup>
<b>Sugarcane Bagasse</b>		
<b>Hemicellulose (Eq.2)</b>	$H_B = -549.82 + 5.53T - 0.013T^2 + 246.02c - 45.49c^2 + 7.14t - 1.18T \cdot c - 0.044T \cdot t$	0.958
<b>Glucose (Eq. 3)</b>	$G_B = -374.85 + 3.64T - 0.009T^2 + 203.75c - 59.20c^2 + 1.86t - 0.057t^2 - 0.81T \cdot c$	0.958
<b>Combined Sugar (Eq. 4)</b>	$CS_B = -871.48 + 8.45T - 0.019T^2 + 385.76c - 93.29c^2 + 12.60t - 0.073t^2 - 1.67T \cdot c - 0.063T \cdot t$	0.974
<b>Sugarcane Harvest Residues</b>		
<b>Hemicellulose (Eq. 5)</b>	$H_T = -115.04 + 0.655T + 91.05c + 2.89t - 0.397T \cdot c - 0.014T \cdot t - 0.65c \cdot t$	0.881
<b>Glucose (Eq. 6)</b>	$G_T = -59.86 + 0.432T + 15.35c + 0.332t$	0.741
<b>Combined Sugar (Eq. 7)</b>	$CS_T = -99.50 + 0.718T + 30.41c + 0.593t$	0.809

Relatively high  $R^2_{adj}$  values (Table 3-6) confirmed a small degree of variation, and implied that variations of the results could be explained by the variables of the process. Despite the lower  $R^2_{adj}$  values for ST, the lack-of-fit for each model was not significant (Appendix A-2). The low  $R^2_{adj}$  values for glucose and combined sugar of ST could be attributed to the fact that the model was constrained by the maximum experimental temperature of 190°C.

The predicted response models were plotted as three-dimensional contour plots and used to optimise for the maximum hemicellulose, glucose and combined sugar yields for SB and ST. Diedericks (2013) proposed a peak theory for the three responses, whereby xylose, CSY and glucose can be optimised individually due to the various conditions of pretreatment required. From the proposed optima for SB (Table 3-7, Figure 3-6 A - C), the peak theory can be applied as three unique pretreatment conditions were obtained for the response variables. However, when the response variables for ST were optimised (Table 3-7, Figure 3-6 D - F), a different response to the peak theory was observed. Each peak of the optimised conditions for ST were within closer proximity of one another compared to SB. Therefore, instead of pretreating the material at three different conditions, pretreating the material at the CSY optimum would be more favourable as this would result in an

acceptable trade-off between hemicellulose yield and readily digestible WIS (Benjamin, Cheng and Görgens, 2014; Agudelo Aguirre, 2016).

Table 3-7: Proposed optimised conditions from predicted response models for hemicellulose, glucose and combined sugar yield for sugarcane bagasse and harvest residues

	<b>Hemicellulose</b>		<b>Glucose</b>		<b>Combined Sugar</b>	
	SB	ST	SB	ST	SB	ST
Temperature (°C)	165	170	186.6	190	180.1	180.1
[Acid] (% w/w)	0.5	0.48	0.5	0.84	0.5	0.61
Time (min)	15	16.7	18.4	23.4	13.3	12.2
Predicted value (g/100 g DM)	24.5	17.4	32.4	42.9	63.0	66.7

From the above results, for SB, different pretreatment conditions are required to obtain maximum hemicellulose and glucose. The pretreatment conditions for maximum CSY, are closer to the glucose optimum as the glucose contribution towards the CSY is higher in comparison to hemicellulose. The compromise between choosing to pretreat material at CSY or maximum hemicellulose would be determined from an economic perspective. The hydrolysate generated at maximum CSY has a higher inhibitor concentration compared to the maximum hemicellulose hydrolysate (Appendix A-4). This could have a negative impact on downstream processing if the hydrolysate were to be used for fermentation purposes, in which case a lower inhibitor concentration would be favoured. More severe pretreatment conditions, especially regarding temperature, were observed for ST.



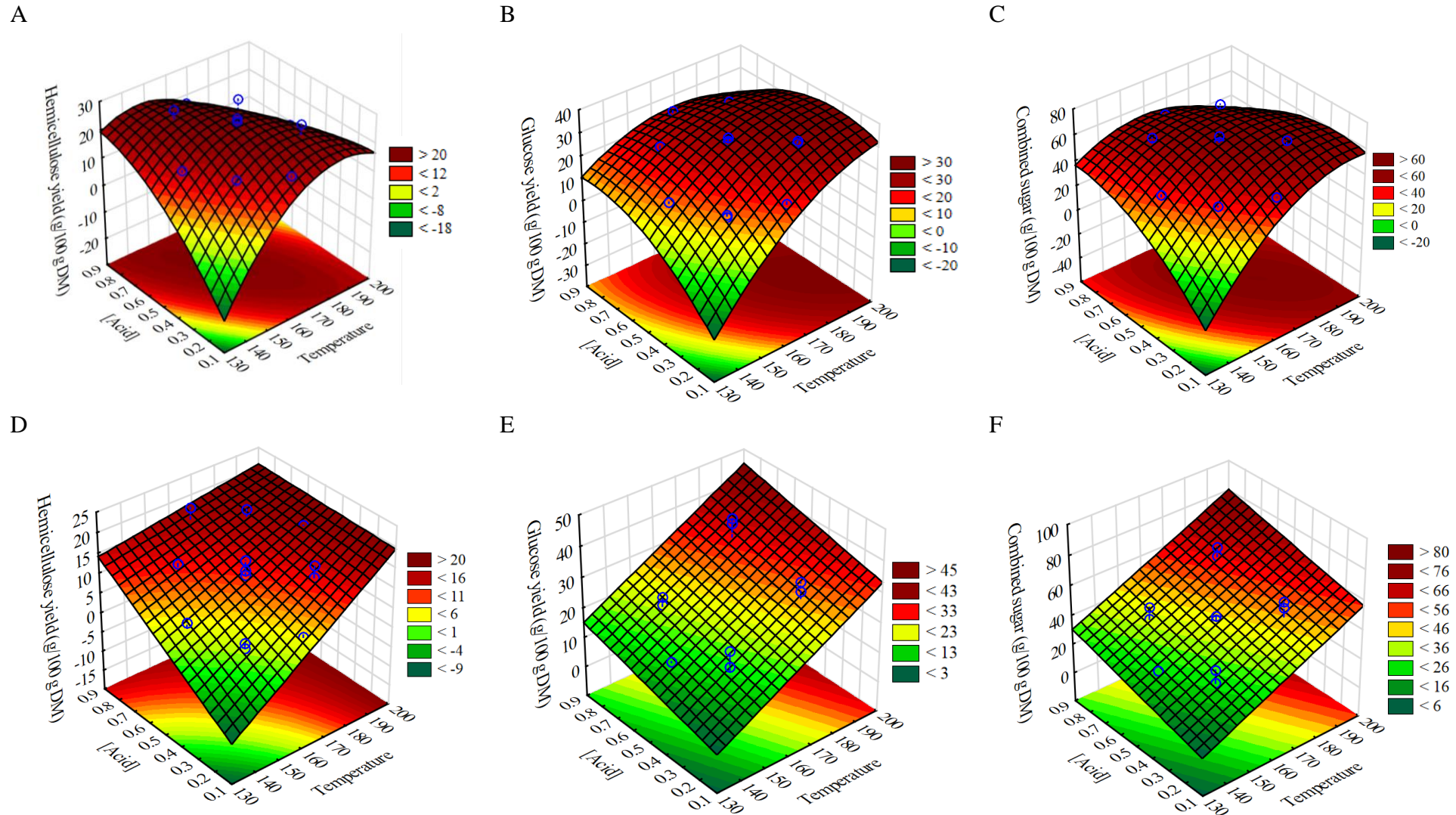


Figure 3-6: Estimated response surface plots for sugarcane bagasse (A-C) and harvesting residues (D-F) as optimised for hemicellulose, glucose and combined sugar yield, showing the influence of temperature and sulphuric acid concentration for a reaction time of 15 min.

*Effect of pretreatment conditions on hemicellulose yield*

The main objective of the acid pretreatment was to obtain the hemicellulose fraction, of which xylose is the major component. The pretreatment conditions brought about changes in hemicellulose yield (/100 g DM) ranging from 2.46 g to 19.44 g for ST and 11.54 g to 27.4 g for SB. Two opposing trends were observed for both feedstocks. At the lower temperatures (< 155 °C), higher acid concentrations (> 0.5% (w/w) H<sub>2</sub>SO<sub>4</sub>) and longer reaction times (> 15 min) were favoured. Whereas at higher temperatures, lower acid concentrations and shorter reaction times were favoured.

The trade-off between the two opposing trends would be seen in the digestibility of the material and the inhibitor concentration due to sugar (xylose and glucose) degradation and the release of acetyl group. Higher temperature reactions favoured an increased digestibility from the resulting WIS but also resulted in higher inhibitor concentrations in the supernatant (Benjamin, Cheng and Görgens, (2014)). Whereas at lower temperatures, a decreased concentration of inhibitors was present in the supernatant, however, the resulting WIS was less digestible.

For ST, the variation between the low and high values in the star point conditions were more noticeable in comparison to SB (Table 3-5). This can be confirmed from the statistical analysis of the experimental data for ST, as temperature and acid concentration were more significant ( $p < 0.05$ ) in hemicellulose released during acid hydrolysis (Appendix A-2, Appendix A-3). For ST, higher acid concentrations (> 0.5% (w/w) H<sub>2</sub>SO<sub>4</sub>) were required to solubilise the hemicellulose fraction, whereas SB favoured low to moderate acid concentrations ( $\leq 0.5\%$  (w/w) H<sub>2</sub>SO<sub>4</sub>).

The higher acid loading requirements of ST could be explained by the higher ash content of the material (Table 3-3). Higher ash content in biomass has been linked to an increase in neutralising capacity of biomass and affects the effectiveness of sugars released during pretreatment (Esteghlalian *et al.*, (1997); Lindedam *et al.*, (2012); Agudelo Aguirre, (2016)).

*Effect of pretreatment conditions on glucose yield after enzymatic hydrolysis*

To evaluate the effectiveness of the pretreatment to deliver a digestible WIS, the glucose yield after EH was measured. The glucose yield (/ 100 g DM) varied from 13.62 g to 39.86 g for the ST, while the glucose yield for SB varied from 12.75 g to 34.08 g. More severe conditions, i.e. higher temperatures, higher acid concentrations and longer reaction times, were favoured to obtain a more digestible material, as can be seen from the increase in glucose yield between the various star points conditions of the CCRD (Table 3-5). However, the increased digestibility of the solids was countered by the decrease in gravimetric material recovery after PT. Solids recovery varied from 60% to 94% for ST and 60% to 89% for SB. The lower solids recovery would negatively impact the overall

process as more raw material would be required to obtain a specific product yield downstream. From the statistical analysis of the data (Appendix A-2, Appendix A-3), the three independent variables had a significant impact ( $p < 0.05$ ) on the glucose yield for SB. For ST, only temperature and acid concentration had a significant effect, while time had a moderately significant effect ( $0.05 < p < 0.1$ ).

In agreement with the present results, Pereira *et al.* (2015) and (Moutta, Ferreira-Leitão and Bon, 2014) reported that sugarcane straw showed a higher enzymatic digestibility than sugarcane bagasse. This could be due to the morphological composition of the feedstocks, as well as the differences in chemical composition (Moutta, Ferreira-Leitão and Bon, 2014).

#### *Effect of pretreatment conditions on combined sugar yield*

The combined sugar yield (/100 g DM) varied from 24.81 g – 66.62 g for the ST, and from 31.82 g – 65.25 g for SB. For SB, the hemicellulose fraction recovered after dilute acid PT contributed 27 – 47% towards the CSY whereas glucose recovered after EH contributed 36 – 58%. Runs 2, 3, 4, and 9 (Table 3-5), where the temperature was below 150°C, resulted in a hemicellulose contribution that was higher than the glucose yield. But as the temperature and severity increased, the contribution from glucose yielded after EH increased; although the digestibility of the material improved, sugar degradation occurred in the hemicellulose hydrolysate fraction. Based on work reported by Benjamin (2014), the potential CSY for industrial SB (collected from TSB mills, Malelane) was up to 50.4 g/100 g raw material and could be increased by up to 34.1% by optimising the pretreatment conditions and improving feedstock selection.

The following was observed from the statistical analysis (Appendix A-2): For SB, the three independent variables had a significant effect on the CSY. A high  $R^2$  of 0.974 indicated that most of the variance in the data could be explained by the model and the lack-of-fit was insignificant. However, for ST, only temperature and acid had a significant effect on the CSY, and even though the  $R^2$  value (0.809) was not as high in comparison to SB, the lack-of-fit was found to be statistically insignificant. The regression model also resulted in a linear model compared to the second-order polynomial for SB (Table 3-7). This could be due to the temperature limitation of the experiment, and a higher acid loading being required due to the neutralisation capacity of ST. The resulting WIS, favoured EH, however, the hemicellulose fraction recovered contained less degradation inhibitors compared to the hemicellulose fraction of the SB. This could be due to the higher acetyl group concentration found in the raw SB (Table 3-3). A decrease in CSY was more prominently observed for SB than for ST, indicating to a negative effect on the hemicellulose fraction and increase degradation of sugars at higher severities.

#### 3.4.4 Upscaling for industrial application

The aim of this study was to evaluate ST as a feedstock for a biorefinery. However, the small scale of the experiments executed in Phase one and Phase two would be impractical to use as basis for industrial application. Thus, the question arises whether the results obtain at gram scale could be directly replicated at bench and/or pilot scale without re-evaluating the entire optimisation study.

Based on the research performed by Benjamin (2014), the experiment can be scaled up to bench-scale using a Parr reactor vessel. It was noted that the Parr reactor required more severe pretreatment conditions (i.e. higher temperature and longer residence time), due to heat and mass transfer limitations. A maximum solid loading of 10% was achievable at bench scale compared to 30% at gram scale used in this study. This was due to mixing problems encountered with the viscosity of the slurry. One solution to the mixing problem could be to use a different impellor that is more suited to a slurry and would ensure homogenous mixing. However, Benjamin (2014) noted that, despite all these differences, the results obtained in the two systems were statistically similar.

### 3.5 Conclusions

One of the main objectives of this study was to investigate the difference between SB and ST, including chemical composition and variations in pretreatment requirements. Along with SB, ST could serve as a potential feedstock to be integrated into a biorefinery. Both feedstocks were optimised for maximum hemicellulose, glucose and combined sugar yield. When evaluating the fermentable sugars released during the dilute acid pretreatment process and after enzymatic hydrolysis, the pretreatment of ST resulted in comparable sugar concentrations to SB. The ST WIS generated across the optimisation study, resulted in > 85% glucose yield after standard enzymatic hydrolysis (based on raw material composition). These sugars can be further used for fermentation of ethanol or LA.

The optimised pretreatment conditions for hemicellulose, glucose and combined sugar of the two feedstocks were within proximity and can potentially be combined and pretreated as one feedstock. In a biorefinery, the pretreatment conditions used will depend on downstream process requirements:

- Maximising for hemicellulose yield obtained in the hydrolysate results in a decreased glucose yield after EH. This would not be favourable for downstream processing such as ethanol fermentation as more enzymes would be required. However, the concentration of inhibitors released during the pretreatment would not render the hydrolysate toxic to biological fermentation, such as lactic acid production.

- Maximising for glucose yield and digestibility of WIS, will result in a hemicellulose hydrolysate with a higher inhibitor concentration and less fermentable sugars. However, higher inhibitor concentrations will be less favourable in simultaneous saccharification fermentation whereby the enzymes and microorganism would need to display innate inhibitor tolerance.
- In the instance of co-generation of ethanol and LA, it would be more ideal to maximise for combined sugar as this results in a digestible WIS with approximately 80% to 90% of glucose yield and a hemicellulose hydrolysate that is still suitable for downstream processing.

## Chapter 4: Lactic acid production from steam-pretreated sugarcane bagasse and harvesting residue hydrolysate

### 4.1 Preface

Hemicellulose-rich hydrolysates generated from steam-explosion and dilute acid pretreatment of the two feedstocks were assessed for viable use of lactic acid production. Based on the HPLC analysis results, the hydrolysate obtained from steam explosion could be used instead of dilute acid (Chapter 3:). The steam explosion had comparable and/or higher sugar concentrations (g/L) as well as inhibitor concentrations (g/L) required for the sub-lethal toxicity testing in this chapter. Due to the small scale of the dilute acid pretreatment, sufficient volumes of hydrolysate could not be generated and hence steam-explosion was used as an alternative to generate the volumes required for fermentation testing.

### 4.2 Abstract

The fermentation performance and tolerance to inhibitors of six different lactic acid bacteria were compared during micro-aerobic and anaerobic grown in hemicellulose-rich hydrolysates from steam-pretreated sugarcane bagasse and harvesting residues. *Bacillus coagulans* P38, *Bacillus coagulans* MXL-9, and *Lactococcus lactis* IO-1 displayed the highest degree of tolerance at a hydrolysate concentration of 75% (v/v), especially under anaerobic conditions. Final lactic acid concentration produced by *B. coagulans* P38 in 75% sugarcane bagasse hydrolysate was 4.18 g/L and in harvesting residue hydrolysate it was 20.42 g/L. *B. coagulans* MXL-9 could produce up 5.58 g/L (bagasse) and 16.97 g/L (harvesting residue) lactic acid and *L. lactis* IO-1 produced 8.68 g/L and 17.44 g/L lactic acid in the respective hydrolysates.

**Keywords:** lactic acid production, lignocellulosic hemicellulose hydrolysate, inhibitor tolerance, lactic acid bacteria

### 4.3 Introduction

Lactic acid (LA) is a valuable platform chemical with widespread application in various industries. The increased interest in the production of LA has largely been driven by the production of polylactic acid (PLA), a biodegradable and biocompatible plastic alternative to petroleum-based plastic (Ye *et al.*, 2013). World demand for LA is estimated to reach 600 000 ton per annum by 2020 and is expected to keep increasing (Dusselier *et al.*, 2013). Over 90% of LA is commercially produced through fermentation (Dusselier *et al.*, 2013) using starch-based materials and refined sugars (mainly glucose) as carbon source. Pure sugar mixtures significantly contribute to the production cost of LA

but also compete with food supply (Hofvendahl and Hahn-Hägerdal, 2000; Ye *et al.*, 2013). Therefore, it is essential to find a cheaper alternative feedstock for feasible LA production.

Lignocellulosic biomass is a cheap, abundant and renewable carbon source, consisting of cellulose (35 to 50%), hemicellulose (20 to 40%) and lignin (10 to 30%) (Saha, 2003). Sugarcane bagasse (SB) and harvesting residues (ST) are by-products of the sugar production industry and can be hydrolysed to fermentable sugars from which LA can be produced. Various pretreatment methods for lignocellulose hydrolysis have been investigated, including dilute acid (Neureiter *et al.*, 2004; Laopaiboon *et al.*, 2010) and alkali pretreatment (Maas *et al.*, 2008; Cui, Li and Wan, 2011), and steam explosion (van der Pol *et al.*, 2015). Hydrolysis of lignocellulose under acid conditions generates hydrolysate rich in pentose sugars, predominantly xylose. However, the harsh conditions to which the cellulose and hemicellulose polymers are exposed during pre-treatment could result in the formation of inhibitory compounds. At high severity treatment conditions, the inhibitory compounds could adversely affect fermentation performance, such as a decrease in product yield and hence, increased cost of production from detoxification (Palmqvist and Hahn-Hägerdal, 2000). Therefore, microorganisms used in hydrolysate fermentation to lactic acid should exhibit two key traits, namely the capability to utilise C<sub>5</sub> and C<sub>6</sub> sugars in the hydrolysate and tolerance towards the inhibitors arising from pretreatment.

Numerous studies have been done regarding the fermentation of LA from glucose and most bacteria are able to ferment glucose. However, very few strains can simultaneously ferment glucose and xylose. From the literature, 12 different strains were identified capable of fermenting xylose under optimal cell culture and anaerobic conditions. Most of these strains are facultatively anaerobic, which implies a higher LA yield at low oxygen tensions where the bacterium would exhibit a fermentative metabolism. However, co-factor imbalances could occur when xylose is assimilated via the pentose phosphate pathway (Kwak and Jin, 2017) under low oxygen conditions (Garde *et al.*, 2002; Tanaka *et al.*, 2003).

The aim of this work was to identify LA producing bacteria (LAB) viable for potential use in industrial application and capable of fermenting pentose and hexose sugars in the hemicellulose-rich hydrolysate generated after steam pretreatment of SB and ST. At large scale production strict anaerobic conditions would be difficult and costly to achieve, along with using refined sugars mixtures as carbon source. The capability of various *Lactobacillus* and *Bacillus* to ferment xylose, was tested under micro-aerobic condition in shake flasks. Based on the xylose pre-screening results, the innate inhibitor tolerance of the selected strains was tested in various SB and ST hydrolysate concentrations. The SB and ST hydrolysates were compared to see if the strains performed differently regarding LA production and inhibitor tolerance.

## 4.4 Materials and Methods

### 4.4.1 Production of steam-explosion hemicellulose hydrolysate

A batch pilot steam-explosion unit (IAP GmbH, Graz, Austria) equipped with a 19 L stainless steel reactor vessel fitted with a 40-bar high pressure boiler delivering saturated steam was used to pretreat SB and ST. The SB and ST were pretreated at conditions resulting in optimal combined sugar yield (CSY), as determined by Hamann (unpublished results). The SB was pretreated at 202.4 °C for 5 minutes and ST was pretreated at 199.6 °C for 9.44 minutes. The pretreated slurry was separated into solid and liquid fractions using a spindel dryer (AEG, GmbH) at 2800 rpm for 15 min.

The hemicellulose hydrolysate generated from steam-explosion pretreatment of SB and ST contained mostly oligomeric sugars, which had to be converted to monomers before use in the subsequent fermentation experiments. The mild acid hydrolysis procedure described by Sluiter *et al.*, (2006) was modified to investigate the effect of post-hydrolysis on the conversion of oligomeric sugars to monomers. The mild dilute acid hydrolysis was optimised by adding 72% H<sub>2</sub>SO<sub>4</sub> to achieve a final concentration of 1% to 4% H<sub>2</sub>SO<sub>4</sub> before being autoclaved at 121 °C for 1 h. The hydrolysate collected was analysed using HPLC for glucose, xylose, arabinose, formic acid, acetic acid, 5-hydroxylfurfural and furfural. Acid hydrolysis was performed for 1 h at 121 °C in an autoclave by adding 72% H<sub>2</sub>SO<sub>4</sub> to a final acid concentration of 1% to 4% (w/v) H<sub>2</sub>SO<sub>4</sub>. The post-hydrolysis was completed in triplicate.

The conversion of oligomeric to monomeric sugars was optimised (data not shown) and post-hydrolysis completed in 500 mL batches. Prior to fermentation, the pH of the hydrolysate was readjusted to pH 6 or 6.5 using KOH pellets (Sigma-Aldrich, South Africa), depending on bacterial strain requirements. Suspended particles were removed from the hydrolysate by centrifugation at 8000 rpm for 10 min and vacuum filtration, before sterilisation using a 0.22 µm nylon filter (Anatech, South Africa).

### 4.4.2 Microorganisms

*Lactobacillus pentosus* (ATCC® 8041™), *Lactobacillus brevis* (ATCC® 367™) and *Lactococcus lactis subsp. lactis* (ATCC® 15577™ renamed *Lactobacillus xylosus* for this study) were acquired from American Type Culture Collection (Manassas, VA). *Lactococcus lactis* IO-1 (JCM 7638) was obtained from RIKEN BioResource Center (Ibaraki, Japan). *Bacillus coagulans* MXL-9 (NRRL B-50549) was obtained from the National Center for Agricultural Utilization Research (United States Department of Agriculture, Agricultural Research Station, Peoria, IL). *Bacillus coagulans* P38 (CGMCC No. 7312) was obtained under a material transfer agreement with the Chinese Academy of



Sciences (Beijing, China). Strains were stored in 0.5 mL aliquots at -85 °C using 18% (v/v) glycerol as cryoprotectant.

#### 4.4.3 Fermentation media

Complete media for cultivation of *L. brevis*, *L. pentosus* and *L. xylosus* consisted of (per litre): 5 g yeast extract, 10 g peptone, 5 g sodium acetate, 2 g sodium citrate, 2 g K<sub>2</sub>HPO<sub>4</sub>, 0.58 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.12 g MnSO<sub>4</sub>·7H<sub>2</sub>O and 0.05 g FeSO<sub>4</sub>·7H<sub>2</sub>O (all from Sigma-Aldrich SA, Kempton Park, South Africa except salts purchased from Merck, Darmstadt, Germany). Basal media used for *L. lactis* IO-1 and *B. coagulans* MXL-9 consisted of (per litre) 5 g yeast extract and 10 g tryptone (Merck). *B. coagulans* P38 was grown in a 10 g/L yeast extract solution (Sigma). All fermentation media were supplemented with 20 g/L xylose. A 100 mM potassium phosphate buffer of pH 6 or 6.5 (See Table 4-1) was added to the corresponding strain media (except the complete media). Media, potassium phosphate buffer and xylose were sterilised as separate solutions and added aseptically to sterilised flasks. To assist with reducing oxygen present in media for anaerobic fermentations, sodium thioglycolate (Sigma-Aldrich, South Africa) (0.02%, w/v) was added to the fermentation media and buffer.

#### 4.4.4 Fermentation conditions

The fermentation temperature and pH of each strain are summarised in Table 4-1.

Table 4-1: Fermentation conditions for bacterial strains

Bacterial strain	Conditions		Literature
<i>Lactobacillus pentosus</i>	37 C	pH 6.0	Garde <i>et al.</i> (2002) Moldes <i>et al.</i> (2006)
<i>Lactobacillus brevis</i>	37 C	pH 6.0	Garde <i>et al.</i> (2002) Zhang and Vadlani (2015)
<i>Lactobacillus xylosus</i>	37 C	pH 6.0	Tyree, Clausen and Gaddy (1990) Sreenath <i>et al.</i> (2001)
<i>Lactococcus lactis</i> IO-1	37 C	pH 6.0	Ishizaki <i>et al.</i> (1992)
<i>Bacillus coagulans</i> MXL-9	50 C	pH 6.5	Bischoff <i>et al.</i> (2010) Walton <i>et al.</i> (2010)
<i>Bacillus coagulans</i> P38	45 C	pH 6.0	Peng <i>et al.</i> (2013) Modified temperature as per personal communication

#### 4.4.5 Fermentation method

##### *Strain selection*

Bacterial strains were inoculated into 10 mL test tubes containing 5 mL medium and incubated for 24 h in an orbital shaker, at 100 rpm, before transferring 4.5 mL of the fermentation broth to a 250 mL flask containing 50 mL of the corresponding medium. During the final culturing step, 40 mL of the fermentation broth was then transferred to 400 mL of media in a 1 L flask to obtain an OD<sub>600nm</sub> reading of 0.1. Strain selection experiments were carried out under micro-aerobic conditions; however, no additional oxygen was added during the fermentation process, and oxygen transfer was limited by using Erlenmeyer flasks with foil cotton plugs and low orbital shaking speeds. Samples were taken at regular intervals for HPLC analysis of residual xylose and LA production. The microbial growth was measured turbidimetrically using a spectrophotometer adjusted to 600 nm. All experiments were carried out in triplicate, and average results with standard deviations are reported.

##### *Determination of inhibitor tolerance*

Under micro-aerobic conditions, 250 mL Erlenmeyer flasks with a working volume of 100 mL were used to determine the inhibitor tolerance of the strains. Colonies grown on MRS plates were transferred to fermentation media with xylose in 100 mL flasks (40 mL working volume) for 24 h, prior to sub culturing into pre-conditioning (PC) flasks containing 25% hydrolysate, supplemented with media and topped with xylose to 30 g/L. The PC flasks were incubated for no longer than 36 h. Cellular growth and pH were continuously monitored. Consequently, each strain in the 25% PC flask was transferred to 50% and 75% PC flasks yielding an initial OD<sub>600nm</sub> of 0.5. The pH was adjusted using 3 N KOH. Triplicate samples were taken every 6 h for HPLC analysis.

For the anaerobic conditions, experiments were conducted in 100 mL serum bottles with a working volume of 80 mL. Strains were cultivated in the same process as described above. Serum bottles were closed with aluminium crimp caps and rubber stoppers (Sigma-Aldrich, South Africa). After cultures were transferred to 25% PC serum bottles, the bottles were sparged with N<sub>2</sub> to remove excess O<sub>2</sub>, before incubating for 36 h. Like the micro-aerobic fermentation, cellular growth and pH were monitored for sugars depletion and the pH adjusted accordingly with 3 N KOH. Consequently after 36 h, cultures were transferred to a falcon tubes and cells pelleted using centrifugation at 8000 rpm for 5 min. The supernatant was decanted to limit the amount of residual sugars and additional fermentation broth transferred to the next set of experiments. From the falcon tubes, each strain was transferred to the 50% and 75% serum bottles and inoculated to an initial OD<sub>600nm</sub> of 0.5. Triplicate samples were taken every 6 h for HPLC analysis.

#### 4.4.6 Analytical procedure

The fermentation broth of the experiments was analysed for glucose, xylose and arabinose as well as weak acids (lactic, formic and acetic acid) and furans (5-HMF and furfural) using high performance liquid chromatography (HPLC) fitted with an Aminex HPx-87 column equipped with a cation-H Micro Guard Cartridge and an AS3000 AutoSampler (all Bio-Rad, Johannesburg, South Africa) at a column temperature of 65 °C using 5 mM H<sub>2</sub>SO<sub>4</sub> as a mobile phase at a flow rate of 0.6 mL/min. A Refractive Index detector (Shodex, RI-101, Munich, Germany) adjusted to 45°C, was used to measure sugar concentrations. 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, was used for the analysis of 5-HMF and furfural.

### 4.5 Results and Discussions

#### 4.5.1 Xylose fermentation: Screening and selection of bacteria

An initial screen was performed to establish a baseline of the xylose fermentation performance to LA by the selected bacterial strains. These baseline fermentations were performed in the presence of oxygen and therefore the bacteria strains favoured cellular growth. Cell growth, pH and fermentation were monitored during the fermentation process. The growth curves for each strain can be seen in Figure 4-1.

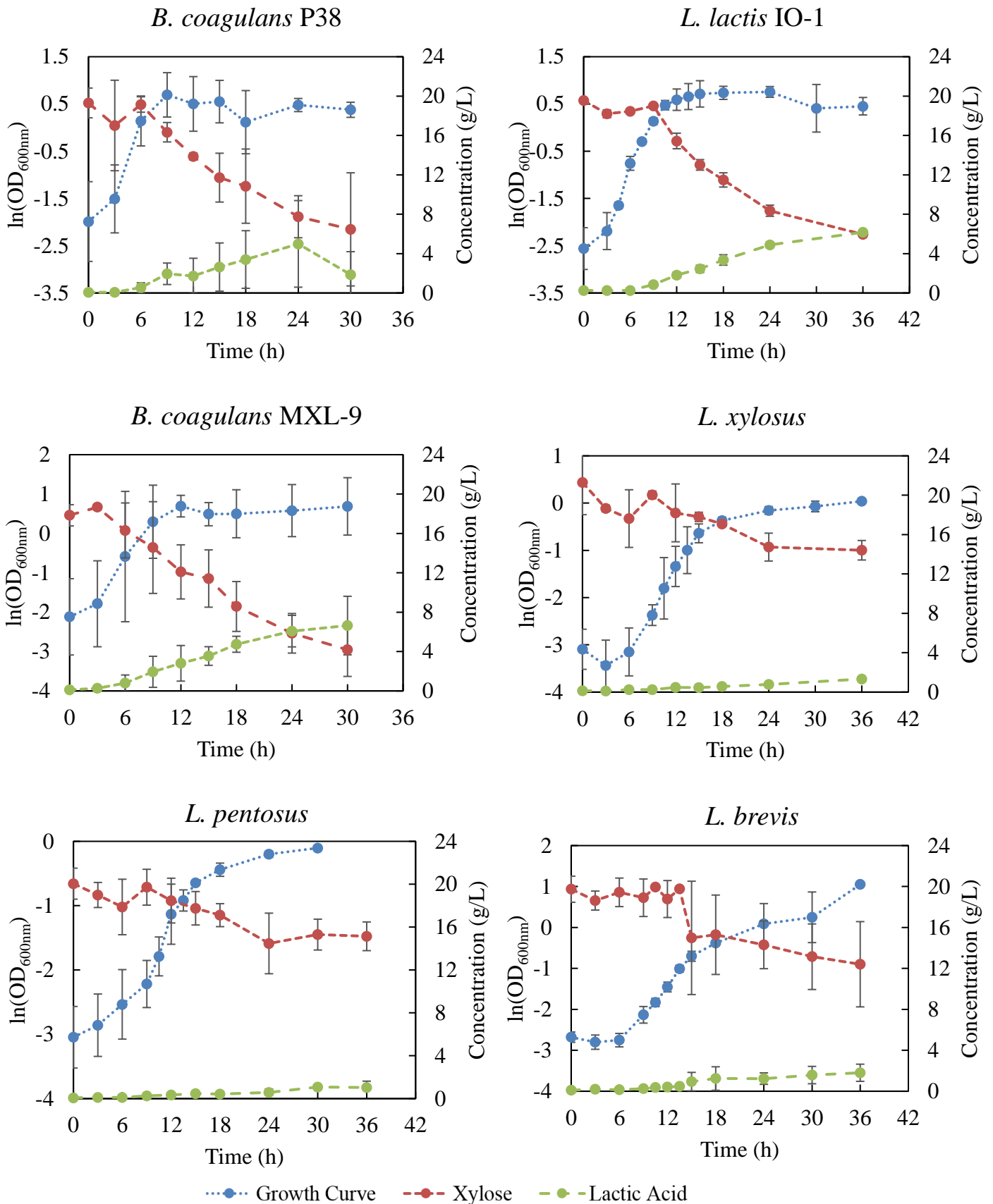


Figure 4-1: Fermentation parameters of the six selected LAB strains. The primary y-axis represents cellular growth and lactic acid production, whereas residual xylose is plotted on the secondary y-axis. Error bars represent the standard deviation of samples of triplicate cultures sampled at each time point.

Cultures of *L. pentosus*, *L. xylosus* and *L. brevis* exhibited poor xylose fermentation performance since less than 2 g/L LA was produced by these strains over a 36 h period. The low levels of lactic acid production by these strains pointed to the requirement for anaerobic conditions to improve the LA yield. By contrast, *L. lactis* IO-1, *B. coagulans* P38 and *B. coagulans* MXL-9 performed substantially better under the micro-aerobic conditions compared to three other strains with LA titres ranging between 5 and 6.6 g/L with up to 14 g/L xylose consumed.

Compared to values in literature, it is evident that each strain's fermentation capability was hindered by the presence of oxygen. Under anaerobic conditions, metabolism of xylose to lactic acid by *L. lactis* IO-1 consumed 93% of the supplied xylose (30 g/L), to produce 13.64 g/L of LA (Tanaka *et al.*, 2003). Similarly, a yield 0.37 (g LA/g xylose) for *L. lactis* IO-1 was reported by Ishizaki *et al.*, (1992) when 24.3 g/L xylose supplied as substrate. In contrast, Garde *et al.* (2002) showed that *L. brevis* could converted 20.9 g/L xylose to 11.5 g/L LA, with a yield of 0.92 (mol LA/mol xylose) based on pentose assimilation through the phosphoketolase (PK) pathway. Under the same conditions, *L. pentosus* attained a yield of 1.13 (mol LA/mol xylose) (Garde *et al.*, 2002). Alternatively, *L. xylosus* under anaerobic conditions produced 13 g/L LA from 31 g/L xylose, with an overall product yield 0.41 (g LA/g xylose) (Tyree, Clausen and Gaddy, 1990). Primary characterisation of *B. coagulans* P38 by Peng *et al.*, (2013) under static conditions with xylose as sole carbon source, resulted in a product yield of 0.89 (g LA/g xylose) from the 80 g/L xylose supplied. Finally, *B. coagulans* MXL-9 produced 18 g/L LA from 20 g/L of xylose within 14 h (Walton *et al.*, 2010). Based on these literature values, *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 were selected for future experiments.

#### 4.5.2 Preparation of steam explosion hydrolysates to liberate monomeric sugars for fermentation

Based on the composition of the hydrolysate after mild dilute acid hydrolysis (Table 4-2), 1% H<sub>2</sub>SO<sub>4</sub> was the optimal final acid concentration. The pre-hydrolysis hydrolysate of the SB contained 8.5 g/L total sugars (glucose, xylose and arabinose) and ST contained 5.2 g/L total sugars. After the application of the mild dilute acid hydrolysis, the total sugar concentration for SB and ST increased to 37.1 g/L and 25.7 g/L respectively. The total inhibitor concentration, however, also increased from 4.526 g/L to 9.974 g/L for SB and from 4.082 g/L to 6.719 g/L for ST. Acetic acid contributed to 76% and 69% of the total inhibitor concentration for SB and ST respectively.

Table 4-2: Pre- and post-hydrolysis composition (g/L) of the sugarcane bagasse and harvesting residue hydrolysate generated from steam-explosion pretreatment using mild dilute acid hydrolysis at 121 °C for 1 h.

<b>Sugarcane Bagasse</b>					
% Acid	0	1	2	3	4
Glucose	0.173 ± 0.001	2.382 ± 0.054	2.719 ± 0.181	2.263 ± 0.647	2.233 ± 0.747
Xylose	7.197 ± 0.544	33.752 ± 0.804	33.245 ± 0.301	30.036 ± 0.270	28.134 ± 1.235
Arabinose	1.133 ± 0.290	0.939 ± 0.003	1.174 ± 0.038	1.161 ± 0.050	1.296 ± 0.105
Formic acid	0.518 ± 0.005	0.651 ± 0.088	0.625 ± 0.004	0.672 ± 0.009	0.721 ± 0.015
Acetic acid	3.095 ± 0.011	7.550 ± 0.132	7.649 ± 0.173	7.442 ± 0.323	7.577 ± 0.098
HMF	0.060 ± 0.000	0.072 ± 0.002	0.055 ± 0.001	0.040 ± 0.002	0.033 ± 0.001
Furfural	0.853 ± 0.003	1.701 ± 0.033	2.320 ± 0.022	2.650 ± 0.074	n.d.
<b>Sugarcane Harvesting Residues</b>					
% Acid	0	1	2	3	4
Glucose	0.163 ± 0.103	2.317 ± 0.061	2.519 ± 0.041	2.590 ± 0.121	2.444 ± 0.004
Xylose	4.127 ± 0.027	21.748 ± 0.057	20.854 ± 0.170	20.520 ± 0.925	18.403 ± 0.094
Arabinose	0.944 ± 0.009	1.655 ± 0.006	1.652 ± 0.018	1.662 ± 0.078	1.549 ± 0.008
Formic acid	0.753 ± 0.005	0.772 ± 0.006	0.812 ± 0.036	0.917 ± 0.059	0.917 ± 0.018
Acetic acid	2.477 ± 0.004	4.629 ± 0.013	4.676 ± 0.044	4.946 ± 0.206	4.893 ± 0.005
HMF	0.084 ± 0.015	0.125 ± 0.008	0.098 ± 0.003	0.081 ± 0.002	0.068 ± 0.003
Furfural	0.768 ± 0.005	1.165 ± 0.026	1.573 ± 0.025	1.775 ± 0.020	2.090 ± 0.090

n.d. – furfural concentration was outside of the HPLC detection range

#### 4.5.3 Tolerance to hydrolysate inhibitors and effect of oxygen on microorganisms

The ability of the strains to ferment xylose under toxic conditions was tested to identify potential LAB for industrial application. To establish the innate tolerance of each strain selected to the presence of inhibitors found in hemicellulose hydrolysate and the ability to ferment in the presence of oxygen, the strains were tested under various conditions. Research has been done regarding the inhibitory effects of acetic acid (Walton *et al.*, 2010; Zhang, Chen, Luo, *et al.*, 2014)), and furfural (Bischoff *et al.*, 2010; Peng *et al.*, 2013; Zhang, Chen, Luo, *et al.*, 2014) on various *Lactococcus* and *Bacillus coagulans* strains. However, to date, very little has been done where detoxification was not applied to the hydrolysate and the effect of oxygen was tested along with the inhibitory compounds, without prior genetic modification or strain evolution. Bacteria were tested under both micro-aerobic and anaerobic conditions. Under each set, the bacteria were also exposed to various hydrolysate concentrations, i.e. 25% (v/v) pre-conditioning (PC), 50% and 75% (remaining volume presents

fermentation media). Each experiment was supplemented with fermentation medium and xylose (Section 4.4.5).

#### *Micro-aerobic fermentation*

*B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 were cultivated under micro-aerobic conditions at two different concentrations of hydrolysate derived from SB and ST. Key fermentation data are shown in Table 4-3 with total sugar consumption and LA production as a function of time of the three strains depicted in Figure 4-2. Generally, *B. coagulans* MXL-9 outperformed the other two strains as evident from a two to four-fold greater concentration of lactic acid production, irrespective of the concentration of the inhibitors supplemented to the culture medium. An increase in the hydrolysate concentration from 50% to 75% only marginally affected LA production as evident from the lactic acid yield from sugars consumed (Table 4-3) as well as from the rate of sugar consumption, evident from time point at which sugars in the culture medium were depleted. These values, however, were quite low, which could be attributed to the presence of oxygen, which probably resulted in an increase in the biomass yield. Finally, cessation in the increase in LA generally coincided with depletion of the total sugars supplied to the culture, which suggested that inhibitors were at a sufficiently sub-lethal concentration and hence, tolerance of the cultures to hydrolysate under aerobic conditions. Product yield coefficient ( $Y_{P/S}$ ) was calculated as total gram LA produced over gram of total measured sugars consumed, i.e. glucose, xylose and arabinose. Maximum theoretical LA production was calculated based on one gram of LA produced per gram sugar consumed.

Table 4-3: Parameters from micro-aerobic fermentation of various hydrolysate concentrations by *L. lactis* IO-1, *B. coagulans* MXL-9 and *B. coagulans* P38. Data represent average  $\pm$  SD (n = 3).

Strain	B/T	Hydrolysate %	Duration	Total Sugars Initial (g/L)	Lactic Acid produced (g/L)	$Y_{P/S}$ (g/g)	q (g/L/h)
IO-1	B	50%	48	23.39 $\pm$ 0.041	4.05 $\pm$ 0.20	0.18 $\pm$ 0.011	0.08
	T	50%	48	16.84 $\pm$ 1.33	4.90 $\pm$ 0.25	0.29 $\pm$ 0.05	0.10
	B	75%	54	24.82 $\pm$ 1.57	2.31 $\pm$ 1.12	0.12 $\pm$ 0.07	0.04
	T	75%	54	24.96 $\pm$ 6.08	4.24 $\pm$ 0.37	0.25 $\pm$ 0.06	0.08
MXL-9	B	50%	54	22.68 $\pm$ 1.96	7.45 $\pm$ 0.54	0.49 $\pm$ 0.06	0.14
	T	50%	54	26.61 $\pm$ 0.47	7.30 $\pm$ 0.18	0.30 $\pm$ 0.012	0.14
	B	75%	54	24.14 $\pm$ 0.41	8.16 $\pm$ 0.58	0.35 $\pm$ 0.02	0.15
	T	75%	54	25.06 $\pm$ 5.73	5.60 $\pm$ 0.13	0.23 $\pm$ 0.009	0.10
P38	B	50%	54	24.17 $\pm$ 0.23	4.86 $\pm$ 0.11	0.21 $\pm$ 0.096	0.09
	T	50%	54	25.50 $\pm$ 0.74	6.14 $\pm$ 0.47	0.39 $\pm$ 0.06	0.11
	B	75%	54	24.45 $\pm$ 0.21	3.64 $\pm$ 0.05	0.14 $\pm$ 0.006	0.07
	T	75%	54	27.80 $\pm$ 0.20	3.96 $\pm$ 0.58	0.24 $\pm$ 0.01	0.07

Table 4-4: Initial inhibitor concentrations present prior to bacterial inoculation of micro-aerobic and anaerobic fermentation experiments. Data represent average  $\pm$  SD (n = 3).

Strain	B/T	Hydrolysate %	Formic acid (g/L)	Acetic Acid (g/L)	HMF (g/L)	Furfural (g/L)
IO-1	B	50%	1.01 $\pm$ 0.005	5.88 $\pm$ 0.02	0.10 $\pm$ 0.001	0.98 $\pm$ 0.01
	T	50%	0.54 $\pm$ 0.01	3.36 $\pm$ 0.02	0.07 $\pm$ 0.002	0.67 $\pm$ 0.00
	B	75%	1.22 $\pm$ 0.25	7.85 $\pm$ 0.31	0.15 $\pm$ 0.005	1.30 $\pm$ 0.02
	T	75%	0.91 $\pm$ 0.10	4.82 $\pm$ 0.40	0.11 $\pm$ 0.01	1.03 $\pm$ 0.13
MXL-9	B	50%	1.06 $\pm$ 0.04	6.05 $\pm$ 0.19	0.10 $\pm$ 0.01	0.91 $\pm$ 0.08
	T	50%	0.71 $\pm$ 0.08	3.91 $\pm$ 0.02	0.08 $\pm$ 0.001	0.72 $\pm$ 0.01
	B	75%	1.58 $\pm$ 0.01	8.51 $\pm$ 0.04	0.15 $\pm$ 0.003	1.33 $\pm$ 0.00
	T	75%	0.97 $\pm$ 0.31	4.59 $\pm$ 0.98	0.10 $\pm$ 0.02	0.94 $\pm$ 0.20
P38	B	50%	1.04 $\pm$ 0.02	5.68 $\pm$ 0.05	0.11 $\pm$ 0.001	0.98 $\pm$ 0.01
	T	50%	0.82 $\pm$ 0.03	3.48 $\pm$ 0.12	0.08 $\pm$ 0.002	0.73 $\pm$ 0.02
	B	75%	1.56 $\pm$ 0.00	8.02 $\pm$ 0.07	0.16 $\pm$ 0.001	1.34 $\pm$ 0.01
	T	75%	1.27 $\pm$ 0.04	4.95 $\pm$ 0.02	0.12 $\pm$ 0.0003	1.10 $\pm$ 0.00



The fermentation curves for the three strains are shown in Figure 4-2 below. The total sugar concentration (glucose, xylose and arabinose) (TS) and lactic acid concentration (LA) were plotted over the fermentation period.

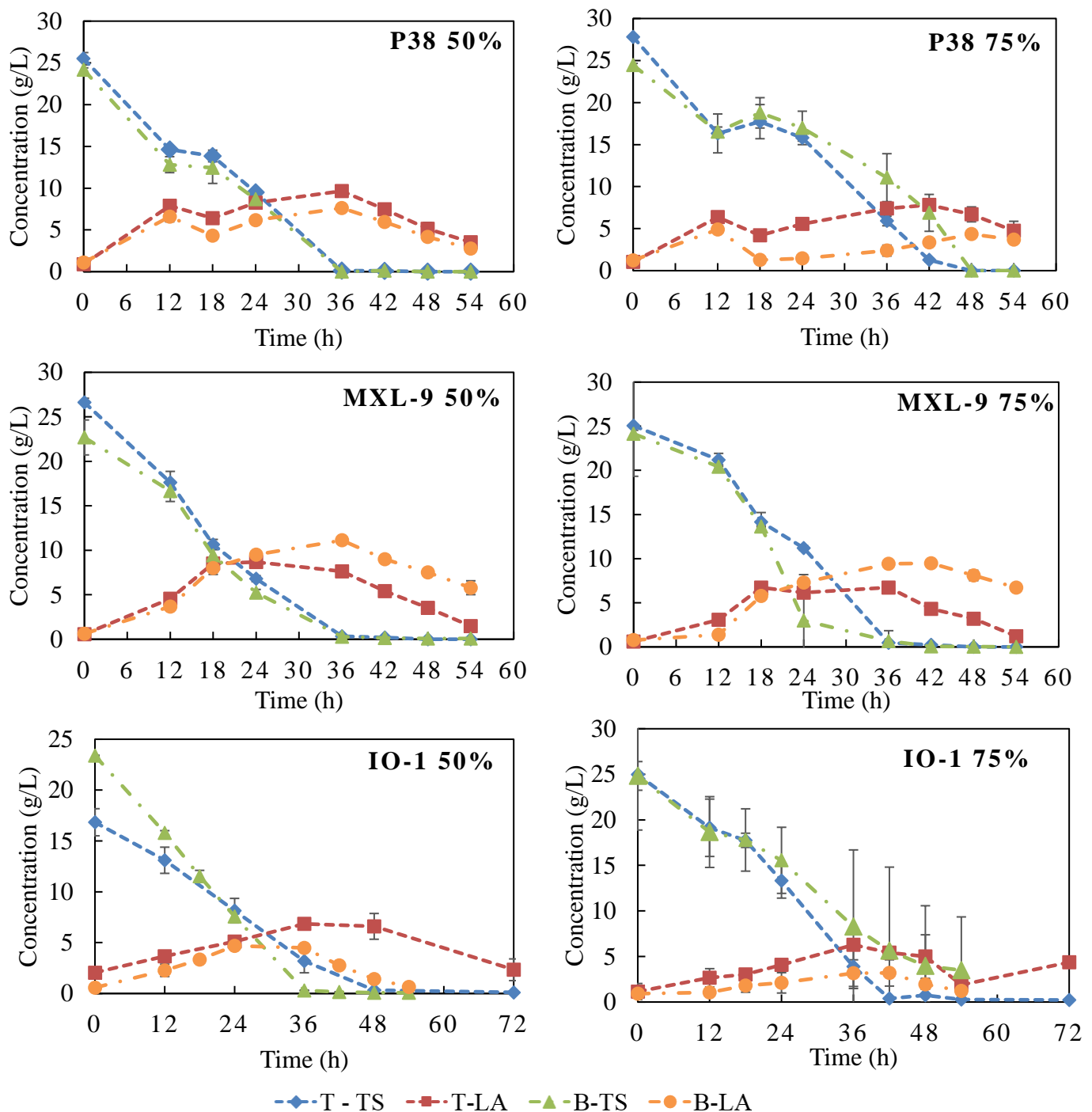


Figure 4-2: Micro-aerobic fermentation curves for *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 at 50% and 75% hydrolysate concentration of sugarcane bagasse hydrolysate (B) and harvesting residues hydrolysate (T). Data represent average  $\pm$  SD (n = 3).

To maximise the LA produced from the hydrolysate, the bacterial strains needed to be capable of utilising all available sugars (Saha, 2003; Peng *et al.*, 2013). Instead most bacteria show a phenomenon called carbon catabolite repression (CCR) that represses the consumption of other sugars in the presence of glucose. To date few bacteria have been reported to be capable of metabolising mixed sugars simultaneously with little to no CCR (Guo *et al.*, 2010; Peng *et al.*, 2013; Zhang, Chen, Qi, *et al.*, 2014). *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 could simultaneously consume the mixed sugars with no noticeable sign of CCR when shifting from glucose/arabinose to xylose.

*L. lactis* IO-1 was more susceptible to the presence of inhibitory compounds in the presence of oxygen. However, the strain performed better in ST hydrolysate at 50% and 75% in comparison to SB hydrolysate, where the LA concentration decreased from 4.05 g/L to 2.31 g/L. Likewise, the product yield coefficients (g LA produced/g TS consumed) were also higher for ST hydrolysate in the 50% (0.29) and 75% (0.25) hydrolysate compared to SB hydrolysate (0.18 and 0.12). At 50% hydrolysate, the strain took 36 h to ferment the available sugars in ST hydrolysate and SB hydrolysate, whereas at 75% hydrolysate fermentation duration increased to 42 h for ST and 54 h for SB.

*B. coagulans* MXL-9 performed better overall when examining the fermentation parameters, in comparison to *B. coagulans* P38 and *L. lactis* IO-1. Product yield coefficients varying from 0.23 to 0.49 (g/g) were observed for the various conditions while LA production ranged from 5.60 to 8.13 g/L. In 36 h the strain metabolised all the available sugars under the various fermentation conditions. *B. coagulans* P38 in comparison only yielded coefficients ranging from 0.14 to 0.39 (g/g) with a LA production content of 3.64 to 6.14 g/L. A similar trend as was observed for *L. lactis* IO-1.

In summary, all three strains showed improved performance in the ST hydrolysate compared to the SB hydrolysate. This could be attributed to lower inhibitor concentrations initially present in the hemicellulose hydrolysate. Nonetheless, all strains could convert the available sugars to LA in the presence of the reported inhibitor concentrations.

#### *Anaerobic fermentation*

Anaerobic experiments were performed as described in Section 4.4.5. *B. coagulans* P38 and *B. coagulans* MXL-9 showed improved performance regarding LA production in comparison to *L. lactis* IO-1 under the ascribed conditions (Table 4-5, Figure 4-3). This can be attributed to the fact that, as from literature, *B. coagulans* P38 and *B. coagulans* MXL-9 are reported as a homo-fermenters, while *L. lactis* IO-1 is a hetero-fermenter. Reports on *B. coagulans* MXL-9 show that it is able to ferment xylose via the PK pathway at low by-product concentrations, however, these are

dependent on culture conditions and the type of hemicellulose hydrolysate used (Bischoff *et al.*, 2010; Walton *et al.*, 2010). In contrast, Tanaka *et al.* (2003) reported that IO-1 utilises two different pathways for fermenting xylose, depending on the available xylose concentration. According to the authors, glucose is homo-fermentatively metabolised via the PP/glycolytic pathway, whereas xylose can either be hetero-fermentatively metabolised via the PK pathway or alternatively homo-fermentatively via the PP pathway. The pathway utilised results in different final LA yields, as the PP/glycolytic pathway results in a theoretical maximum of one gram LA produced per gram sugar consumed, in comparison to the PK pathway where only 0.6 gram LA is produced per gram sugar consumed (Tanaka *et al.*, 2003). Evaluating the results in Table 4-5, the high product yield coefficients ( $Y_{P/S}$ ) ( $> 0.6$  g/g) obtained for the various experiments indicate that the strains metabolised the sugars via the PP pathway.

Table 4-5: Parameters from anaerobic fermentation of various hydrolysate concentrations by *L. lactis* IO-1, *B. coagulans* MXL-9 and *B. coagulans* P38. Data represent average values + SD (n=3).

Strain	SB/ ST	Hydr. %	Total Sugars Consumed (g/L)	Lactic Acid produced (g/L)	$Y_{P/S}$ (g LA/g TS)	q (g/L/h)
IO-1	SB	25%	15.64 ± 0.13	14.47 ± 1.39	0.91 ± 0.11	0.38
	ST	25%	16.08 ± 2.16	14.06 ± 1.12	0.87 ± 0.11	0.40
	SB	50%	16.94 ± 0.73	13.72 ± 0.29	0.85 ± 0.01	0.20
	ST	50%	18.47 ± 1.83	15.48 ± 0.43	0.90 ± 0.03	0.23
	SB	75%	12.21 ± 3.75	7.56 ± 3.76	0.75 ± 0.16	0.12
	ST	75%	19.60 ± 0.33	15.83 ± 1.47	0.92 ± 0.03	0.24
MXL-9	SB	25%	19.00 ± 0.48	16.06 ± 0.81	0.89 ± 0.01	0.48
	ST	25%	20.20 ± 1.12	17.02 ± 0.74	0.95 ± 0.03	0.50
	SB	50%	9.43 ± 0.58	6.70 ± 0.09	0.59 ± 0.03	0.11
	ST	50%	17.82 ± 0.56	20.80 ± 2.33	0.90 ± 0.08	0.29
	SB	75%	8.45 ± 1.65	4.89 ± 0.33	0.51 ± 0.19	0.08
	ST	75%	21.09 ± 0.62	16.12 ± 0.37	0.79 ± 0.04	0.24
P38	SB	25%	18.14 ± 0.45	16.58 ± 0.04	0.87 ± 0.02	0.48
	ST	25%	18.90 ± 0.44	18.48 ± 0.83	0.97 ± 0.06	0.53
	SB	50%	18.83 ± 2.16	14.76 ± 1.48	0.85 ± 0.04	0.19
	ST	50%	20.63 ± 0.16	21.45 ± 1.03	0.98 ± 0.10	0.31
	SB	75%	7.49 ± 1.62	3.39 ± 1.33	0.46 ± 0.20	0.06
	ST	75%	20.71 ± 0.82	19.40 ± 2.67	1.01 ± 0.15	0.28

The fermentation curves for the three strains are shown in Figure 4-3. The total sugar concentration (glucose, xylose and arabinose) (TS) and lactic acid concentration (LA) were plotted over the fermentation period.

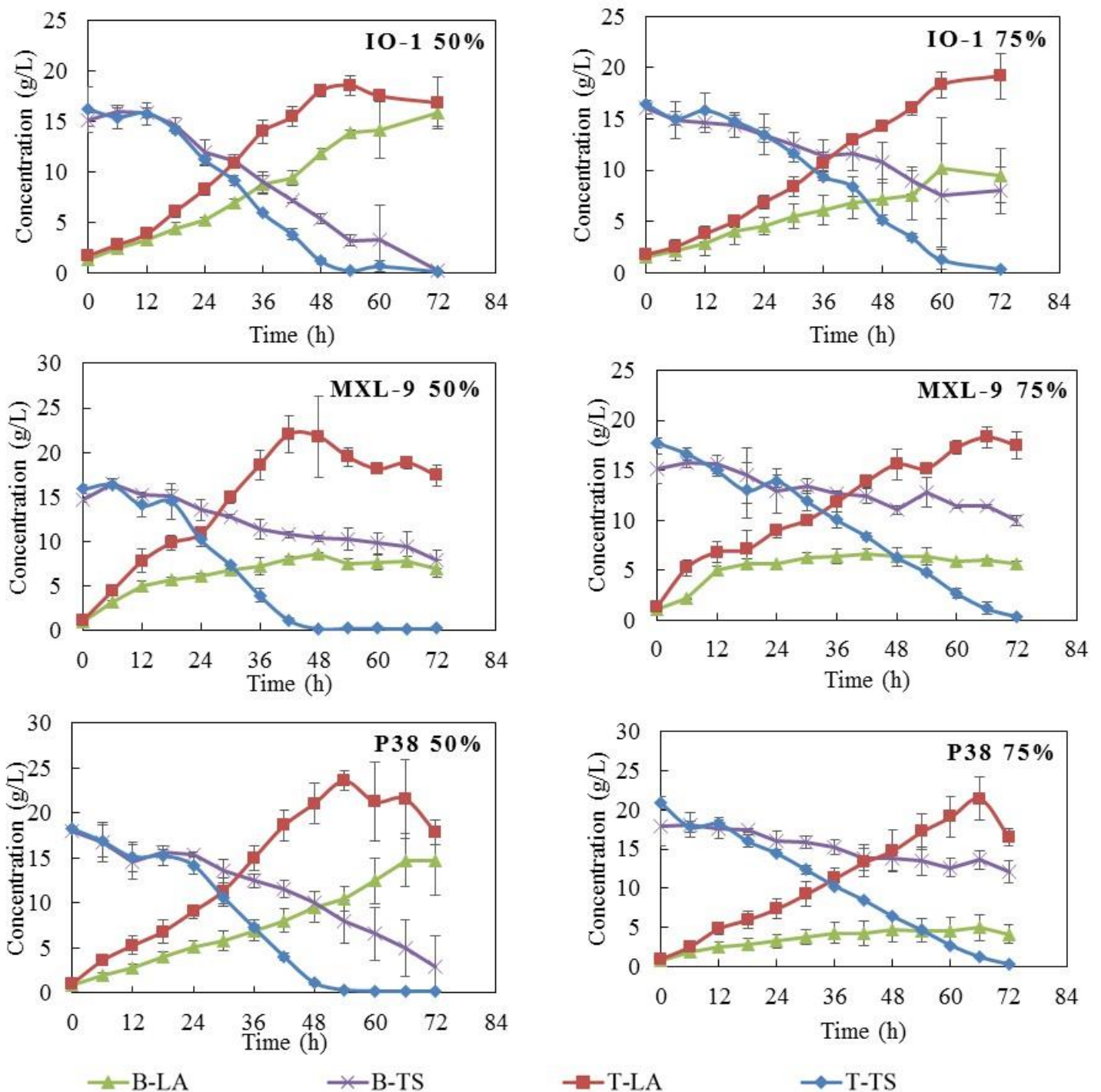


Figure 4-3: Anaerobic fermentation curves for *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 at 50% and 75% hydrolysate concentration of sugarcane bagasse hydrolysate (B) and harvesting residues hydrolysate (T). Data represent average  $\pm$  SD (n = 3).

A noticeable improvement in LA production was observed when comparing results from the micro-aerobic conditions to the anaerobic conditions (Figure 4-2, Figure 4-3). LA production yields for *B. coagulans* P38 and *B. coagulans* MXL-9 drastically improved from 10 to 40% and 49 to 101% respectively, which is comparable to yields reported in literature (Walton *et al.*, 2010; Peng *et al.*, 2013). Almost all the sugars were consumed within 72 h. However, a lower LA production rate is more noticeable in the fermentations using SB hydrolysate, which can be attributed higher inhibitor concentrations present (Figure 4-3).

In comparison to the micro-aerobic fermentation conditions (Table 4-3), the higher LA production and product yield coefficients for *L. lactis* IO-1 (Table 4-5) across the various anaerobic experiments are indicative that the strain consumed xylose via the PP/glycolytic pathway whereby a product yield of 1.0 g LA/g xylose is obtained (Tanaka *et al.*, 2003). From previous research conducted with *L. lactis* IO-1, it has been reported by Ishizaki *et al.*, (1990) that the strain can grow in both anaerobic and microaerophilic conditions. Under anaerobic conditions as reported by Ishizaki and Ueda, (1995), a yield coefficient of 0.404 (g LA/g Xyl) was reported in the presence of 5.43 g/L acetic acid concentration. The xylose and acetic acid concentration used in the present study were similar to those reported by Ishizaki and Ueda, (1995), however, xylose was the only carbon source and acetic acid was the only inhibitor present during the fermentation.

The ST hydrolysate had a lower concentration of inhibitors present especially acetic acid compared to SB hydrolysate (Table 4-4). Therefore, the strains performed better in the ST hydrolysate compared to SB hydrolysate. To date, little has been reported on the use of ST hydrolysate for use in LA fermentation. Furthermore, a decrease in furfural and 5-HMF concentrations was observed throughout the micro-aerobic and anaerobic fermentations experiments. This could be indicative of the bacteria metabolising the inhibitors, as was observed by Walton *et al.*, (2010) when using *B. coagulans* MXL-9.

Similar research to the present work was published in 2016 by Jiang *et al.* whereby *B. coagulans* NL01 was modified through atmospheric and room temperature plasma mutation and evolution experiments using undetoxified condensed dilute-acid hydrolysate. These experiments resulted in an inhibitor-tolerant strain, *B. coagulans* GKN316. The strain could produce LA under aerobic and anaerobic conditions and in the presence of inhibitory compounds. This work highlights the importance for pre-cultivation of strains prior to use in fermentation as it could assist with adaptation and consequently improved inhibitor tolerance. However, compared to the work from Jiang *et al.* (2016) no long term strain adaptation or evolutionary work was performed on the three selected bacterial strains used in the present study. The hemicellulose hydrolysate used was also not concentrated prior to use in fermentations, nonetheless, it is interesting to note that *B. coagulans*

MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 were all able to produce comparable results to *B. coagulans* GKN316.

In summary, *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 could ferment in ST and SB hydrolysate with minimal signs of inhibitor inhibition and deliver a high LA product yield (> 0.6 g/g). This could be attributed to the shift in metabolic pathways whereby LA fermentation was produced via the PP pathway.

#### **4.6 Conclusion**

Six strains were obtained from various research groups and culture collections. All could ferment xylose in the presence of oxygen. *B. coagulans* MXL-9, *B. coagulans* P38 and *L. lactis* IO-1 were selected for further experiments based on higher LA production in the presence of oxygen, to determine innate inhibitor tolerance. *B. coagulans* P38, *B. coagulans* MXL-9 and *L. lactis* IO-1 could ferment sugars under micro-aerobic and anaerobic conditions and in the presence of inhibitors found in the hydrolysate generated from sugarcane bagasse and harvesting residues. Furthermore, sugarcane harvesting residues is a novel feedstock investigated for the use of lactic acid production and could be a promising addition to be used alongside conventional sugarcane bagasse. Finally, *B. coagulans* MXL-9 and *B. coagulans* P38 are promising thermophiles that could be investigated for industrial use.

#### **Acknowledgements**

The author of this work is grateful to Chinese Academy of Sciences (Beijing, China) and the National Center for Agricultural Utilization Research (United States Department of Agriculture, Agricultural Research Station, Peoria, IL) for kindly providing *Bacillus coagulans* P38 and *Bacillus coagulans* MXL-9 under material transfer agreement.

## Chapter 5: Conclusions

This study formed part of a broader group project that investigated various scenarios of a biorefinery. Sugarcane bagasse and harvesting residues are used as feedstock for the co-production of lactic acid, ethanol (not evaluated) and electricity (not evaluated). The experimental conditions for dilute acid pretreatment of sugarcane bagasse and harvesting residues were investigated to establish the key differences between the two feedstocks. More specifically, dilute acid pretreatment was optimised for maximum hemicellulose, glucose and combined sugar yield. Lactic acid production from hemicellulose hydrolysates obtained by steam explosion pretreatment (separate study) was also investigated. The innate inhibitor tolerance of various *Lactobacillus* and *Bacillus* were tested at micro-aerobic and anaerobic conditions.

The conclusions from this study are given below with reference to the aims and objectives given in Section 1.3.

### *Differences in chemical composition and sugarcane harvesting residues as a feedstock*

The feedstocks were evaluated for potential use in a biorefinery for co-production of ethanol, lactic acid and generation of electricity from residual waste. On a dry mass basis, sugarcane bagasse consisted of 38% glucan, 15% arabinoxylan, 27% lignin, 7% extractives, 9% acetyl groups and 3% ash. In comparison, the harvesting residues consisted of 33% glucan, 17.5% arabinoxylan, 20% lignin, 16% extractives, 5 % acetyl groups and 9% ash.

### *Optimisation of hemicellulose, glucose and combined sugar yield*

The optimised PT conditions for hemicellulose, glucose and combined sugar of the two feedstocks were within proximity and the two feedstocks could potentially be combined and pretreated as one. In a biorefinery, the PT conditions used will depend on downstream process requirements. Maximising for hemicellulose yield would result in higher concentrations of fermentable sugars for lactic acid fermentation. Whilst optimising for maximum glucose yield would favour ethanol production. Therefore, the trade-off in economics between the two variables would need to be evaluated.

### *Lactic acid production from xylose*

It is important to select the correct bacterial strain for lactic acid production to be maximised. From the six bacterial strains capable of fermenting xylose, *Lactobacillus lactis* IO-1, *Bacillus coagulans* MXL-9 and *Bacillus coagulans* P38 were capable of fermenting xylose in the presence of oxygen.

*Sub-lethal hydrolysate toxicity and tolerance in micro-aerobic vs anaerobic fermentations*

*B. coagulans* P38, *B. coagulans* MXL-9 and *L. lactis* IO-1 could ferment sugars under micro-aerobic and anaerobic conditions and in the presence of inhibitors found in the hydrolysate generated from sugarcane bagasse and harvesting residues. All three strains could produce lactic acid in fermentations containing up to 75% (v/v) hemicellulose hydrolysate. Final lactic acid concentration produced by *B. coagulans* P38 in 75% sugarcane bagasse hydrolysate was 4.18 g/L and in harvesting residue hydrolysate it was 20.42 g/L. *B. coagulans* MXL-9 could produce up to 5.58 g/L (bagasse) and 16.97 g/L (harvesting residue) lactic acid and *L. lactis* IO-1 produced 8.68 g/L and 17.44 g/L lactic acid in the respective hydrolysates. Furthermore, sugarcane harvesting residues is a novel feedstock investigated for the use of lactic acid production and could be a promising addition to be used alongside conventional sugarcane bagasse. Finally, *B. coagulans* MXL-9 and *B. coagulans* P38 are promising thermophiles that could be investigated for industrial use.

*Use of sugarcane harvesting residues in a biorefinery concept alongside sugarcane bagasse*

Based on the results from the two experimental sections, harvesting residues could be used in a biorefinery concept. Two potential scenarios were identified from the study:

- (i) The two feedstocks can be pretreated separately. The pretreatment of sugarcane bagasse is optimised to obtain cellulose rich solids for ethanol production and that of the harvesting residues is optimised for a hemicellulose-rich hydrolysate (with low inhibitor concentration) for lactic acid production. The solids waste stream obtained from both processes can then be combined and used to produce electricity.
- (ii) The two feedstocks are combined and pretreated to maximise combined sugar yield whereby ethanol is produced from the cellulose rich solids and lactic acid is produced from the hemicellulose hydrolysate and the solid waste is used to produce electricity.

Software, such as Aspen, can be used to simulate the various scenarios and determine the trade-off between variables such as the cost of raw material, operating expenses and overall product yield.



## References

- Abdel-Rahman, M. A., Tashiro, Y. and Sonomoto, K. (2011) 'Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits.', *Journal of biotechnology*, 156(4), pp. 286–301.
- Abdel-Rahman, M. A., Tashiro, Y. and Sonomoto, K. (2013) 'Recent advances in lactic acid production by microbial fermentation processes.', *Biotechnology advances*. Elsevier Inc., 31(6), pp. 877–902.
- Adnan, A. and Tan, I. (2007) 'Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential', *Bioresource technology*, 98, pp. 1380–1385.
- Adsul, M. G. *et al.* (2011) 'Development of biocatalysts for production of commodity chemicals from lignocellulosic biomass.', *Bioresource technology*. Elsevier Ltd, 102(6), pp. 4304–12.
- Adsul, M. G., Varma, A. J. and Gokhale, D. V. (2007) 'Lactic acid production from waste sugarcane bagasse derived cellulose', *Green Chemistry*, 9(1), p. 58.
- Agudelo Aguirre, R. (2016) *Integrated optimization of pretreatment conditions for bioethanol production from steam treated triticale straw*. Stellenbosch University.
- Alonso-Pippo, W. *et al.* (2009) 'Energy recovery from sugarcane biomass residues: Challenges and opportunities of bio-oil production in the light of second generation biofuels', *Journal of Renewable and Sustainable Energy*, 1(6).
- Antonio Bizzo, W. *et al.* (2014) 'The generation of residual biomass during the production of bio-ethanol from sugarcane, its characterization and its use in energy production', *Renewable and Sustainable Energy Reviews*. Elsevier, 29, pp. 589–603.
- Bailey, B. (1996) 'Performance of ethanol as a transportation fuel', in *Handbook on bioethanol: production and utilization*. C.E. Wyman. Washington: Taylor & Francis, pp. 37–60.
- Behera, S. *et al.* (2014) 'Importance of chemical pretreatment for bioconversion of lignocellulosic biomass', *Renewable and Sustainable Energy Reviews*. Elsevier, 36, pp. 91–106.
- Benjamin, Y. (2014) *Sugarcane cultivar selection for ethanol production using dilute acid pretreatment, enzymatic hydrolysis and fermentation*. Stellenbosch University.
- Benjamin, Y., Cheng, H. and Görgens, J. F. (2014) 'Optimization of dilute sulfuric acid pretreatment to maximize combined sugar yield from sugarcane bagasse for ethanol production.', *Applied*

*biochemistry and biotechnology*, 172(2), pp. 610–30.

Bischoff, K. M. *et al.* (2010) ‘Fermentation of corn fiber hydrolysate to lactic acid by the moderate thermophile *Bacillus coagulans*.’, *Biotechnology letters*, 32(6), pp. 823–8.

Bocci, E., Di Carlo, A. and Marcelo, D. (2009) ‘Power plant perspectives for sugarcane mills’, *Energy*, 34(5), pp. 689–698.

Budhavaram, N. and Fan, Z. (2009) ‘Production of lactic acid from paper sludge using acid-tolerant, thermophilic *Bacillus coagulans* strains’, *Bioresource Technology*, 100, pp. 5966–5972.

Canilha, L. *et al.* (2011) ‘A study on the pretreatment of a sugarcane bagasse sample with dilute sulfuric acid.’, *Journal of industrial microbiology & biotechnology*, 38(9), pp. 1467–75.

Chandel, A. K. *et al.* (2012) ‘Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products’, *Journal of Chemical Technology & Biotechnology*, 87(1), pp. 11–20.

Chandel, A. and Singh, O. (2011) ‘Weedy lignocellulosic feedstock and microbial metabolic engineering: advancing the generation of “Biofuel”’, *Applied Microbiology and Biotechnology*, 89(5), pp. 1289–1303.

Chandra, R. *et al.* (2007) ‘Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics?’, *Advances in Biochemical Engineering/Biotechnology*, 108, pp. 67–93.

Cherubini, F. (2010) ‘The biorefinery concept: Using biomass instead of oil for producing energy and chemicals’, *Energy Conversion and Management*, 51(7), pp. 1412–1421.

Claassen, P. *et al.* (1999) ‘Utilisation of biomass for the supply of energy carriers’, *Applied Microbiology and Biotechnology*, 52(6), pp. 741–755.

Clomburg, J. and Gonzalez, R. (2010) ‘Biofuel production in *Escherichia coli* : the role of metabolic engineering and synthetic biology’, *Applied Microbiology and Biotechnology*, 86(2), pp. 419–434.

Cui, F., Li, Y. and Wan, C. (2011) ‘Lactic acid production from corn stover using mixed cultures of *Lactobacillus rhamnosus* and *Lactobacillus brevis*.’, *Bioresource technology*, 102(2), pp. 1831–6.

Diedericks, D. (2013) *Extraction and Recovery of Precursor Chemicals From Sugarcane Bagasse , Bamboo and Triticale Bran Using Conventional , Advanced and Fractionation Pretreatment*. Stellenbosch University.

Diedericks, D., van Rensburg, E. and Görgens, J. F. (2013) ‘Enhancing sugar recovery from sugarcane bagasse by kinetic analysis of a two-step dilute acid pretreatment process’, *Biomass and*

*Bioenergy*, 57, pp. 149–160.

Dobbins, J. J. (2010) ‘Prescott’s Microbiology, Eighth Edition’, *Journal of Microbiology & Biology Education : JMBE*, 11(1), pp. 64–65.

DOE (US Department of Energy) (2006) *Biomass feedstock composition and property database*. Available at: <http://www.afdc.energy.gov/biomass/progs/search1.cgi> (Accessed: 12 March 2014).

Dusselier, M. *et al.* (2013) ‘Lactic acid as a platform chemical in the biobased economy: the role of chemocatalysis’, *Energy & Environmental Science*, 6(5), p. 1415.

Eliasson, A. *et al.* (2000) ‘Anaerobic Xylose Fermentation by Recombinant *Saccharomyces cerevisiae* Carrying XYL1, XYL2, and XKS1 in Mineral Medium Chemostat Cultures’, *Applied and Environmental Microbiology*, 66(8), pp. 3381–3386.

Erdei, B. *et al.* (2013) ‘Glucose and xylose co-fermentation of pretreated wheat straw using mutants of *S. cerevisiae* TMB3400’, *Journal of Biotechnology*, 164(1), pp. 50–58.

Esteghlalian, A. *et al.* (1997) ‘Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass’, *Bioresource Technology*, 59(2–3), pp. 129–136.

FAOSTAT (2014) *Food and Agriculture Organization Statistical Database*. Available at: <http://faostat3.fao.org/faostat-gateway/> (Accessed: 2 March 2014).

FitzPatrick, M. *et al.* (2010) ‘A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products.’, *Bioresource technology*. Elsevier Ltd, 101(23), pp. 8915–22.

Focher, B., Marzett, A. and Crescenzi, V. (1991) *Fundamentals and industrial applications in Steam Explosion Techniques*. Philadelphia: Gordon & Breach.

Gao, Y. *et al.* (2013) ‘Effects of different pretreatment methods on chemical composition of sugarcane bagasse and enzymatic hydrolysis.’, *Bioresource technology*, 144, pp. 396–400.

García-Aparicio, M. *et al.* (2011) ‘Evaluation of triticale bran as raw material for bioethanol production’, *Fuel*, 90(4), pp. 1638–1644.

Garde, A. *et al.* (2002) ‘Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*.’, *Bioresource technology*, 81(3), pp. 217–23.

Goldemberg, J. (2007) ‘Ethanol for a sustainable energy future’, *Science*, 315, pp. 808–810.

- Guo, W. *et al.* (2010) ‘Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics.’, *Applied biochemistry and biotechnology*, 161(1–8), pp. 124–36.
- Hahn-Hägerdal, B. *et al.* (2001) ‘Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization’, *Advances in Biochemical Engineering/Biotechnology*, 73, pp. 53–84.
- Hames, B. *et al.* (2003) ‘Rapid biomass analysis: New tools for compositional analysis of corn stover feedstocks and process intermediates from ethanol production’, *Applied biochemistry and biotechnology*, 105(1–3), pp. 5–16.
- Hatti-Kaul, R. *et al.* (2007a) ‘Industrial biotechnology for the production of bio-based chemicals--a cradle-to-grave perspective.’, *Trends in biotechnology*, 25(3), pp. 119–24.
- Hatti-Kaul, R. *et al.* (2007b) ‘Industrial biotechnology for the production of bio-based chemicals – a cradle-to-grave perspective’, *Trends in Biotechnology*, 25(3), pp. 119–124.
- Hendriks, A. and Zeeman, G. (2009) ‘Pretreatments to enhance the digestibility of lignocellulosic biomass’, *Bioresource Technology*, 100(1), pp. 10–18.
- Hofvendahl, K. and Hahn-Hägerdal, B. (2000) ‘Factors affecting the fermentative lactic acid production from renewable resources(1).’, *Enzyme and microbial technology*, 26(2–4), pp. 87–107.
- Ishizaki, A. *et al.* (1990) ‘Biochemical Characterization of *Lactococcus lactis* IO-1 whose optimal temperature is as high as 37C’, *Journal of General and Applied Microbiology*, 36, pp. 1–6.
- Ishizaki, A. *et al.* (1992) ‘L-Lactate production from xylose employing *Lactococcus lactis* IO-1’, *Biotechnology Letters*, 14(7), pp. 599–604.
- Ishizaki, A. and Ueda, T. (1995) ‘Growth kinetics and product inhibition of *Lactococcus lactis* IO-1 culture in xylose medium’, *Journal of Fermentation and Bioengineering*, 80(3), pp. 287–290.
- Jiang, T. *et al.* (2016) ‘Lactic acid production from pretreated hydrolysates of corn stover by a newly developed bacillus coagulans strain’, *PLoS ONE*, 11(2), pp. 1–13.
- Johnson, F. *et al.* (2007) ‘Cane energy for sustainable development and economic competitiveness in southern Africa’, *Proceedings International Society of Sugar Cane Technologists Congress, Durban*, 26, pp. 1246–1255.
- Kamm, B. and Kamm, M. (2004) ‘Biorefinery-systems’, *Chemical and biochemical engineering quarterly*, 18(1), pp. 1–6.
- Kim, Y. *et al.* (2011) ‘Comparative study on enzymatic digestibility of switchgrass varieties and

- harvests processed by leading pretreatment technologies’, *Bioresource Technology*, 102(24), pp. 11089–11096.
- Knauf, M. and Moniruzzaman, M. (2004) ‘Lignocellulosic biomass processing: A perspective’, *International Sugar Journal*, 106, pp. 147–150.
- Kwak, S. and Jin, Y.-S. (2017) ‘Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective’, *Microbial Cell Factories*. BioMed Central, 16(1), p. 82.
- Laopaiboon, P. *et al.* (2010) ‘Acid hydrolysis of sugarcane bagasse for lactic acid production.’, *Bioresource technology*, 101(3), pp. 1036–43.
- Larsen, S. U., Bruun, S. and Lindedam, J. (2012) ‘Straw yield and saccharification potential for ethanol in cereal species and wheat cultivars’, *Biomass and Bioenergy*, 45(Supplement C), pp. 239–250.
- Larsson, S. *et al.* (1999) ‘Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce’, *Applied Biochemistry and Biotechnology*, 77(1), pp. 91–103.
- Li, X., Kim, T. H. and Nghiem, N. P. (2010) ‘Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF).’, *Bioresource technology*, 101(15), pp. 5910–5916.
- Lindedam, J. *et al.* (2012) ‘Cultivar variation and selection potential relevant to the production of cellulosic ethanol from wheat straw’, *Biomass and Bioenergy*, 37, pp. 221–228.
- Lloyd, T. A. and Wyman, C. E. (2005) ‘Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids.’, *Bioresource Technology*, 96(18), pp. 1967–77.
- Maas, R. H. W. *et al.* (2008) ‘Lactic acid production from lime-treated wheat straw by *Bacillus coagulans*: neutralization of acid by fed-batch addition of alkaline substrate.’, *Applied microbiology and biotechnology*, 78(5), pp. 751–8.
- Mackie, K. L. *et al.* (1985) ‘Effect of Sulphur Dioxide and Sulphuric Acid on Steam Explosion of Aspenwood’, *Journal of Wood Chemistry and Technology*, 5(3), pp. 405–425.
- Martin, C. *et al.* (2007) ‘Dilute Sulfuric Acid Pretreatment of Agricultural and Agro-Industrial Residues for Ethanol Production’, *Applied biochemistry and biotechnology*, 136-, pp. 339–352.

- Martín, C. *et al.* (2002) 'Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*', *Enzyme and Microbial Technology*, 31(3), pp. 274–282.
- Mazumdar, S., Clomburg, J. and Gonzalez, R. (2010) 'Escherichia coli strains engineered for homofermentative production of D-lactic acid from glycerol', *Applied Environ. Microbiol.*, 76, pp. 4327–4336.
- Menon, V. and Rao, M. (2012) 'Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept', *Progress in Energy and Combustion Science*. Elsevier Ltd, 38(4), pp. 522–550.
- Moldes, A. B. *et al.* (2006) 'Complete bioconversion of hemicellulosic sugars from agricultural residues into lactic acid by *Lactobacillus pentosus*', *Applied Biochemistry and Biotechnology*, 135(3), pp. 219–227.
- Mosier, N. *et al.* (2005) 'Features of promising technologies for pretreatment of lignocellulosic biomass.', *Bioresource technology*, 96(6), pp. 673–86.
- Moutta, R. D. O., Ferreira-Leitão, V. S. and Bon, E. P. D. S. (2014) 'Enzymatic hydrolysis of sugarcane bagasse and straw mixtures pretreated with diluted acid', *Biocatalysis and Biotransformation*, 32(1), pp. 93–100.
- Moutta, R. O. *et al.* (2012) 'Statistical Optimization of Sugarcane Leaves Hydrolysis into Simple Sugars by Dilute Sulfuric Acid Catalyzed Process', *Sugar Tech*, 14(1), pp. 53–60.
- nee' Nigam, P., Gupta, N. and Anthwal, A. (2009) 'Pre-treatment of Agro-Industrial Residues', in nee' Nigam, P. and Pandey, A. (eds) *Biotechnology for Agro-Industrial Residues Utilisation: Utilisation of Agro-Residues*. Dordrecht: Springer Netherlands, pp. 13–33.
- Neureiter, M. *et al.* (2002) 'Dilute-acid hydrolysis of sugarcane bagasse at varying conditions.', *Applied biochemistry and biotechnology*, 98–100, pp. 49–58.
- Neureiter, M. *et al.* (2004) 'Lignocellulose feedstocks for the production of lactic acid', *Chemical and biochemical engineering quarterly*, 18(1), pp. 55–63.
- Octave, S. and Thomas, D. (2009) 'Biorefinery: Toward an industrial metabolism.', *Biochimie*, 91(6), pp. 659–64.
- Ohara, H. (2003) 'Biorefinery – a mini review', *Applied microbiology and biotechnology*, 62, pp. 474–477.

- Olofsson, K., Bertilsson, M. and Lidén, G. (2008) 'A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks', *Biotechnology for Biofuels*, 1, p. 7.
- Ou, M. S., Ingram, L. O. and Shanmugam, K. T. (2011) 'L (+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*', *Journal of industrial microbiology & biotechnology*, 38(5), pp. 599–605.
- Ouyang, J. *et al.* (2012) 'Efficient non-sterilized fermentation of biomass-derived xylose to lactic acid by a thermotolerant *Bacillus coagulans* NL01.', *Applied biochemistry and biotechnology*, 168(8), pp. 2387–97.
- Ouyang, J. *et al.* (2013) 'Open fermentative production of L-lactic acid by *Bacillus* sp. strain NL01 using lignocellulosic hydrolyzates as low-cost raw material.', *Bioresource technology*. Elsevier Ltd, 135, pp. 475–80.
- Palmqvist, E. and Hahn-Hägerdal, B. (2000) 'Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification', *Bioresource Technology*, 74(1), pp. 17–24.
- Pandey, A. *et al.* (2000) 'Biotechnological potential of agro-industrial residues. I: sugarcane bagasse', *Bioresource Technology*, 74(1), pp. 69–80.
- Patel, M. *et al.* (2004) 'Fermentation of sugar cane bagasse hemicellulose hydrolysate to L(+)-lactic acid by a thermotolerant acidophilic *Bacillus* sp.', *Biotechnology letters*, 26(11), pp. 865–8.
- Patel, M. a *et al.* (2005) 'Simultaneous saccharification and co-fermentation of crystalline cellulose and sugar cane bagasse hemicellulose hydrolysate to lactate by a thermotolerant acidophilic *Bacillus* sp.', *Biotechnology progress*, 21(5), pp. 1453–60.
- Peng, L. *et al.* (2013) 'Bacillus sp. strain P38: an efficient producer of L-lactate from cellulosic hydrolysate, with high tolerance for 2-furfural.', *Bioresource Technology*, 149, pp. 169–176.
- Pengilly, C. *et al.* (2015) 'Enzymatic hydrolysis of steam-pretreated sweet sorghum bagasse by combinations of cellulase and endo-xylanase', *Fuel*. Elsevier Ltd, 154, pp. 352–360.
- Pereira, S. *et al.* (2015) '2G ethanol from the whole sugarcane lignocellulosic biomass', *Biotechnol. Biofuels*, 8(1), p. 44.
- Pippo, W. A. *et al.* (2011) 'Energy recovery from sugarcane-trash in the light of 2nd generation biofuels. Part 1: Current situation and environmental aspects', *Waste and Biomass Valorization*, 2(1), pp. 1–16.

van der Pol, E. *et al.* (2015) ‘Analysis of by-product formation and sugar monomerization in sugarcane bagasse pretreated at pilot plant scale: Differences between autohydrolysis, alkaline and acid pretreatment’, *Bioresource Technology*, 181, pp. 114–123.

Qin, J. *et al.* (2009) ‘Non-sterilized fermentative production of polymer-grade L-lactic acid by a newly isolated thermophilic strain *Bacillus* sp. 2-6.’, *PloS one*, 4(2), p. e4359.

*Renewable Fuel Association (RFA)* (2017) *World fuel ethanol production*. Available at: <http://www.ethanolrfa.org/resources/industry/statistics/#1454099103927-61e598f7-7643> (Accessed: 22 March 2017).

Rodríguez-Moyá, M. and Gonzalez, R. (2010) ‘Systems biology approaches for the microbial production of biofuels’, *Biofuels*, 1(2), pp. 291–310.

Saha, B. C. (2003) ‘Hemicellulose bioconversion.’, *Journal of industrial microbiology & biotechnology*, 30(5), pp. 279–91.

Sanchez, O. and Cardona, C. (2008) ‘No Trends in biotechnological production of fuel ethanol from different feedstocks’, *Bioresource technology*, 99, pp. 5270–5295.

Selig, M., Weiss, N. and Ji, Y. (2008) *Enzymatic Saccharification of Lignocellulosic Biomass*, *National Renewable Energy Laboratory (NREL)*. 1617 Cole Boulevard, Golden, Colorado 80401-3393.

Sindhu, R. *et al.* (2014) ‘Physicochemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology’, *Renewable Energy*. Elsevier Ltd, 62, pp. 362–368.

Singh, P. *et al.* (2008) ‘Biological pretreatment of sugarcane trash for its conversion to fermentable sugars’, *World Journal of Microbiology and Biotechnology*, 24(5), pp. 667–673.

Slininger, P. J. *et al.* (1985) ‘Comparative evaluation of ethanol production by xylose-fermenting yeasts presented high xylose concentrations’, *Biotechnology Letters*, 7(6), pp. 431–436.

Sluiter, A. *et al.* (2006) *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples*, *National Renewable Energy Laboratory (NREL)*. 1617 Cole Boulevard, Golden, Colorado 80401-3393.

Sluiter, A. *et al.* (2011) *Determination of Structural Carbohydrates and Lignin in Biomass*, *National Renewable Energy Laboratory (NREL)*. 15013 Denver West Parkway Golden, Colorado 80401.



- Smeets, E., Faaij, A. and Lewandowski, I. (2004) *A quickscan of global bio-energy potentials to 2050 An analysis of the regional availability of biomass resources for export in relation to the underlying factors*. Netherlands: Copernicus Institute.
- Smithers, J. (2014) 'Review of sugarcane trash recovery systems for energy cogeneration in South Africa', *Renewable and Sustainable Energy Reviews*, 32, pp. 915–925.
- Socol, C. R. *et al.* (2010) 'Bioethanol from lignocelluloses: Status and perspectives in Brazil.', *Bioresource technology*, 101(13), pp. 4820–4825.
- Somerville, C. *et al.* (2010) 'Feedstocks for Lignocellulosic Biofuels', *Science*, 329(5993), pp. 790–792.
- Sreenath, H. K. *et al.* (2001) 'Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber.', *Journal of bioscience and bioengineering*, 92(6), pp. 518–23.
- Sun, Y. and Cheng, J. (2002) 'Hydrolysis of lignocellulosic materials for ethanol production: a review.', *Bioresource technology*, 83(1), pp. 1–11.
- Szczerbowski, D. *et al.* (2014) 'Sugarcane biomass for biorefineries: Comparative composition of carbohydrate and non-carbohydrate components of bagasse and straw', *Carbohydrate Polymers*, 114, pp. 95–101.
- Taherzadeh, M. and Karimi, K. (2007) 'Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review', *BioResources*, 2(2007), pp. 472–499.
- Talukder, M., Das, P. and Wu, J. (2012) 'Microalgae (*Nannochloropsis salina*) biomass to lactic acid and lipid', *Biochemical Engineering Journal*, 68, pp. 109–113.
- Tanaka, K. *et al.* (2003) 'Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1', *Applied Microbiology and Biotechnology*, 60(1–2), pp. 160–167.
- Taniguchi, M. *et al.* (2004) 'Production of L-lactic acid from a mixture of xylose and glucose by co-cultivation of lactic acid bacteria.', *Applied microbiology and biotechnology*, 66(2), pp. 160–5.
- Tyree, R. W., Clausen, E. C. and Gaddy, J. L. (1990) 'The fermentative characteristics of *Lactobacillus xylosus* on glucose and xylose', *Biotechnology Letters*, 12(1), pp. 51–56.
- Uppugundla, N. *et al.* (2014) 'A comparative study of ethanol production using dilute acid, ionic liquid and AFEX™ pretreated corn stover.', *Biotechnology for biofuels*, 7(1), p. 72.

- Walton, S. L. *et al.* (2010) 'Production of lactic acid from hemicellulose extracts by *Bacillus coagulans* MXL-9.', *Journal of industrial microbiology & biotechnology*, 37(8), pp. 823–30.
- Wang, K. and Sun, R.-C. (2010) 'Chapter 7.5 - Biorefinery Straw for Bioethanol', in Sun, R.-C. (ed.) *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels*. Amsterdam: Elsevier, pp. 267–287.
- Wang, Q. *et al.* (2011) 'Isolation, characterization and evolution of a new thermophilic *Bacillus licheniformis* for lactic acid production in mineral salts medium.', *Bioresource technology*, 102(17), pp. 8152–8158.
- Wyman, C. (1999) 'Biomass ethanol: technical progress, opportunities, and commercial challenges', *Annu. Rev. Energy Environ.*, 24, pp. 189–226.
- Xiang, Q., Kim, J. S. and Lee, Y. Y. (2003) 'A comprehensive kinetic model for dilute-acid hydrolysis of cellulose.', *Applied biochemistry and biotechnology*, 105–108, pp. 337–352.
- Xu, K. and Xu, P. (2014) 'Efficient production of l-lactic acid using co-feeding strategy based on cane molasses/glucose carbon sources.', *Bioresource technology*, 153, pp. 23–9.
- Yang, B. and Wyman, C. E. (2008) 'Pretreatment: the key to unlocking low-cost cellulosic ethanol', *Biofuels, Bioproducts and Biorefining*, 2, pp. 26–40.
- Yang, B. and Wyman, C. E. (2009) 'Dilute Acid and Autohydrolysis Pretreatment', in Mielenz, J. R. (ed.) *Biofuels: Methods and protocols, Methods in Molecular Biology*. Totowa, NJ: Humana Press (Methods in Molecular Biology), pp. 103–114.
- Ye, L. *et al.* (2013) 'Highly efficient production of L-lactic acid from xylose by newly isolated *Bacillus coagulans* C106.', *Bioresource technology*, 132, pp. 38–44.
- Ye, L. *et al.* (2014) 'Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for l-lactic acid production by *Bacillus coagulans* JI12', *Biochemical Engineering Journal*. Elsevier B.V., 83, pp. 16–21.
- Zeitsch, K. J. (ed.) (2000) '2. The reactions leading to furfural', in *the chemistry and technology of furfural and its many by-products*. Elsevier (Sugar Series), pp. 3–7.
- Zhang, Y., Chen, X., Luo, J., *et al.* (2014) 'An efficient process for lactic acid production from wheat straw by a newly isolated *Bacillus coagulans* strain IPE22.', *Bioresource technology*, 158, pp. 396–399.

Zhang, Y., Chen, X., Qi, B., *et al.* (2014) 'Improving lactic acid productivity from wheat straw hydrolysates by membrane integrated repeated batch fermentation under non-sterilized conditions.', *Bioresource technology*, 163, pp. 160–6.

Zhang, Y.-H. P. *et al.* (2007) 'Fractionating recalcitrant lignocellulose at modest reaction conditions.', *Biotechnology and bioengineering*, 97(2), pp. 214–223.

Zhang, Y. and Vadlani, P. V (2015) 'Lactic acid production from biomass-derived sugars via co-fermentation of *Lactobacillus brevis* and *Lactobacillus plantarum*.' , *Journal of bioscience and bioengineering*, 119(6), pp. 694–699.

Zhao, B. *et al.* (2010) 'Repeated open fermentative production of optically pure L-lactic acid using a thermophilic *Bacillus* sp. strain.', *Bioresource technology*. Elsevier Ltd, 101(16), pp. 6494–8.

Zhu, L. *et al.* (2008) 'Structural features affecting biomass enzymatic digestibility', *Bioresource Technology*, 99(9), pp. 3817–3828.

## Appendices

Appendix A Results related to Chapter 3:

Appendix A-1 Average sugar yields (g/100 g DM) of pretreatment screening after dilute acid pretreatment and enzymatic hydrolysis at 15 FPU/g WIS of sugarcane bagasse and harvest residues

Temperature (°C)	Time (min)	Pretreated Liquor			Enzymatic hydrolysis		Combined Sugar
		Glucose	Xylose	Arabinose	Glucose	Xylose	
<b>Sugarcane Bagasse</b>							
120	5	0.71	1.49	0.44	8.75	3.47	14.86
120	10	0.77	1.86	0.61	8.86	3.98	16.09
120	15	0.64	1.33	0.46	8.32	3.48	14.22
120	20	0.72	1.62	0.62	10.12	3.90	16.99
120	40	1.11	6.21	1.07	11.33	4.44	24.16
137.5	30	1.00	8.27	1.25	12.54	4.65	27.70
155	5	1.32	12.08	1.57	15.56	4.46	34.99
155	10	1.43	13.14	1.60	18.67	4.55	39.40
155	15	1.10	12.67	1.20	19.65	4.47	39.08
155	20	1.16	12.97	1.22	19.86	4.64	39.86
155	40	1.97	16.22	1.06	19.24	3.16	41.66
190	5	2.64	15.82	1.18	28.53	2.58	50.74
190	10	3.06	12.16	0.68	35.34	1.32	52.55
190	15	3.59	8.11	0.54	34.32	2.09	48.65
<b>Sugarcane Harvest Residues</b>							
120	5	0.36	0.85	0.35	11.62	3.41	16.75
120	10	0.25	1.01	0.35	12.69	3.36	17.80
120	15	0.64	2.07	0.91	12.33	3.39	19.47
120	20	0.59	1.98	0.73	12.29	3.60	19.32
120	40	0.53	2.04	0.85	12.52	3.69	19.75
137.5	30	0.91	6.31	1.72	16.91	4.42	30.33
155	5	0.49	3.31	1.12	18.99	3.27	27.18
155	10	0.79	5.79	1.43	17.95	4.05	30.05
155	15	0.90	5.08	1.23	17.26	4.45	28.91
155	20	3.48	6.84	1.24	21.21	3.24	36.01
155	40	2.40	8.51	1.61	19.79	4.19	36.59
190	5	2.07	7.81	1.44	18.05	4.43	33.90
190	10	1.56	9.28	1.64	22.87	4.11	39.47
190	15	4.71	8.99	1.16	23.61	2.30	40.78

## Appendix A-2 Analysis of Variance for CCRD of sugarcane bagasse and harvest residues

<b>Sugarcane Bagasse</b>															
Factor	<b>Hemicellulose yield; R<sup>2</sup> = 0.942; R<sup>2</sup><sub>Adj</sub> = 0.897</b>					<b>Glucose yield; R<sup>2</sup> = 0.979; R<sup>2</sup><sub>Adj</sub> = 0.952</b>					<b>Combined sugar yield; R<sup>2</sup> = 0.9698; R<sup>2</sup><sub>Adj</sub> = 0.939</b>				
	SS	df	MS	F	p	SS	df	MS	F	p	SS	df	MS	F	p
(1)Temperature(L)	3.450	1	3.450	6.541	0.125	422.529	1	422.529	6998.666	0.000	587.605	1	587.605	301.659	0.003
Temperature(Q)	103.088	1	103.088	195.490	0.005	57.234	1	57.234	948.013	0.001	229.933	1	229.933	118.041	0.008
(2)[Acid](L)	7.466	1	7.466	14.158	0.064	41.670	1	41.670	690.205	0.001	71.576	1	71.576	36.745	0.026
[Acid](Q)	57.821	1	57.821	109.648	0.009	91.913	1	91.913	1522.423	0.001	270.404	1	270.404	138.818	0.007
(3)Time (L)	0.096	1	0.096	0.183	0.711	2.689	1	2.689	44.545	0.022	1.059	1	1.059	0.544	0.538
Time (Q)						27.044	1	27.044	447.943	0.002	41.581	1	41.581	21.346	0.044
1L by 2L	86.680	1	86.680	164.375	0.006	40.339	1	40.339	668.162	0.001	160.365	1	160.365	82.327	0.012
1L by 3L	98.928	1	98.928	187.600	0.005	6.427	1	6.427	106.460	0.009	167.439	1	167.439	85.959	0.011
2L by 3L						1.984	1	1.984	32.859	0.029					
Lack of Fit	19.223	7	2.746	5.207	0.171	13.385	5	2.677	44.340	0.022	39.015	6	6.502	3.338	0.248
Pure Error	1.055	2	0.527			0.121	2	0.060			3.896	2	1.948		
Total SS	349.489	16				647.521	16				1418.389	16			

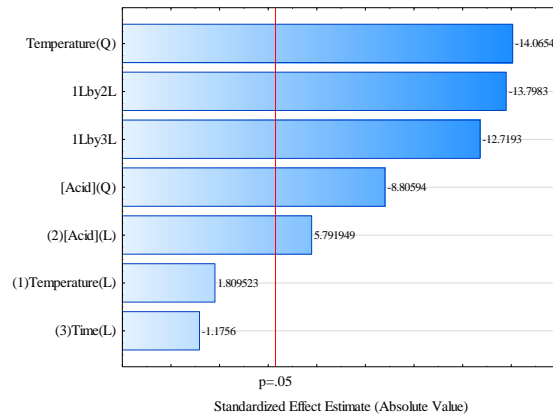
  

<b>Sugarcane Harvest Residues</b>															
Factor	<b>Hemicellulose yield; R<sup>2</sup> = 0.881; R<sup>2</sup><sub>Adj</sub> = 0.809</b>					<b>Glucose yield; R<sup>2</sup> = 0.741; R<sup>2</sup><sub>Adj</sub> = 0.681</b>					<b>Combined sugar; R<sup>2</sup> = 0.809; R<sup>2</sup><sub>Adj</sub> = 0.766</b>				
	SS	df	MS	F	p	SS	df	MS	F	p	SS	df	MS	F	p
(1)Temperature(L)	200.812	1	200.812	160.541	0.006	572.806	1	572.806	133.129	0.007	1582.110	1	1582.110	188.502	0.005
(2)[Acid](L)	135.847	1	135.847	108.605	0.009	128.722	1	128.722	29.917	0.032	505.082	1	505.082	60.178	0.016
(3)Time (L)	21.837	1	21.837	17.457	0.053	37.732	1	37.732	8.769	0.098	119.961	1	119.961	14.293	0.063
1L by 2L	11.365	1	11.365	9.086	0.095										
1L by 3L	8.846	1	8.846	7.072	0.117										
2L by 3L	3.362	1	3.362	2.688	0.243										
Lack of Fit	49.205	8	6.151	4.917	0.180	250.304	11	22.755	5.289	0.170	502.656	11	45.696	5.444	0.165
Pure Error	2.502	2	1.251			8.605	2	4.303			16.786	2	8.393		
Total SS	433.776	16				998.169	16				2726.594	16			

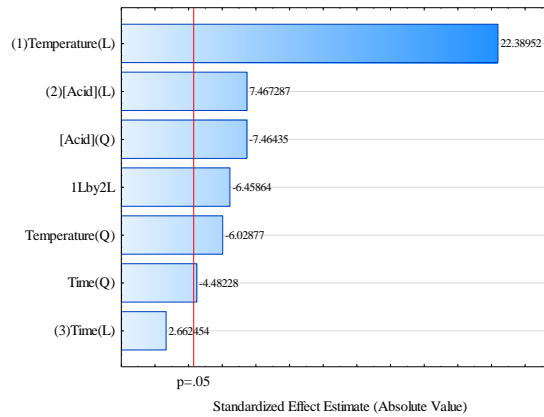
Appendix A-3 Pareto Chart of Standardised Effects for Sugarcane bagasse and harvesting residues

**Sugarcane bagasse**

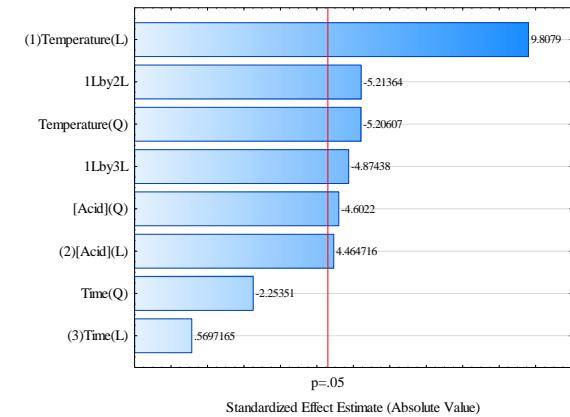
**Hemicellulose**



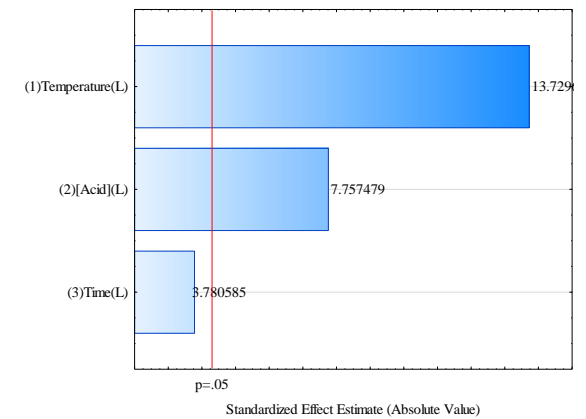
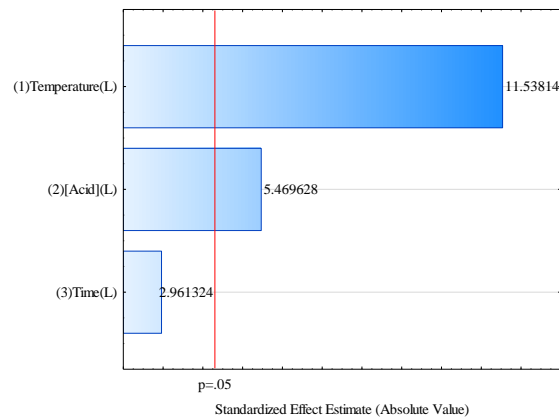
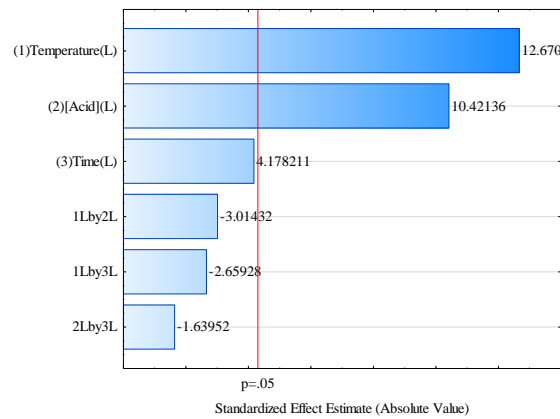
**Glucose**



**Combined Sugar Yield**



**Sugarcane harvesting residues**



Appendix A-4 Average sugar degradation product yields (g/100 g DM) for pretreatment optimisation after dilute acid pretreatment of sugarcane bagasse and harvest residues

No.	Pretreatment Conditions			Sugarcane Bagasse		Sugarcane Harvest Residues	
	Temperature °C	[Acid] % (w/w)	Time min	Formic Acid & Acetic Acid g/100 g DM	HMF & Furfural g/100 g DM	Formic Acid & Acetic Acid g/100 g DM	HMF & Furfural g/100 g DM
<b>Factorial Points</b>							
1	150	0.3	10	1.24 ± 0.01	0.58 ± 0.13	0.43 ± 0.01	0.22 ± 0.01
2	150	0.3	20	1.88 ± 0.02	0.94 ± 0.08	0.53 ± 0.03	0.29 ± 0.01
3	150	0.7	10	2.46 ± 0.60	1.34 ± 0.38	0.80 ± 0.05	0.62 ± 0.01
4	150	0.7	20	3.46 ± 0.17	2.14 ± 0.22	1.33 ± 0.05	0.82 ± 0.05
5	180	0.3	10	4.45 ± 0.06	2.93 ± 0.01	1.46 ± 0.0	1.31 ± 0.27
6	180	0.3	20	3.71 ± 0.02	2.75 ± 0.14	1.84 ± 0.13	1.12 ± 0.36
7	180	0.7	10	5.08 ± 0.65	2.98 ± 0.99	2.91 ± 0.29	2.20 ± 0.60
8	180	0.7	20	5.11 ± 0.11	4.17 ± 1.18	2.53 ± 0.01	1.48 ± 0.11
<b>Star Point: Temperature</b>							
9	140	0.5	15	1.96 ± 0.11	1.11 ± 0.04	0.78 ± 0.06	0.53 ± 0.12
10	190	0.5	15	6.02 ± 0.06	4.44 ± 0.15	3.48 ± 0.17	2.89 ± 0.38
<b>Star Point: Acid Concentration</b>							
11	165	0.16	15	2.27 ± 0.06	1.63 ± 0.08	0.93 ± 0.01	0.40 ± 0.02
12	165	0.84	15	4.12 ± 0.62	3.63 ± 0.79	2.57 ± 0.06	1.81 ± 0.29
<b>Star Point: Time</b>							
13	165	0.5	6.6	3.15 ± 0.20	2.11 ± 0.01	0.84 ± 0.01	0.75 ± 0.01
14	165	0.5	23.4	3.53 ± 0.19	2.64 ± 0.30	1.84 ± 0.17	1.52 ± 0.27
<b>Centre Point</b>							
15	165	0.5	15	3.00 ± 0.42	2.48 ± 0.16	1.60 ± 0.22	1.18 ± 0.27
16	165	0.5	15	4.34 ± 0.03	3.04 ± 0.21	1.27 ± 0.17	0.99 ± 0.12
17	165	0.5	15	3.94 ± 0.20	2.39 ± 0.80	1.06 ± 0.01	1.07 ± 0.09