# INTEGRATED BIOENERGY AND ANIMAL FEED PRODUCTION FROM AFEX$^{TM}$ AND STEAM EXPLODED SUGARCANE RESIDUES

*by*

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# ABSTRACT

Current and future trends demonstrate that the increasing world population, dwindling arable land, changing human diets and increased demand for (bio)energy present an opportunity to redesign the way land is used to meet the future food, feed and bioenergy demands. The sustainable integration of bioenergy and highly digestible livestock feed production systems has been touted as a potential avenue to increase the economic returns to agriculture and simultaneously promote energy security, particularly in developing countries. To this end, post-harvest residues from sugarcane processing (*i.e.* sugarcane bagasse (SCB) and cane leaf matter (CLM)) have emerged as candidate feedstock for integrated bioenergy (*e.g.* bio-ethanol and biogas) and animal feeds production in South Africa and Brazil. The principal aim of this dissertation was to perform a systematic comparison of the potential use of steam explosion (StEx) and ammonia fiber expansion (AFEX™) as pretreatment technologies to overcome biomass recalcitrance, thereby generating highly digestible animal feeds, and cellulosic ethanol and biogas production feedstocks from sugarcane residues for future integrated biofuel-animal feed systems.

A side-by-side comparison of the effect of StEx and AFEX™ pretreatment of sugarcane residues revealed AFEX™ to be the better pretreatment for maximising ethanol yields per Mg raw dry material (RDM) from both SCB and CLM. Under industrially relevant solids loadings of 16% and dosages of 9.8 mg protein/g RDM, AFEX™ pretreated sugarcane residues generated ethanol yields up to 324 litres/Mg RDM, the highest ethanol yields reported in literature from sugarcane residues. In contrast, ethanol yields from steam exploded sugarcane residues were limited to the range 205 to 257 litres/Mg RDM primarily due to the compounded effect of carbohydrate degradation during pretreatment, enzyme inhibition and microbial inhibition of recomninant *Saccharomyces cerevisiae* 424A (LNH-ST) during fermentation.



To debottleneck microbial inhibition during the fermentation of non-detoxified StEx whole slurry's, the potential use of industrial xylose-fermenting *S. cerevisiae* strains as efficient and inhibitor tolerant ethanologens was evaluated. *S. cerevisiae* strains CelluX™ 4 and TP-1 demonstrated near complete glucose and xylose consumption, with high acetate resistance, furan detoxification and phenolic aldehyde detoxification phenotypes. Ultimately, both strains facilitated the generation of 224 litres/Mg RDM from non-detoxified StEx SCB whole slurry under a pre-hydrolysis simultaneous saccharification and co-fermentation (PSSCF) configuration. In comparison, the same yeast strains generated moderately higher ethanol yields (254 litres/Mg RDM) during the PSSCF of highly fermentable AFEX™-treated SCB, demonstrating that the difference in the potential ethanol yields that can be recovered from the two pretreatment technologies can be significantly reduced by using inhibitor-tolerant ethanologens.

With both AFEX™ and StEx-treated sugarcane residues requiring enzyme dosages of 9.8mg protein/g RDM to achieve high ethanol yields, the potential use a room-temperature Cellulose $III_l$-activation ($CIII_l$-activation) process to enhance the digestibility of StEx- or AFEX™-treated sugarcane residue pellets was investigated as a potential strategy to minimise the enzyme cost contribution per unit volume ethanol produced. Coupling AFEX™ sugarcane lignocelluloses with $CIII_l$-activation reduced of the enzyme dosage requirements by more than 60% (to ~3 mg protein/g RDM), whilst achieving ethanol yields greater than 280 litres/Mg RDM. These results represented the lowest enzyme dosage to achieve ethanol yields of 280 L/Mg RDM reported in literature. In contrast, upgrading StEx-treated sugarcane residue pellets could only facilitate ethanol yields up to 201 litres/Mg RDM at an enzyme dosage of ~3 mg protein/g RDM.

Besides ethanol production, both AFEX™ and StEx also demonstrated significant improvements in the animal feed value of SCB and CLM. AFEX™-treated sugarcane residues were characterized by 230% increase in the non-protein nitrogen content of the biomass, and up to 69% and 26% improvement in the *in-vitro* true digestibility (IVTD) and metabolizable energy (ME), respectively, relative to untreated controls ($P < 0.05$). Although StEx did not increase the nitrogen



content of the pretreated sugarcane residues, the IVTD and ME of StEx-treated SCB and CLM were improved by 54% and 7%, respectively ($P < 0.05$). These results demonstrated that both AFEX and StEx pretreatment can simultaneously generate highly digestible animal feeds and enhanced cellulosic ethanol feedstocks from sugarcane residues.

The combination of the near optimal C/N ratios and structural modifications of AFEX™-treated sugarcane residues also facilitated biogas production with methane yields up to 299 L $CH_4$/kg VS, with or without co-digestion with dairy cow manure (DCM). To obtain comparable methane yields, untreated and steam exploded (StEx) sugarcane residues had to be co-digested with DCM, at mass ratios providing initial C/N ratios in the range of 18 to 35. Furthermore, the solid digestates recovered from the co-digestion of the sugarcane lignocelluloses with DCM were enriched in nitrogen-phosphate-potassium (NPK), suggesting that they could be used as biofertilizers or partial replacements for the CLM that is typically left on the field during green cane harvesting.

The results from this dissertation showed that both AFEX™ and StEx successfully enhanced the ethanol production potential, methane production potential, and animal feed value of sugarcane residues, providing alternative models for the sugarcane industry to create bioenergy and food value from sugarcane residues. Ultimately, these results provide essential information and insights for future techno-economic and life-cycle analyses that are required to establish the preferred pretreatment technology and processing strategies to enable economically viable and environmentally sustainable integrated bioenergy and animal feed production from South African sugarcane residues.



# OPSOMMING

Huidige en toekomstige tendense dui daarop dat die toename in die wêreldbevolking, drastiese afname in bewerkbare grond, verandering in menslike diëte en verhoging in die vraag na (bio-)energie ’n geleentheid bied vir die herontwerp van grondgebruik om in toekomstige voedsel-, voer- en bio-energiebehoeftes te voorsien. Die volhoubare integrasie van produksiestelsels vir bio-energie en hoogs verteerbare veevoer word as ’n moontlikheid beskou om die ekonomiese opbrengs van landbou te verhoog en terselfdertyd energiesekerheid te bevorder, veral in ontwikkelende lande. Die naoesreste van suikerrietverwerking (d.w.s. suikerrietbagasse (SRB) en rietblaarmateriaal (RBM)) word as kandidaatvoerstof vir geïntegreerde bio-energie- (bv. bio-etanol- en biogas-) en veevoerproduksie in Suid-Afrika en Brasilië beskou. Die hoofdoel van hierdie verhandeling was om ’n stelselmatige vergelyking te onderneem van die moontlike gebruik van stoomontploffing (“StEx”) en ammoniakveseluitsetting (AFEX$^{TM}$) as voorbehandelingstegnologieë om enersyds die biomassaweerspannigheid van suikerrietreste te bowe te kom en sodoende hoogs verteerbare veevoer te skep, en andersyds sellulosiese etanol- en biogasproduksievoerstof uit suikerrietreste te vervaardig vir toekomstige geïntegreerde biobrandstof-veevoerstelsels.

Wanneer die uitwerking van StEx- en AFEX$^{TM}$-voorbehandeling van suikerrietreste naas mekaar beskou word, blyk AFEX$^{TM}$ die beter voorbehandeling te wees vir maksimum etanolproduksie per Mg onverwerkte droëmateriaal (ODM) vir sowel SRB as RBM. Met industrieel relevante vastestofladings van 17% en ’n dosis van 9,8 mg proteïen/g ODM, bied AFEX$^{TM}$-voorbehandelde suikerrietreste ’n etanollewering van tot 324 liter/Mg ODM, synde die hoogste etanollewering uit suikerrietreste wat tot nog toe in die literatuur aangemeld is. Daarteenoor is die etanollewering van stoomontplofte suikerrietreste beperk tot tussen 205 en 257 liter/Mg ODM, hoofsaaklik weens die



saamgestelde uitwerking van koolstofafbreking gedurende voorbehandeling, ensieminhibisie en mikrobiese inhibisie van *Saccharomyces cerevisiae* 424A (LNH-ST) gedurende fermentasie.

Om die bottelnek van mikrobiese inhibisie gedurende die fermentasie van nie-gedetoksifiseerde StEX-ru-flodder uit die weg te probeer ruim, is die potensiële gebruik van industriële xilose-fermenterende *S. cerevisiae*-stamme ook ondersoek. Die *S. cerevisiae*-stamme CelluX™ 4 en TP-1 het byna volledige glukose- en xiloseverbruik, hoë asetaatweerstandigheid, furaandetoksifikasie- én fenoliese-aldehied-detoksifikasiefenotipes getoon, en het uiteindelik ’n etanollewering van 224 liter/Mg ODM gebied in ’n konfigurasie van pre-hidrolise- gelyktydige versuikering en gesamentlike fermentasie (“PSSCF”). Daarteenoor het die PSSCF van hoogs fermenteerbare AFEX™-behandelde SRB ’n effens hoër etanollewering getoon (254 liter/Mg ODM), wat daarop dui dat die verskil in die moontlike etanollewering van die twee voorbehandelingstegnologieë beduidend verminder kan word met behulp van inhibitorverdraagsame etanologene.

Daarbenewens is daar ondersoek ingestel na die opgradering van verpilde StEx- of AFEX™-behandelde suikerrietreste deur middel van ’n $CIII_l$-aktiveringsproses ($CIII_l$) by kamertemperatuur om die bottelnek van die hoë ensiemdosisvereistes verbonde aan hoë etanollewering te probeer verwyder. Die kombinasie van AFEX™-suikerrietlignosellulose en $CIII_l$ het die ensiemdosisvereistes met meer as 60% verlaag (tot ~3 mg proteïen/g ODM) en etanollewering tot meer as 280 liter/Mg ODM verhoog. Hierdie resultaat is die laagste ensiemdosis vir ’n etanollewering van 280 L/Mg ODM wat tot dusver in die literatuur aangemeld is. Daarteenoor het die opgradering van verpilde StEx-behandelde suikerrietreste ’n etanollewering van slegs 201 liter/Mg ODM by ’n ensiemdosis van ~3 mg proteïen/g ODM teweeggebring.

Benewens die uitwerking op etanolproduksie, blyk sowel AFEX™ as StEx ook aansienlike verbeteringe in die veevoerwaarde van SRB en RBM tot gevolg te hê. Die biomassa van AFEX™-behandelde suikerrietreste het tipies oor ’n 230% hoër nieproteïenstikstofinhoud beskik, en *in vitro*-ware verteerbaarheid (IVWV) en metaboliseerbare energie (ME) was onderskeidelik 69% en 26% hoër as by onbehandelde kontroles ($P < 0,05$). Hoewel StEx nie die stikstofinhoud van die voorbehandelde



suikerrietreste verhoog het nie, het die IVWV en ME van StEx-behandelde SRB en RBM met onderskeidelik 54% en 7% verhoog ($P$ < 0,05). Hierdie resultate toon dat sowel AFEX- as StEx-voorbehandeling terselfdertyd hoogs verteerbare veevoer én beter sellulosiese etanolvoerstof uit suikerrietreste kan oplewer.

Die kombinasie van die byna optimale C/N-verhoudings en strukturele aanpassings van AFEX™-behandelde suikerrietreste het ook biogasproduksie teweeggebring, met ’n metaanlewering van tot 299 L $CH_4$/kg VS, met óf sonder gesamentlike vertering met melkbeesmis (MBM). Om vergelykbare metaanlewering te verkry, moes onbehandelde en stoomontplofte (StEx-) suikerrietreste saam met MBM verteer word, wat op massaskaal aanvanklike C/N-verhoudings van tussen 18 en 35 gelewer het. Daarbenewens was die vaste digestate wat uit die gesamentlike vertering van die suikerrietlignosellulose en MBM herwin is, ryk in stikstof-fosfaat-kalium (NPK), wat daarop dui dat dit as biobemesting of gedeeltelike plaasvervanger kan dien vir die RBM wat gewoonlik gedurende groen oesting op landerye agterbly.

Die resultate van hierdie studie toon dat sowel AFEX™ as StEx die etanolproduksiepotensiaal, metaanproduksiepotensiaal en veevoerwaarde van suikerrietreste suksesvol verhoog, en sodoende die suikerrietbedryf van alternatiewe modelle voorsien om bio-energie en voedselwaarde te skep. Die bevindinge bied noodsaaklike inligting en insigte vir toekomstige tegno-ekonomiese en lewensiklusontledings om te bepaal watter voorbehandelingstegnologie en verwerkingstrategieë die beste sal werk om geïntegreerde bio-energie- en veevoerproduksie uit Suid-Afrikaanse suikerrietreste ekonomies lewensvatbaar en omgewingsvolhoubaar te maak.



# DEDICATION

This work is dedicated to my late grandparents,

**John Simelo Mokomele and Mmathabo Rosina Mokomele**



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# NOMENCLATURE

| Abbreviations | | Units |
|---|---|---|
| 1G | First generation | |
| 2G | Second generation | |
| AD | Anaerobic digestion | |
| ADF | Acid detergent fiber | [g/kg DM] |
| ADIN | Acid detergent insoluble nitrogen | [g/kg DM] |
| AFEX™ | Ammonia fiber expansion | |
| AIC | Akaike's information criterion | |
| aNDF | amylase-treated Neutral detergent fiber | [g/kg DM] |
| ANOVA | Analysis of variance | |
| ASTM | American society for testing materials | |
| ATR-FTIR | Attenuated total reflectance Fourier Transform Infrared | |
| BECCS | Bioenergy carbon Capture and Storage | |
| BMP | Biomethane potential | |
| CCD | Central composite design | |
| CHP | Combined heat and power | |
| CLM | Cane leaf matter | |
| DCM | Dairy cow manure | |
| DM | Dry matter | |
| DOE | Design of experiment | |
| EtOH | Ethanol | |
| GC-MS | Gas chromatography-mass spectroscopy | |
| GHG | Greenhouse gas | |
| G-OS | Gluco-oligosaccharides | |
| GDP | Gross domestic product | |
| HDI | Human development index | |
| HHV | Higher heating value (gross calorific energy) | [MJ/kg] |
| HPLC | High performance liquid chromatography | |
| HSL-EH | High solids loading enzymatic hydrolysis | |
| IBS | Industrial Biofuel Strategy | |
| IRR | Internal rate of return | [%] |



| | | |
|---|---|---|
| ILUC | Indirect land use exchange | |
| IVTD | *In-vitro* true digestibility | [g/kg DM] |
| LCC | Lignin carbohydrate complex | |
| LCFA | Long chain fatty acids | |
| LHV | Lower heating value (net calorific energy) | [MJ/kg] |
| MBI | Michigan Biotechnology Institute | |
| ME | Metabolizable energy | [MJ/kg DM] |
| MGY | Million gallons per year | [Mgal/year] |
| MLY | million litres per year | [ML/year] |
| NPK | Nitrogen-phosphorus-potassium | [g/kg DM] |
| NREL | National Renewable Energy laboratory | |
| OD | Optical density | |
| PRESS | Prediction sum of squares | |
| PSSCF | Pre-hydrolysis simultaneous saccharification and co-fermentation | |
| RDM | Raw dry material | |
| RMSE | Root mean square error | [L $CH_4$/kg $VS_{added}$] |
| SER | Standard error of regression | |
| SCB | Sugarcane bagasse | |
| SHF | Separate hydrolysis and fermentation | |
| SSF | Simultaneous saccharification and fermentation | |
| StEx | Steam explosion | |
| STP | Standard temperature pressure | [273 K, 101.325 kPa] |
| TDN | Total digestible nutrients | [g/kg DM] |
| TGA | Thermogravimetric analysis | |
| TS | Total solids | [%] |
| VFA | Volatile fatty acids | [mg/L] |
| VS | Volatile solids | |
| WIS | Water insoluble solids | [g/100g RDM] |
| WSS | Water soluble solids | [g/100g RDM] |
| X-OS | Xylo-oligosaccharides | |
| XRD | X-ray diffraction | |
| YEP | Yeast extract peptone | |

| List of Symbols | | Units |
|---|---|---|
| $CI_\beta$ | Cellulose $I_\beta$ | |
| $CIII_I$ | Cellulose $III_I$ | |
| CrI | Crystallinity index | |
| $D_{enzyme}$ | Enzyme Dosage | [kg protein/Mg RDM] |



| | | |
|---|---|---|
| $EC_i$ | Energy conversion factor (gross) | [%] |
| *k* | Cone model biodegradation rate constant | [1/day] |
| $Log(R_o)$ | Severity factor | |
| *n* | Cone model dimensionless shape constant | |
| $P_{enzyme}$ | Enzyme Production/Purchase cost | [US$/kg protein] |
| $R^2$ | Coefficient of determination | [%] |
| $R^2_{adjusted}$ | Adjusted $R^2$ | [%] |
| $R^2_{predicted}$ | Predicted $R^2$ | [%] |
| $V_{methaneSTP}$ | Specific methane yield at STP | [L $CH_4$/kg $VS_{added}$] |
| $Y_{ethanol}$ | Ethanol Yield | [L EtOH/Mg RDM) |
| $Y_{p/s}$ | Ethanol yield | [g EtOH/g sugar added] |
| $Y_{x/s}$ | Cell biomass yield | [g CDW/g sugar consumed] |

| **Greek Letters** | | **Units** |
|---|---|---|
| β | Accumulated methane yield at time t | [L $CH_4$/kg $VS_{added}$] |
| $\beta_0$ | Accumulated methane yield at time infinity | [L $CH_4$/kg $VS_{added}$] |
| $\rho_{methaneSTP}$ | Methane density at STP | [kg/$m^3$] |
| $\mu_{max}$ | Maximum specific growth rate | [$h^{-1}$] |

# RESEARCH OUTPUTS

## Peer-reviewed & Submitted Publications

I. **Mokomele, T**., da Costa Sousa L., Balan, B., van Rensburg, E., Dale, B.E., Görgens, J.F., *Exploring the Ethanol Production Potential from AFEX$^{TM}$ and Steam Exploded Sugarcane Residues for Sugarcane Biorefineries*. Biotechnology for Biofuels, 11:127, 1-21 (2018)

II. **Mokomele, T**., da Costa Sousa L., Balan, B., Goosen, N., Bals, B., Dale, B.E., Görgens, J.F., *Using Steam explosion or AFEX$^{TM}$ to produce Animal Feeds and Biofuel Feedstocks in a Biorefinery Based on Sugarcane Residues*. Biofuels, Bioproducts, Biorefining, 2018

III. **Mokomele, T**., da Costa Sousa L., Balan, B., van Rensburg, E., Dale, B.E., Görgens, J.F., *Incorporating Anaerobic Co-digestion of Sugarcane Residues with manure into a sugarcane-based bioenergy-livestock nexus*. Bioresource Technology, 272, 326-336 (2019)

IV. **Mokomele, T**., da Costa Sousa L., Balan, B., Dale, B.E., Görgens, J.F., *CIII$_l$-activation of AFEX$^{TM}$ and steam exploded sugarcane residue pellets for low enzyme loading ethanol production from decentralized sugarcane biorefineries*. Energy conversion and management, 2018 (manuscript prepared for submission).

V. Brandt, B.A., García-Aparicio, M.D.P., **Mokomele, T**., Görgens, J.F., van Zyl, W.H., *Rational engineering of Saccharomyces cerevisiae towards improved tolerance to multiple inhibitors in lignocellulose fermentations*. Biotechnology for Biofuels, 2018 (manuscript prepared for submission)

## Conference poster presentations

***VI.*** **Mokomele, T**., da Costa Sousa L., Hamann, M., Balan, B., van Rensburg, E., Dale, B.E., Görgens, J.F., A *comparative study on the enzymatic digestibility of AFEX$^{TM}$ and steam exploded sugarcane bagasse and sugarcane tops & leaves.* Symposium on Biotechnology for Biofuels & Chemicals, Baltimore, Maryland (USA), 2016

VII. **Mokomele, T**., da Costa Sousa L., Balan, B., Dale, B.E., Görgens, J.F., *Diversifying sugarcane residue based biorefineries towards the co-production of biofuels and animal feeds.* Symposium on Biotechnology for Biofuels & Chemicals, San Fransisco, California (USA), 2016

## Conference oral presentations

***VIII.*** Dale, B.E., Bals, B., Bonomi, A., Cavalett, O., Cortez, L., Maciel Filho, R., Fracarolli, J.A., Görgens, J.F., Junqueira, T., **Mokomele, T.,** Rinke, N., De Souza, RA., *A New Model for Sustainable Expansion of Biofuel Production in Brazil: Sugarcane Biomass to Biofuels and Animal Feed.* Brazilian BioEnergy Science and Technology Conference, Sao Paulo, Brazil, 2017



**Other publication contributions not included in dissertation but completed during this Ph.D. study**

**IX.** Clarke, K.G., **Mokomele, T**., Callanan, L.H., Groenewald, J., *Zymomonas mobilis—Towards Bacterial Biofuel*. In: Leal Filho W., Surroop D. (eds) The Nexus: Energy, Environment and Climate Change. Green Energy and Technology. Springer, pg. 205-219, 2018



# CHAPTER ONE:

# INTRODUCTION

Liquid biofuels are considered as one of the leading alternative transportation fuels with several commercial facilities producing ethanol from first-generation feedstocks such as cereal grains and sugarcane juice. Sugarcane residues have demonstrated significant promise as second-generation bioenergy feedstock, allowing for the integration of biorefineries to existing sugar mills, particularly in developing regions such as Brazil and sub-Saharan Africa. However, current and future trends demonstrate that the increasing world population, demand for animal products in human diets and demand for biofuels will require a reconfiguration of the way land is used to meet the future food, animal feed and biofuels demands. Hence, the fundamental challenge for unlocking the commercial appeal of bioenergy from sugarcane residues lies not only in the economic conversion of these residues, but also on its potential interaction with animal feed, human food, environmental impact, and domestic job creation sectors.

This chapter introduces insights into the use of two mature pretreatment technologies, steam explosion (StEx) and ammonia fiber expansion (AFEX$^{TM}$) to enable the potential integration of biofuel and animal feed production to current biorefinery models, thereby creating more sustainable bioenergy-feed-food production systems.

## 1.1. Background – The Grand Challenge

Worldwide energy consumption has increased significantly in the last century due to increases in the world population and industrialization [1]. As of 2018, the International Energy Agency (IEA) estimated that the total global consumption of crude oil and liquid fuels equated to approximately 99 million barrels per day [2,3]. According to the United Nations Human Development Index (HDI), a nations' per capita rate of energy consumption (including electricity, heating and mobility), is a strong indicator of that society's wealth and potential to develop their human potential [4]. HDI, which is a composite metric of human capital development that aggregates measures of national health, education, and wealth, demonstrates that approximately 5 kilowatts per person per year is required for societies to achieve high level of human development (Figure 1.1) [5]. Hence, developing countries will have to increase their per capita energy consumption to maximise their HDI amid rising oil prices,

national economic instability, and climate change driven by the greenhouse gas (GHG) emissions or embrace low-carbon energy sources as an alternative source of energy [5,7].

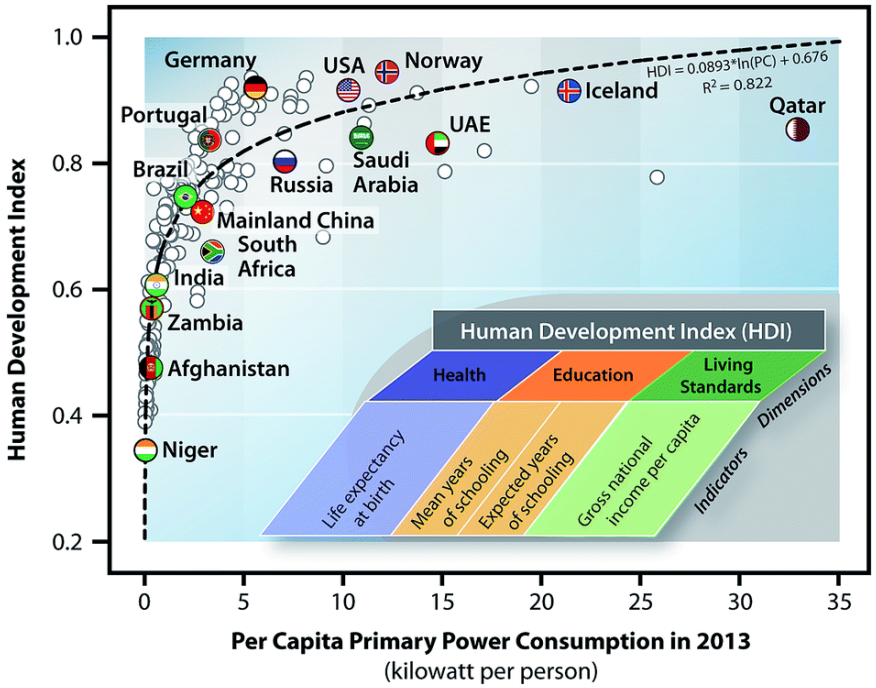



**Figure 1.1:** Relationship between human development index and per capita primary energy consumption study for 70 developed and developing countries for the year 2013. [5]

Energy derived from plant biomass (bioenergy) is widely expected to contribute approximately 25% of the primary energy in future low-carbon energy projections by the year 2050 [6]. Lignocellulosic biomass is considered the only alternative sustainable resource capable of producing liquid biofuels at scales necessary to displace a significant amount of petroleum-based fuels whilst meeting global sustainability goals [7]. Currently, the USA and Brazil already produce more than 50 and 30 million cubic meters of ethanol from edible starch (*i.e.* corn grains) and extractable sugars (*i.e.* cane juice) in first-generation (1G) biorefineries, respectively [5,8,9]. However, in developing countries such as South Africa, the use of edible crops as biorefinery feedstock materials to produce relatively low value biofuels (ethanol) becomes a debatable socio-economic issue due to direct competition with the food market. In addition to competing with the food market, 1G ethanol production feedstock's are generally expensive and cannot be considered as a long-term solution due to the unavailability of sufficient farmland to provide more than 10 percent of developed countries' fuel needs with 1G ethanol [10]. The progressive transition toward indigenous cellulosic second-generation (2G) biofuel



production from first-generation (1G) resources (*e.g.* cereal crops) can potentially facilitate expanded bioenergy production, whilst enabling environmental, economic, and socio-economic benefits in both developing and developed countries.

On the other hand, the potential expansion of the scale of biofuel production from food crops and even crop residues has been subject to debate due to its perceived direct and indirect effects on human food, animal feed and land use [11]. More than 70% of the global agricultural land resources are dedicated to livestock production, particularly pasture and cropland reserved for animal feed crop production [12]. The production of animal products (meat and dairy) is estimated to require more than five-times as much land per unit of nutritional value and twenty-times higher water footprint compared to plant-based equivalents [13,14]. Due to projected changes in human diet, the per capita consumption of animal products in developing countries is expected to increase by more than 70% from current levels by the year 2050, further intensifying pressure to ensure future food security and efficient use of existing agricultural land [15]. Hence, if future biofuel production expansion is not managed properly, it could potentially instigate competition with food crop and animal feed production from the available croplands, resulting the conversion of highly productive cropland and forested areas towards livestock fodder crop production [11,16,17]. Furthermore, recent socio-economic studies for the state of São Paulo (Brazil) have shown that the HDI (particularly the per capita income and education levels) for cattle producing municipalities were significantly lower than municipalities with sugarcane and processing mills, demonstrating the benefit of expanding of the sugarcane sector [18,19]. Hence, the grand challenge for expanding sugarcane based bioenergy in sugarcane and livestock dense regions lies in the economically viable production of bioenergy from existing agricultural land to meet sustainability goals and human development potential, whilst securing future food security [20].



## 1.2. Research motivation

Sugarcane is one of the major agricultural crops mostly planted in developing countries (Brazil, India, China, Southern Africa) and is widely considered as one of the leading candidate bio-energy crops [21]. The sugarcane processing industry typically generates approximately 140 kg dry weight bagasse (fibrous residue after juice extraction) and an equal amount (dry weight) of cane leaf matter (green leaves, tops and trash) per ton of wet harvested cane [22]. Presently, sugarcane bagasse (SCB) is burned in inefficient mill boilers to produce heat and electricity for sugar milling operations, with surplus energy exported to the grid [23,24]. Improvements in the sugar mill operation energy efficiency and investment in more energy efficient power cogeneration technology would liberate surplus bagasse for future biorefinery applications [23,25]. Further, it has previously been common practice to burn sugarcane cane leaf matter (CLM) on the field pre-harvesting to facilitate easier and cheaper sugarcane stalk collection and transportation to the sugar mill [23,26,27]. However, the outlaw of open field cane burning and the adoption of greener mechanical sugarcane harvesting techniques has the potential to release millions of tons of CLM for valorisation to bioethanol, electricity and/or other value-added products in a biorefinery setting [28]. With a saturated global sugar market, the use of these sugarcane residues for bioenergy production or other commodity markets (*e.g.* animal feed, biochemicals) presents an alternative model for adding economic value to sugarcane residues for the sugar industry.

Among the leading thermochemical pretreatment options, steam explosion (StEx) and ammonia fiber expansion (AFEX™, a trademark of MBI International) are two well-studied and scalable technologies (AFEX™ demonstrated at pilot scale and StEx at industrial scale) that are being considered for overcoming biomass recalcitrance, given their different biomass deconstruction patterns (acidic vs alkaline) and potential for near-term integration into existing sugarcane mills [29,30]. Numerous techno-economic estimations have comprehensively shown that integrating 2G biorefineries to existing sugar mills or autonomous distilleries (1G biorefineries) can significantly reduce 2G biofuel production costs by leveraging on existing process utilities including co-generation



(steam and electricity) and wastewater treatment operations [23,31,32]. However, the some of these techno-economic simulations are based on process yields that are projected to be possible in the future and not experimentally validated at industrially relevant conditions. In particular, the experimental data necessary for understanding the pervasive impacts of StEx and AFEX™ industrially relevant downstream enzymatic hydrolysis and fermentation with commercially available and efficient enzyme cocktails and xylose-fermenting ethanologens is lacking in literature. Moreover, the experimental validation of the primary downstream processing bottlenecks for maximising ethanol yields per unit sugarcane cultivation land for StEx and AFEX™-treated SCB and CLM have not been evaluated. The availability of this experimental data would provide valuable insights for developing future techno-economic and life-cycle analysis models that are necessary for comparing StEx or AFEX™-based sugarcane residue 2G biorefineries.

Furthermore, the same leading pretreatment technologies used for improving lignocellulose fungal enzyme digestibility for ethanol production may also be used for improving ruminant (incl. cattle) digestibility of agricultural residues [10,33,34]. Currently, there is no experimental data comparing the effect of StEx and AFEX™ on the animal feed value of SCB and CLM. Given the synergies between the sugarcane production chains for biofuels and livestock production, simultaneously enhancing the ruminant digestible energy content and ethanol yields of AFEX™ and StEx treated sugarcane residues presents an opportunity for more efficient land use for sustainably producing animal feeds (and animal products) and bioenergy compared to the current cereal grain based agricultural system (see Figure 1.2) [35].

The adoption of intensified livestock production practices is another strategy that is being considered for improving the land use efficiency for the livestock production sector whilst allowing the sustainable expansion of biofuel production [39]. However, intensive livestock farms are characterized by significant manure production, which contribute to GHG emissions by the livestock industry. Biogas production from the anaerobic digestion of livestock manure is well-established technology that serves numerous purposes, including: odour management, bioenergy production, and

reduction of GHG emissions from the manures [40]. However, the low C/N ratio in animal manures contributes to anaerobic digestion instability due to nutrient imbalance and ammonia toxicity, thus resulting in low biogas production per unit mass of manure. Alternatively, co-digesting lignocelluloses with animal manures is widely considered a promising strategy to harness the synergies between the two substrates [41]. However, the anaerobic biodegradability of lignocelluloses is limited by its slow rate of hydrolysis, requiring pretreatment to enhance its rate and extent of anaerobic biodegradability [40]. Although pretreatment technologies such as StEx have been investigated for enhancing the anaerobic biodegradability of lignocelluloses, there are no studies investigating the biogas production potential from AFEX™ pretreated lignocelluloses, neither in mono-digestion nor co-digestion with animal manure. The assessment of the co-digestion potential of these pretreated substrates would deepen insights into the potential use of sugarcane residues in intensive animal farms to co-produce bioenergy in the form of biogas, and nutrient rich digestates.

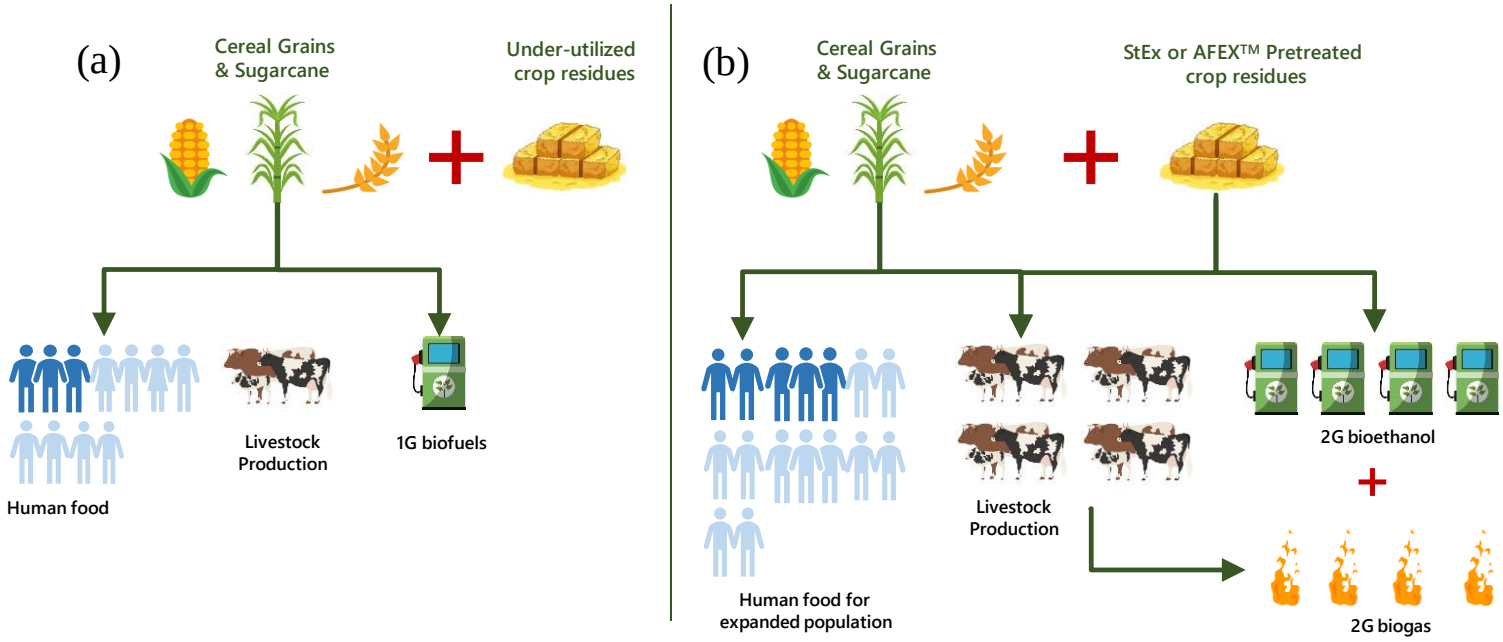



**Figure 1.2:** (**a**) Current agricultural system for producing food, feed and 1G biofuels from cereal grains and sugarcane. (**b**) Future scenario whereby StEx or AFEX™ crop residues complement cereal grains and sugarcane for producing food, feed and 2G bioenergy (bioethanol and biogas).



This integrated approach has the potential to promote more sustainable bioenergy production and increased food production to support the expanding human population and dietary changes while simultaneously mitigating the potential for indirect land use changes (ILUC) [36–38].

## 1.3. Global objective

The global objective of this work was to perform a systematic comparison of the potential use of StEx and AFEX™ to valorize sugarcane residues into cellulosic bioenergy production feedstocks (incl. ethanol and biogas) and animal feeds for future integrated biofuel-animal feed systems. Considering the global objective, the principal aims of this work were:

I. To perform a side-by-side comparison of the pervasive impacts of StEx and AFEX™ on ethanol production from SCB and CLM and to identify the effect of the major processing bottlenecks on estimated ethanol yields per unit sugarcane cultivation area.
II. To experimentally evaluate the potential of pilot-scale StEx and AFEX™ pretreatment to generate sugarcane residues with enhanced animal feed value, whilst simultaneously enabling their use as ethanol production feedstock under industrially relevant conditions.
III. To investigate the potential benefit of co-digesting StEx- or AFEX™-pretreated sugarcane residues with livestock manure as a biogas production and manure management strategy for intensified livestock production farms located in sugarcane dense regions
IV. To study the potential use of a room-temperature Cellulose $III_I$-activation process to enhance the digestibility of StEx- or AFEX™-treated sugarcane residue pellets, thereby allowing for efficient high solids loading enzymatic hydrolysis and fermentation at low enzyme dosages (< 10 mg protein/g glucan).
V. To evaluate the fermentability of steam exploded and non-detoxified whole slurry's using industrial xylose-fermenting *Saccharomyces cerevisiae* strains.



## 1.4. Dissertation Outline

This dissertation is organized in nine chapters (Figure 1.3, next page). **CHAPTER 2** presents a critical literature review of the current state of the cellulosic ethanol industry, sugarcane production in the South African context, the fundamentals of StEx and AFEX™ pretreatment, and the major processing bottlenecks of cellulosic ethanol. Moreover, this chapter delves into the potential use of StEx and AFEX™ to enhance the animal feed value of agricultural residues, and the potential use of pretreatment and co-digestion to enhance anaerobic digestion yields from lignocelluloses. **CHAPTER 3** details the research objectives and research contributions synthesized from the gaps identified from the literature survey. **CHAPTER 4** details the study of the effects of StEx and AFEX on downstream enzymatic hydrolysis and fermentation of SCB and CLM and the identification of the major processing bottlenecks for maximising ethanol yields per unit sugarcane cultivation land. **CHAPTER 5** presents insights into the potential used of pilot-scale StEx and AFEX™ pretreatment in integrated biofuel-livestock production systems to simultaneously enhance the animal value and ethanol production potential of SCB and CLM. **CHAPTER 6** presents a study into the potential incorporation of anaerobic co-digestion StEx or AFEX™ treated sugarcane residues with livestock manure as a bioenergy and manure management strategy for intensive animal feeding operations near sugarcane dense regions. **CHAPTER 7** explores the potential use of a Cellulose $III_I$-activation step to upgrade StEx or AFEX™ sugarcane residue pellets to enable low enzyme dosage ethanol production. **CHAPTER 8** presents a preliminary study for de-bottlenecking ethanol production from StEx treated SCB with the use of industrial xylose-fermenting ethanologens. Lastly, **CHAPTER 9** details a summary of the main findings and perspectives into the potential use of StEx or AFEX™ in integrated biofuel-livestock production systems.

Chapter 1: Introduction

Chapter 2: Literature Review

Chapter 3: Research Objectives and Contributions

Chapters 4,5,6,7,8: Research Contributions

Chapter 9: Conclusions & Perspectives

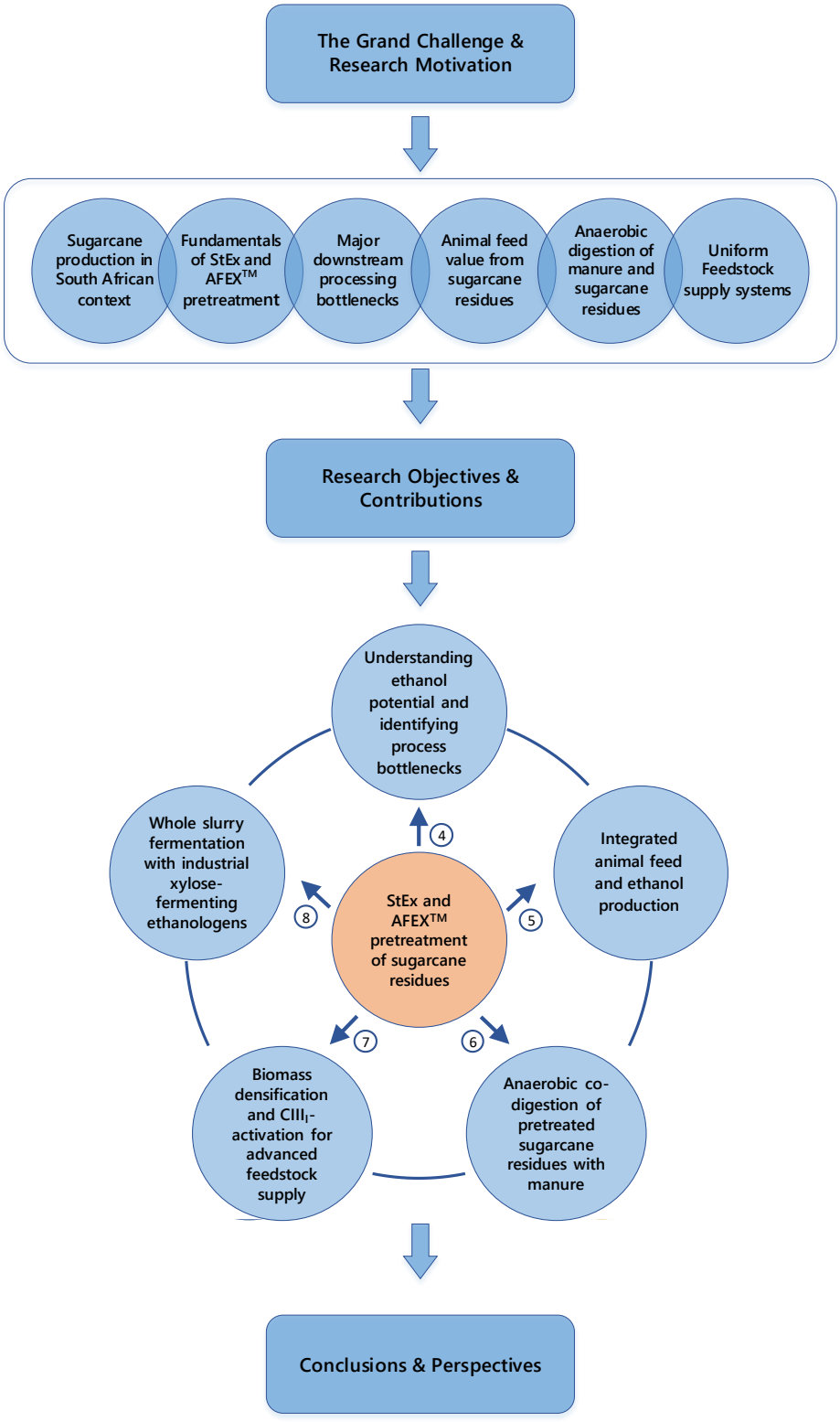



**Figure 1.3:** Layout of the thesis contents



# CHAPTER TWO: LITERATURE REVIEW

This chapter presents an overview of the role of steam explosion and AFEX™ pretreatment as central units in the biochemical production of ethanol, animal feeds and biogas from sugarcane lignocelluloses. Firstly, it introduces the status of the South African sugarcane industry, global cellulosic biorefineries and the South African livestock production sector. Furthermore, the structural composition of lignocellulosic biomass and its contribution to biomass recalcitrance is discussed. The fundamental mechanisms and literature reported progress of overcoming biomass recalcitrance using StEx and AFEX™ are reviewed. For cellulosic ethanol production, this chapter also reviews recent trends to improving the ethanol yield and productivity from agricultural residues.

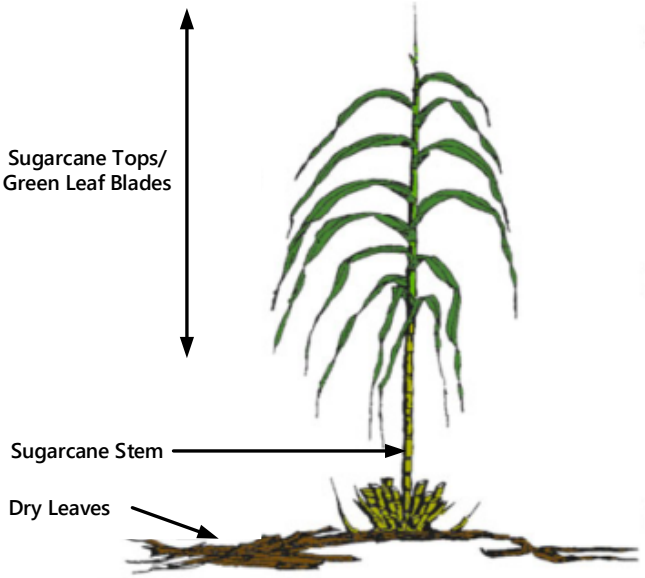
In addition to pretreating sugarcane residues for the ethanol production markets, this chapter also presents insights into the potential use of pretreated sugarcane residues as animal feeds and anaerobic digestion feedstocks for localized bioenergy production. Finally, key gaps in literature identified from the literature survey are summarized.

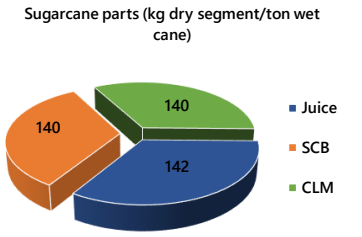
## 2.1 Sugarcane residues based cellulosic biorefineries – South African context

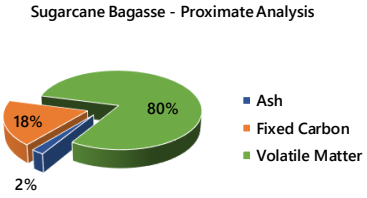
### 2.1.1 *Sugarcane production and residues availability*

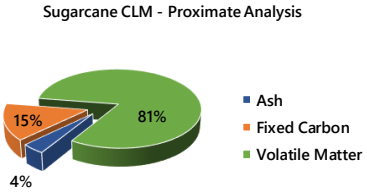
Sugarcane (*Saccharum spp. hybrids*) is one of the major agricultural crops available in South Africa and is widely considered as one of the leading candidate bioenergy crops [21,39]. South Africa is one of the leading cost-competitive sugarcane producers worldwide, with 14 mills distributed in the Kwazulu-Natal, Mpumalanga and Eastern Cape regions and an average mill processing capacity of 300 tons of wet cane per hour [40,41]. Although sugarcane production has declined steadily since 1999 due to land reform, ageing facilities, low global sugar prices and sugar tax tariffs, South African mills still produced approximately 15.07 million tonnes of sugarcane in the 2016/2017 season (Figure 2.1) [42].

In comparison, Brazil, the global leader in sugarcane production, produced 651 million tonnes of sugarcane during the 2016/2017 season (about 35% of the market share), with approximately 52% of the overall cane juice produced used for producing first-generation (1G) ethanol for transportation fuels [43].




| | |
|---|---|
| Commercial Average Yield | 80 tonnes/ha |
| South African Cane Yield (2016/17 season) | 61 tonnes/ha |
| South African Cane Production (2016/17 season) | 15 million tonnes/year |
| Brazilian Sugarcane Yield (2016/17 season) | 75 tonnes/ha |
| Brazilian Sugarcane Production (2016/17 season) | 768 million tonnes/year |
| Global Sugarcane Production (2016/17 season) | 1891 million tonnes/year |

**Figure 2.1:** The sugarcane whole plant morphology, cane yields and sugarcane composition

Mature sugarcane crops are typically composed of approximately 142 kg of dry sugarcane juice (dry basis), 140 kg of SCB, and 140 kg of dry CLM per ton of wet cane [27]. In the South African sugarcane production chain context, CLM has been previously burned on the stalk in open-air to facilitate easier and cheaper bulk stalk collection, storage and transportation to the sugar mill [44,45]. Whilst this is an economically viable CLM disposal option, air burning generates particulate emissions, affects nutrient cycling, and interferes with the soil ecosystem [46,47]. In response to an industry-wide effort to phase-out CLM burning, green-harvesting techniques are now being mandated, with agronomic constraints requiring about 50% of the CLM to be left on the field to sustain soil fertility, and the remaining fraction being available for valorisation into biofuels, bio-chemicals, bio-electricity or animal feeds [26,28,48]. Once harvested, the cane stems are transported to the sugar mills where

they are washed and prepared for cane juice extraction or ethanol fermentation in autonomous ethanol mills (Figure 2.2).

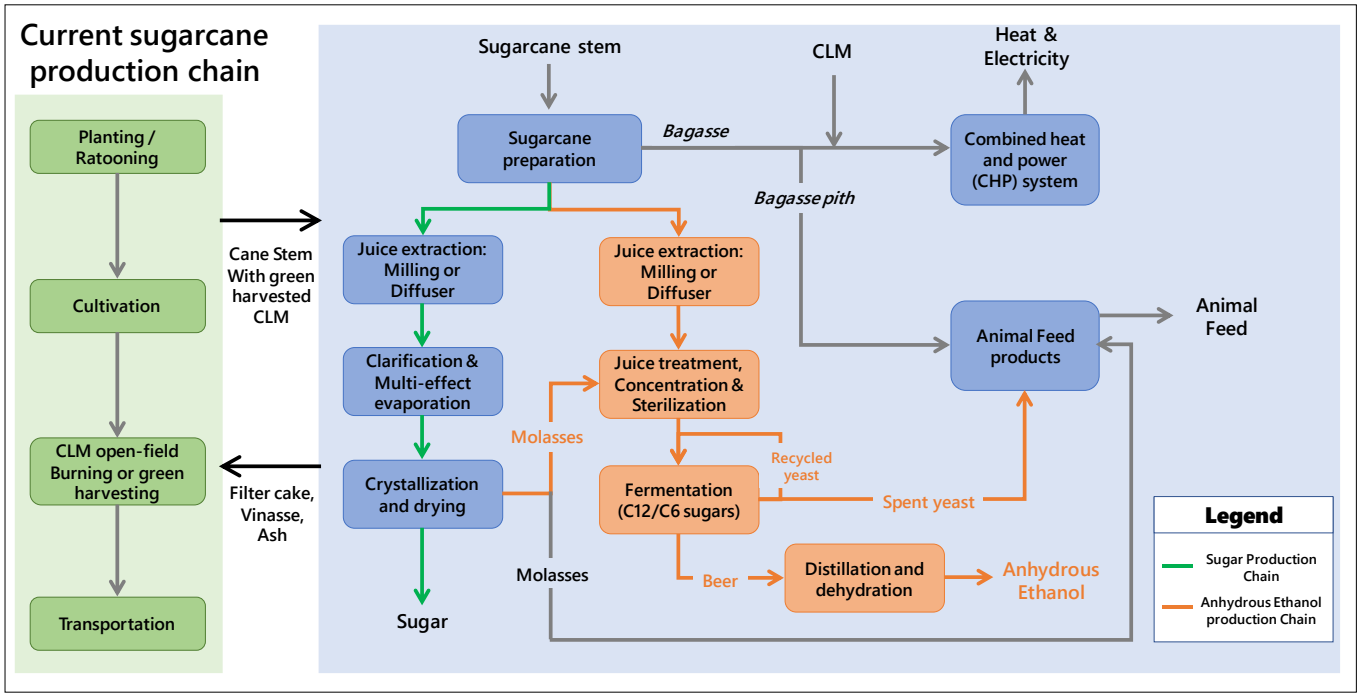



**Figure 1.2:** Sugarcane production value chain for sugar mills and 1G autonomous ethanol distilleries

Sugarcane bagasse is the fibrous residue from the stem after juice extraction [49]. In most Southern African sugar mills, most of the generated bagasse is inefficiently combusted as a solid fuel in cogeneration systems to produce heat and electricity for sugar milling operations with bagasse pith (fine bagasse tissues) used as a molasses carrier for animal feed products, or in the paper-making industry [23,24,50,51]. Improvements in the sugar mill specific steam demand, operation efficiency and investing in more efficient medium-to-high pressure boilers, are recognized as avenues to maximise co-generation of heat and electricity efficiency whilst facilitating the liberation of surplus bagasse for future biorefinery applications [23,25,52]. With the South African sugar industry being under increasing economic pressure due to low global sugar prices, the use of these sugarcane residues (SCB and CLM) for bioenergy production and/or other commodity markets (including animal feeds or biochemicals) presents a variety of alternative greener and sustainable models for adding economic value to sugarcane residues for the long-term survival of the industry.

## 2.1.2 *Current Status and Developments*

### 2.1.2.1 *Commercial second-generation ethanol production sector*

In 2007, the South African Department of Energy announced a five-year Industrial Biofuels Strategy (IBS), to support biofuel production through measures such as fuel tax rebates and mandatory biofuel blending [39]. This strategy identified 1G ethanol production from feedstocks such as sugarcane juice as a starting point to enable a future market penetration of 2% in the domestic liquid transportation fuel sector by 2015. However, the IBS has achieved limited success to date, partly due to insufficient financial incentives to attract investment in 1G ethanol production and high capital costs associated with 2G ethanol production [53].

***Table 2.1:*** Pioneer commercial facilities producing 2G ethanol with capacity ≥ 10 million gallons per year, adapted from Lynd *et al.,* [54] and Obydenkova *et al.,* [55].

| Company | Location | Feedstock | Annual capacity | | | CAPEX (MM $) | CAPEX per Annual Vol. ($/MGY) | Co-products |
|---|---|---|---|---|---|---|---|---|
| | | | Feedstock Capacity (MTY) | Feedstock Supply Radius (km) | Ethanol volume (MGY) ψ | | | |
| Abengoa ɬ | Kansas, USA | Corn stover, straw | 0.33 | 80 | 25 (94.6) | 444.6 | 17.78 | Heat, electricity |
| Beta Renewables ɬ | Crescentino, Italy | *Arundo donax*, rice straw, wheat straw | 0.27 | 70 | 13.4 (50.7) | 171 | 12.76 | Heat, electricity |
| Dow-DuPont ɬ | Iowa, USA | Corn stover | 0.375 | 48 | 30 (113.6) | 500 ɣ | 16.67 ɣ | Solid boiler fuel |
| Granbio | Alagoas, Brazil | SCB/CLM | 0.35 | 20 | 21.6 (81.8) | 265 | 12.27 | Heat, electricity |
| POET/DSM Advanced Biofuels LLC - Project Liberty | Iowa, USA | Corn stover | 0.34 | 56 | 20 (75.7) | 275 | 13.75 | Heat |
| Raizen (expansion of Costa Pinto sugarcane mill) | Piracicaba, Brazil | SCB/CLM | N/A | N/A | 10.6 (40.1) | 102 | 9.62 | Heat, electricity |
| **Average** | - | - | **0.33** | **54.8** | **20.1** | **293** | **13.81** | |

ɬ - Announced plans to exit cellulosic biofuel business; ψ – Ethanol volume in parenthesis in units of Million Litres per Year (MLY)

ɣ - DuPont CAPEX includes feedstock supply infrastructure

MTY – Million bone dry tonnes per year

MGY – Million gallons per year

Over the last 10 years, several pioneer commercial 2G ethanol production biorefineries using agricultural residues as feedstock have been commissioned for operation in the United States and Brazil, with average capital expenditure (CAPEX) per plant amounting to US$293 million (Table 1) [54]. By 2015, six commercial scale cellulosic plants operated by Abengoa (Kansas, USA), Beta-Renewables, (Crescentino, Italy), Dow-DuPont (Iowa, USA), POET/DSM Advanced Biofuels (Iowa, USA), Granbio



(Alagoas, Brazil), and Raizen (Piracicaba, Brazil) were commissioned, featuring an average feedstock capacity of 0.33 million tons per year (MTY) collected from an average radial distance of 54.8 km from the plant [56]. These facilities, which featured dedicated thermochemical biomass pretreatment, downstream enzymatic hydrolysis, fermentation, distillation and dehydration, wastewater treatment and energy cogeneration units, were projected provide an opportunity to assess the readiness of 2G technology and to realise production cost reductions through "learning by doing" [57].

Modest success has been achieved by these pioneer 2G ethanol biorefineries to date. In 2016, global 1G ethanol production from corn grains, sugarcane and sugarcane beet was 98 billion litres, whereas 2G ethanol production was a mere 0.7 billion litres for the same year [58]. The average CAPEX per annual gallon ethanol produced for these standalone 2G pioneer facilities (US$ 13.81/gal) was significantly higher than 1G corn ethanol plants (US$2/gal), demonstrating that high capital costs were a significant impediment to cost-competitive biorefinery replication required to produce high volumes of ethanol to displace a significant fraction of petroleum fuels. Furthermore, Abengoa, Beta-Renewables, and Dow-DuPont have since announced that they were exiting the cellulosic biofuels business, with low oil prices, complex biomass supply chains, cost-competitive lignocellulose conversion (particularly pretreatment and enzyme related costs), bankruptcy, and challenges regarding technology commissioning cited as some of many reasons for departing the cellulosic ethanol production industry [59–61].

However, POET/DSM Advanced Biofuels LCC recently overcame a significant bottleneck in its pretreatment process, allowing them to achieve production yields of 265 litres of ethanol per ton of corn stover (or 70 gal/ton), whilst running pretreatment at 80% uptime [62]. Further, through POET's joint venture with DSM, an on-site enzyme manufacturing facility will be constructed by 2018 to fast-track cellulosic ethanol production in USA. Although these recent developments of POET/DSM provide enthusiasm for the nascent cellulosic biofuel industry, overestimation of technology readiness, complex supply chains, high investment costs (particularly for premature high risk standalone 2G



facilities), and constrained blending mandates are expected to continue to deter the commercial deployment rate of standalone cellulosic biofuel plants [57,61].

Techno-economic valuations of the integration of 2G ethanol production to existing industrial sites (*e.g.* sugar mill or autonomous ethanol distilleries) have comprehensively showed significantly higher Internal Rate of Return (IRR) for the integrated 2G ethanol facilities relative to standalone 2G biorefineries for short-term deployment [44,63]. As observed from Table 2.1, the reported CAPEX per annual gallon of ethanol for the Raizen ethanol production facility integrated to the Costa Pinto sugarcane mill was significantly lower than those for the standalone facilities, demonstrating that the CAPEX disadvantages and overall process economics can be minimized through innovative process integration [64]. Hence, although commercial standalone 2G ethanol biorefineries have achieved limited success to date, research dedicated to the identification of 2G technologies that can be seamlessly integrated to existing industrial sites and their comparison on an economic viability and environmental impact basis may be a key area for stimulating both policy development and investor interest in commercial 2G ethanol production.

#### 2.1.2.2 *Livestock sector*

South Africa is a semi-arid country, with water scarcity, climate change, energy demand and population growth significantly affecting the country's ability to maximise its citizen's HDI [65,66]. According to the South African Bio-Economy Strategy, "14% of the population is already considered vulnerable to food security, with approximately 25% of children younger than six years considered developmentally stunted due to malnutrition" [66]. Consequently, the Bio-Economy Strategy outlines that to successfully transition from fossil to renewable resources, prospective industrial bioeconomies should develop technologies that do not interfere with food production/security [66].

Livestock farming is the largest agricultural sector in South Africa, with price of animal products increasing by 13.3% between the 2008/2009 to 2015/2016 seasons [67]. Approximately half of South Africa's staple cereal agricultural product, maize, is used for animal feed purposes (ruminant and poultry) [65,68]. Whereas the annual maize production has remained relatively constant since the



1970s, maize consumption as human food has steadily increased with the growing population, affecting both the local and regional supply chains. In addition to increasing population, substantial changes in human diets in emerging economies are projected to increase the per capita consumption of animal products (including meat and dairy) by more than 70% by the year 2050 compared to current levels, further intensifying pressure to ensure future food security and efficient land use [15].

Increasing livestock production efficiency through intensification, decreasing the share of animal products in human consumption diets, and reducing livestock feed components that compete with direct human food crop production are three key strategies to curb competition for cereal grains and adverse environmental effects of the livestock sector [38]. For the latter strategy, the utilization of suitably pre-treated lignocelluloses as feedstuffs for animal (especially ruminant) feed could potentially benefit the livestock production sector, particularly in areas whereby supplies of lignocellulose are abundant and areas where cereal grain supplies are limited or primarily utilized for human food [11,69]. According to the South African Department of Agriculture, Forestry & Fisheries, sugarcane producing regions account for a combined 53% (Kwazulu Natal – 20%, Mpumalanga – 10%, Eastern Cape – 23%) of the land used for feeding cattle [67]. Hence, the potential co-generation of biofuel feedstocks and animal feeds from sugarcane residues within the same biorefinery in these regions could be beneficial for minimizing farmland competition with energy crop land production, whilst also freeing cereal grains for monogastric feeds (*e.g.* poultry) or human food [11,33].

## 2.2 Chemical composition of lignocellulosic biomass

The main components of the plant cell wall of lignocellulosic materials are cellulose, hemicellulose, lignin, and ash. Minor components that constitute the remaining fraction of the material include soluble sugars, protein, pectin, lipids, and extractives [70]. The most prominent lignocellulosic materials considered as potential bioenergy sources include hardwood, softwood, forestry residues, agricultural residues, cellulose wastes (*e.g.* recycled paper sludge) and municipal solid waste [71]. Table 2.2 presents a sample of reported chemical compositions of several



lignocellulosic materials obtained from various feedstock groups. The proportion of the main structural components is dependent not only on the feedstock group but on several factors such as the variety of the material, harvest period and season, environmental conditions and geographical location [72].

**Table 2.2:** Chemical composition of different lignocellulosic biomass on a dry weight basis (Redrawn from Davison *et al.,* [73])

| Category | Biomass | Cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|---|
| Agricultural residues | Rice Straw | 34.2 | 24.5 | 11.9 |
| | Corn Stover | 36.4 | 22.6 | 16.6 |
| | Sugarcane bagasse | 35-45 | 23-35 | 15-25 |
| | Sugarcane cane leaf matter | 32-40 | 16-30 | 15-24 |
| Softwood | Spruce | 43 | 26 | 29 |
| | Softwood Stem | 45-50 | 24-40 | 18-25 |
| | Pine | 42-49 | 13-25 | 23-29 |
| Hardwood | Willow | 37 | 23 | 21 |
| | Poplar | 49.9 | 25.1 | 18.1 |
| | Aspen | 51 | 29 | 16 |
| Municipal Solid Waste | Paper | 85-99 | 0-3 | 0-15 |
| | Processed | 47 | 25 | 12 |
| | Newspaper | 40-55 | 25-39 | 18-30 |

The main plant cell wall components form a complex network to render them highly recalcitrant to fungal and bacterial enzymes, hence, it is important to elucidate the fundamental composition and architecture to unlock biomass recalcitrance for bioenergy production [74]. The most important properties of the main structural components of lignocellulose (cellulose, hemicellulose and lignin) are briefly discussed in the subsequent sections.

### 2.2.1 *Cellulose*

Cellulose is the most abundant polysaccharide in biomass available in the biosphere. Celluloses are a linear syndiotactic (alternating spatial arrangement of the side chain) homopolymers consisting of a structural backbone composed of repeating β-(1,4)-linked 4-D glucose units [75]. The degree of polymerization (DP) of the linear chains of glucose units ranges from 100 – 10,000 depending on the source of the lignocellulosic material [76]. Intermolecular and intramolecular hydrogen bonds

involving the hydroxyl groups (OH) and hydrogen atoms of adjacent glucose units enables cellulose to form highly crystalline micro-fibrils with irregular amorphous regions (Figure 2.3) [77]. Furthermore, the presence of hydrogen bonds between the OH groups of cellulose units render the cellulose micro-fibrils hydrophobic [73]. These microfibrils are inter-connected by hemicellulose, pectin and lignin to form larger bundles, *i.e.* macro-fibrils.

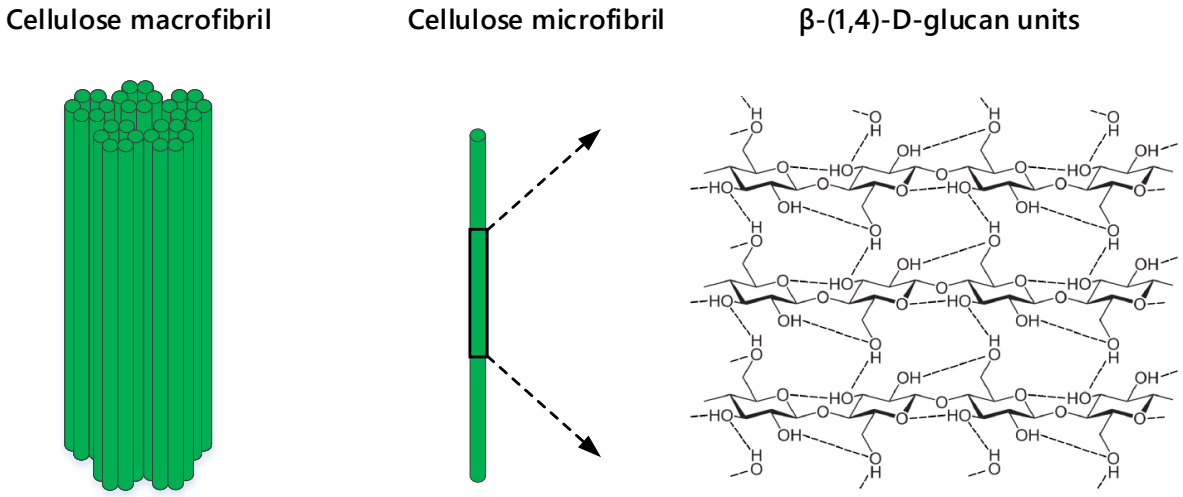



**Figure 2.3:** Graphical representation of the repeating β-(1,4)-D-glucose molecules that compose a cellulose microfibril and macrofibril bundlw

Cellulose of plant species primarily occurs in an allomorph denominated cellulose $I_\beta$ ($CI_\beta$). The $CI_\beta$ regions of the cellulose microfibrils are naturally resistant to biological degradation and therefore require pretreatment to facilitate easier digestion of the polysaccharide to fermentable monosaccharides by fungal or bacterial enzyme attack [78,79]. Some thermochemical pretreatments can transform $CI_\beta$ to other polymorphs with different glucan chain packing. In particular, $CI_\beta$ can be converted to cellulose II by NaOH mercerization or regeneration by ionic liquids, cellulose $III_I$ by amines or liquid ammonia, and cellulose IV by glycerol [74]. However, the amorphous cellulose regions in the native cellulose have higher accessibility, enzyme binding capacity and rate of hydrolysis [80].

### 2.2.2 *Hemicellulose*

Hemicelluloses are polysaccharide polymers that spontaneously bind to the surface of cellulose microfibrils and secure adjacent microfibrils together [81]. They are typically composed of linear and branched heteropolymers of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-

glucose, β-D-galactose) and uronic acids (4-O-methyl-β-D-glucuronic, galacturonic acid) [82]. The composition and frequency of the major backbone and side groups is dependent on the cell tissue and specific plant species. In herbaceous plants and cereal grasses, xyloglucan and arabinoxylan are the most abundant hemicellulose polymers. Xyloglucans consist of a structural backbone of repeating β-(1,4)-linked 4-D glucose units (similar to cellulose) decorated with xylose branches every 3 to 4 glucose units [83]. Arabinoxylans consists of a xylan backbone made up of β-(1,4) bonds of D-xylopyranoside monomer units with frequent branches of *O*-acetyl, xylopyranose, arabinopyranose, galactopyranose and glucoronosyl units as side groups [81]. In monocots, uronic acid (*e.g.* 4-*O*-methyl-glucoronic acid, glucuronic acid) and ferulic acid ester branches are also abundant in arabinoxylans. Ferulic acid residues from arabinoxylan are responsible for cross-linking arabinoxylans to lignin [81,83].

The presence of the arabinose side chains on the arabinoxylan backbone minimizes the presence of hydrogen bonds and as a result, the arabinoxylan fraction of hemicellulose is low in crystallinity [84]. Hence, in contrast to celluloses, hemicelluloses are largely amorphous and are more readily hydrolyzed into the hexose and pentose monomeric sugar components by chemical or enzymatic attack [84].

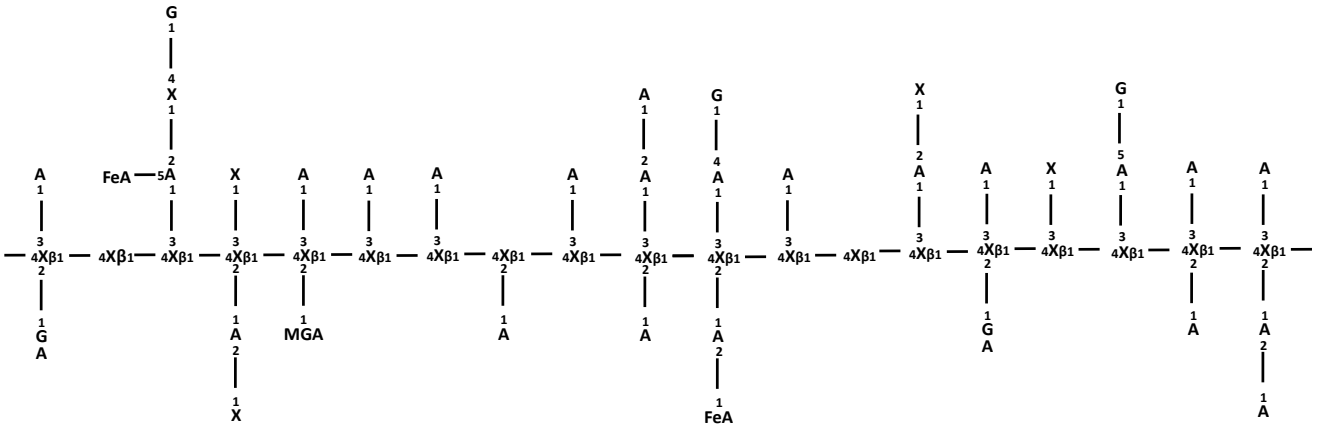



**Figure 2.4:** Representation of hemicellulose (from herbaceous crops and grasses) with major side chains and linkages. (**X** - xylose, **A** – arabinose, **G** – galactose, **GA** – glucoronic acid, **MGA** – 4-O-methyl-glucoronic acid, **FeA** – ferulic acid). Figure redrawn from Deutshmann and Dekker, [82].

Hemicellulose often acts as a physical barrier to cellulose hydrolysis by coating (sheathing) and limiting enzyme accessibility to the cellulose microfibrils in the plant cell wall. For this reason, improvements to enzymatic hydrolysis of cellulose by hemicellulose removal have often been related

to the increase in the accessible surface area [85]. Moreover, other works have reported that acetyl groups inhibit enzyme hydrolytic capacity by interfering with the productive binding of cellulases to the active sites of cellulose in a substrate. Consequently, it has been reported that hemicellulose deacetylation may improve cellulose digestibility through the enhancement of cellulose accessibility to hydrolytic enzymes and minimization of its effect on the enzyme-cellulose binding capacity [86].

### 2.2.3 *Lignin*

Lignin is a heterogeneous polymer of substituted aromatic building blocks and is the largest non-carbohydrate component in the cell wall (typically constitute 10-32% of lignocellulosic biomass) [87]. Lignin is an amorphous polymer composed of phenylpropane units, with their precursors being three monolignol monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2.5) [88]. Lignin arises from the radical coupling reactions of these three primary cinnamyl alcohols, forming a three dimensional highly branched polyphenolic network built of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) moieties, respectively [89,90]. This biosynthesis process produces lignin polymers that lack regular and ordered repeating units, with the relative ratios of the H, G, S units dependent on the taxonomy of the plant species [87]. The phenylpronane units are joined together through various carbon-carbon and carbon-ether bonds, resulting in polymers with DP's of 450-500 units.

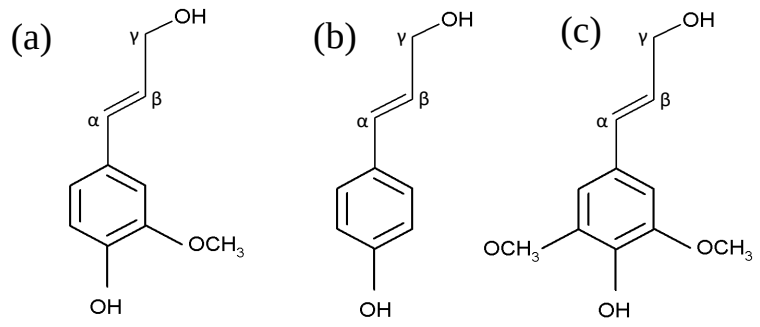



**Figure 2.5:** Lignin monolignols. **(a)** - p-coumaryl alcohol, **(b)** - coniferyl alcohol, **(c)** - sinapyl alcohol (Redrawn from Bunarov and Mazza, [87])

In the plant cell wall, lignin mainly exists as a lignin-carbohydrate complex (LCC). Lignin forms covalent bonds to carbohydrates (particularly hemicellulose polymers) at two sites, *i.e.* α-carbon and C-4 in the benzene ring [87,88,91]. In herbaceous plants, LCC's are formed through the cross-linking of hydrocynnamic acids (ferulic and *p*-coumaric acids) and hemicelluloses (arabinoxylan) to lignin, with ether and ester bonds acting as bridges between them, respectively (Figure 2.6) [90]. As such, the LCC complex acts as a physical barrier in which cellulose microfibrils are interlinked and protected against biological degradation [87,89]. Consequently, the enzymatic digestibility of lignocellulosic biomass can be enhanced with pretreatment methods targeting the cleaving of the ether and ester bonds of the LCC and/or the removal of lignin from the plant cell wall [88].

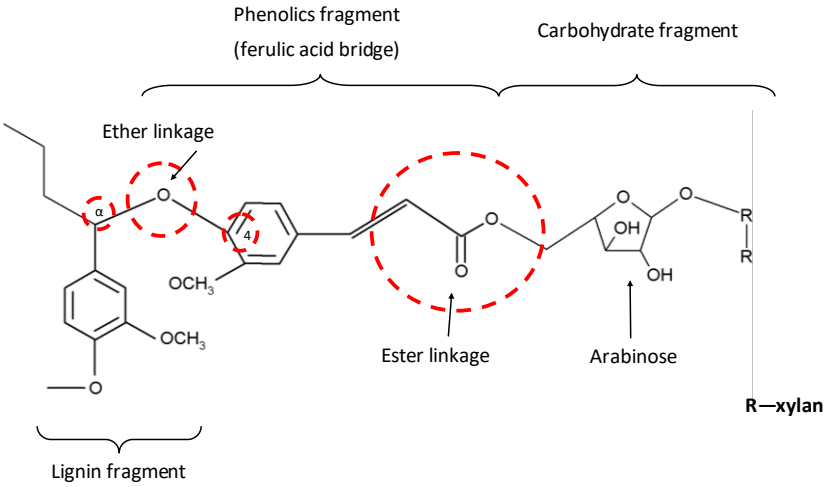



**Figure 2.6:** Lignin carbohydrate complex (LCC) of wheat straw illustrating the polysaccharide - ester - ferulic acid – ether - lignin bonds (Redrawn from Buranov and Mazza, [87])

## 2.3 Overview of cellulosic biorefinery

The production of ethanol from lignocellulosic biomass via a biochemically mediated route typically requires a complex chain of processing steps including: biomass harvesting and transportation, pretreatment of feedstock, on-site or off-site enzyme production, enzymatic hydrolysis of the pretreated biomass to release fermentable sugars, microbial fermentation, ethanol recovery and purification, heat and power cogeneration and wastewater treatment (Figure 2.7) [92]. Many thorough reviews on the overview of cellulosic ethanol production processing steps are available in literature and will only be briefly discussed here [75,93–95]. Biomass, typically harvested

from the field, is transported to the processing plant and pre-processed (*e.g.* reduction of particle size, drying, queuing) in preparation for on-site handling, storage and/or pretreatment. Thereafter, the biomass undergoes pretreatment to alter the structural and/or compositional features thereof to facilitate efficient enzymatic hydrolysis of the carbohydrates embedded in the plant cell wall matrix into fermentable sugars. After pretreatment, the pretreated biomass is converted to ethanol and co-products through enzymatic hydrolysis and fermentation. The enzymes required for hydrolysis can be purchased off-site or produced on-site from fungal strains such as *Trichoderma reesi* or *Aspergillus niger* using sugars generated from pretreatment or purchased purified sugars [95]. Ethanol, the main product from fermentation, is typically recovered via distillation and dehydrated to fuel grade ethanol using advanced technologies such as pervaporation, adsorption, or molecular sieves [93,96]. The distillation bottoms collected are separated into liquid and solid streams, with the solids residues (mostly lignin) used as feed to a power cogeneration plant to provide steam and electricity for the biorefinery. After the biorefinery steam and electricity requirements have been accounted for, surplus electricity is exported onto the grid to generate additional income. The liquid fraction is typically treated via a wastewater treatment unit (*e.g.* anaerobic digestion, polymer resin filtration, coagulation), with the treated water recycled back to the process or released into the environment [97].

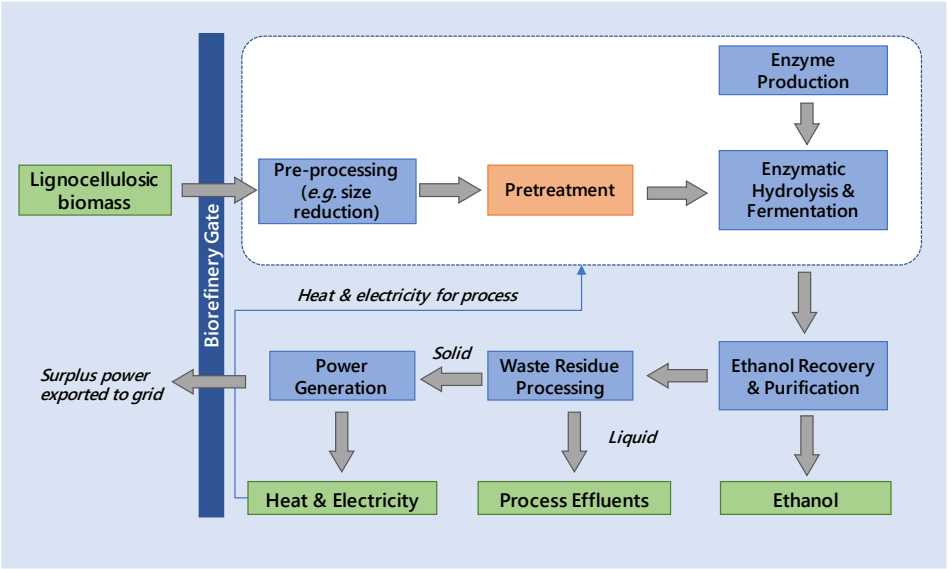



**Figure 2.7:** Process flow diagram illustrating the processing steps for the biologically-mediated conversion of lignocellulosic biomass to ethanol. Redrawn form da Costa Sousa *et al.,* [95]



Several economic models acknowledge that cost-competitive cellulosic ethanol production is dominated by feedstock, capital, and utility (particularly enzyme related) costs and therefore require high product yields [4,44,92,96,98,99]. High product yields reduce (1) feedstock costs by generating more product per unit feedstock, (2) capital costs per unit output by requiring less equipment to process a given amount of feedstock, and (3) operating costs required per unit product [4]. To this end, these techno-economic evaluations have indicated that research-driven improvements in the conversion of biomass to sugars rather than the fermentation of the sugars to ethanol offer greater potential for maximising process yields and subsequently determining the economic feasibility of the biorefinery [99,100]. For this reason, it is important to mature existing pretreatment technologies or to develop technologies that perform critical structural modifications to the plant cell wall to enable easier enzymatic degradation of carbohydrates to fermentable sugars and subsequently curtail the economic impact of overcoming biomass recalcitrance on the overall biorefinery system.

## 2.4 Pretreatment of lignocelluloses for biofuel production

The key technical aims of pretreatment are to cost-effectively generate reactive intermediates for efficient downstream enzymatic hydrolysis and fermentation, whilst minimizing carbohydrate loss due to degradation, inhibitor formation, lignin degradation, and adverse environmental impacts triggered by pretreatment (*e.g.* excess water use, waste generation, greenhouse gas emissions) [95,101,102]. Numerous pretreatment technologies employing either biological, physical, chemical or physio-chemical mechanisms have been investigated, and their advantages and disadvantages thoroughly discussed in several review articles [95,102–104]. Among the leading thermochemical pretreatment options available today, steam explosion (StEx) and ammonia fiber expansion (AFEX™) are two mature and scalable technologies (demonstrated at pilot and industrial scale) that are being considered for overcoming biomass recalcitrance, given their different biomass deconstruction patterns (acidic vs alkaline) and potential for integration into existing sugarcane mills [29,105]. The



differences in AFEX™ and StEx pretreatment mechanisms and previous research findings will be discussed in subsequent sections.

### 2.4.1 *Autocatalyzed Steam Explosion (StEx)*

Autocatalyzed steam explosion (StEx) is a well-known thermochemical pretreatment technology where biomass is exposed to pressurized saturated steam in the temperature range of 160 – 260°C for a period of time followed by a sudden discharge of the biomass to a vessel maintained at atmospheric pressure [104]. The sudden pressure release enables the biomass to undergo an explosive decompression, significantly reducing the biomass particle size and resulting in high surface area fibres. After the pretreatment, a pretreatment slurry composed of a condensed liquid fraction rich in hemicellulose solubilized compounds and a solid fraction enriched in cellulose and lignin [100]. The potential advantages of StEx pretreatment for integration in sugar mill operations include the use of water as a green solvent and catalyst, no chemical recovery costs other than water reclamation, relatively low capital investment, ability to pretreat a wide range of feedstocks (including hardwoods and herbaceous grasses), moderate energy requirements and the ability to accomodate high-moisture content biomass (such as industrial SCB) [100,106–108].

#### 2.4.1.1 *Key reactions and mechanisms during autohydrolysis*

Autoionization of water molecules at high temperatures and pressures leads to the formation of hydronium ($H_3O^+$) and hydroxyl ($OH^-$) ions. Coupled with the high kinetic energy of the system, these protons and hydroxyls act as catalysts to partially cleave acid- and alkali-labile lignin-carbohydrate complexes, hydrogen bonds between hemicellulose and cellulose, and ester-linked *O*-acetyl groups in hemicellulose [106].

The partial de-acetylation of hemicellulose releases acetic acid into the aqueous phase, increasing the proton concentration and catalytic activity of the aqueous solution. The increased acidic hydrothermal conditions facilitate the acid-catalyzed cleavage of glycosidic linkages in arabinoxylan to release short and long-chained xyloologosaccharides (X-OS), arabinooligosacharides (A-OS), and minor amounts of glucooligosaccharides (G-OS) (Figure 2.8) [109]. With increased pretreatment

residence time, temperature or proton concentration, the oligosaccharides are further hydrolysed into pentose monomers (xylose and arabinose) and hexose monomers (glucose, galactose). Under such acidic conditions, xylose and arabinose are both liable to dehydration to furfural, whereas glucose is susceptible to acid-catalyzed dehydration to 5-hydroxymethylfurfural (5-HMF). For harsher pretreatment conditions, 5-HMF degradation yields levulinic acid and formic acid, whereas the furfural can re-absorb onto the solid biomass or further degrade to formic acid and poorly characterized insoluble compounds through polymerization reactions [109].

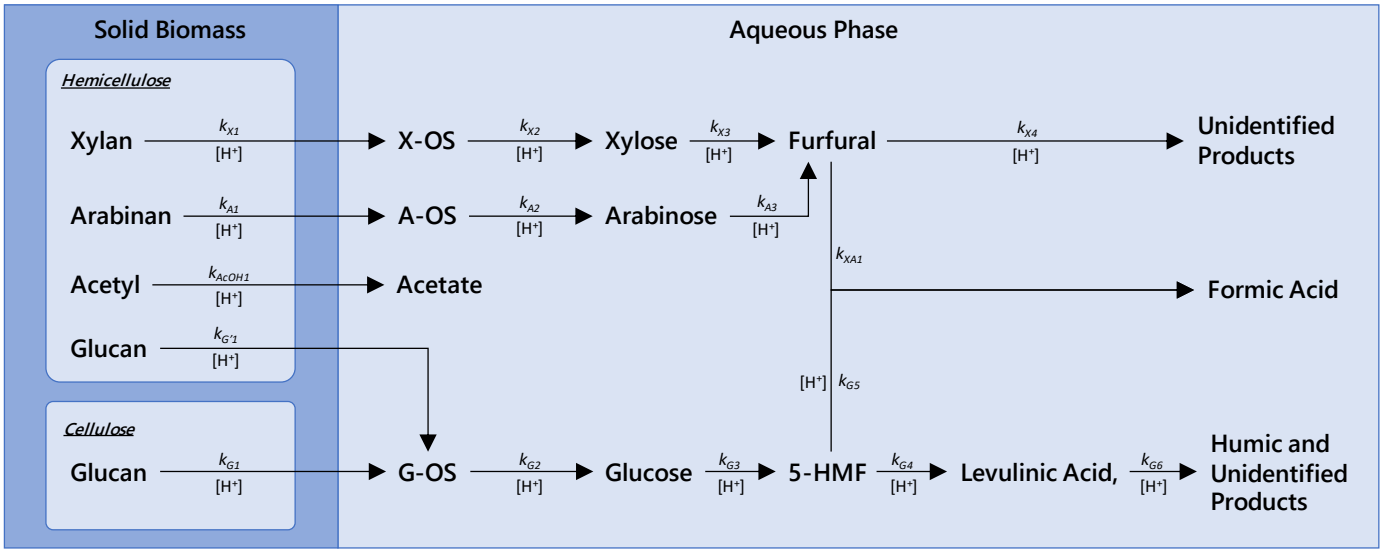



**Figure 2.8:** Schematic representation of the kinetic model of autocatalytic hydrolysis of cellulosic biomass. Modified from Mosier [86]

Recently, the recovery of hemicellulose polysaccharides in predominately oligomeric form has been found when reaction temperatures are low or when the reaction pH was maintained near neutral. The rapid hydrolysis of soluble oligosaccharides to their monomeric constituents under more acidic conditions has been cited as the primary reason for this phenomenon [86]. Hence, to maximise hemicellulose sugar recovery and to limit degradation product formation (*i.e.* furfural,5-HMF), the optimization of pretreatment conditions to maintain the pH close to neutral, the reaction temperature low and the residence long has been suggested [110].

During autocatalyzed pretreatment, lignin melts at temperatures higher than its glass transition temperature ($T_g$), relocates to the outer cell walls, coalesces and redeposits onto the cellulose surface upon cooling as lignin droplets [100]. The formation of lignin droplets on the biomass surface has since



been confirmed with advanced imaging techniques [110]. In addition to morphological changes, lignin is also depolymerized through the acid-catalyzed cleavage of β-*O*-4 ether linkages to form lower molecular weight lignin moieties for hydrothermal pretreatments with higher residence times. However, recent evidence has demonstrated that lignin can repolymerize as evidenced by the presence of large polymers with increased carbon-carbon bonds during infrared-spectrocopy [111]. Moreover, the cleavage of acid-labile lignin macromolecules generates a wide range of phenolic compounds that are inhibitory to downstream enzymatic hydrolysis and microbial fermentation, and therefore need to be minimized during pretreatment. These compounds include: *p*-hydrobenzoic acid, 3,4-dihydrobenzoic acid, 4-hydrobenzaldehyde, vanillin, vanillic acid, dihydroconiferyl alcohol, coniferyl alcohol, syringic acid, syringealdehyde, sinapyl alcohol, *p*-coumaric acid, ferulic acid, and Hibbert's ketones [112].

The proton concentration during StEx can be increased by the addition or impregnation of the biomass with acidifying catalysts such as $H_2SO_3$ (*aq*), $SO_2$ (*g*), or $CO_2$ (*g*). The acidification of the reaction medium reduces thermal energy, pressure, and volume requirements, but also increases capital costs by up to 15% for the reactor design and construction due to the requirement of lining the pretreatment reactor with high-cost corrosion resistant alloys [100]. Furthermore, the addition of acidifying agents increases OPEXs through the requirement of neutralization agents such as ammonia for efficient downstream enzymatic hydrolysis and fermentation.

#### 2.4.1.2 *Impact of StEx on sugar recovery from sugarcane residues*

Among the numerous factors that influence the efficiency of autocatalyzed StEx, the pretreatment temperature, water content and residence time have demonstrated the most significant effect on the sugar recovery in the aqueous phase and after enzymatic hydrolysis. For autocatalyzed StEx, the combined effect of the pretreatment temperature, residence time and the amount of catalyst added can be characterized by a severity factor ($LogR_o$) correlation represented in Equation 2.1

$$log\ (R_o) = log\ \left[t.exp\left(\frac{T-100}{14.75}\right)\right] \qquad \text{Equation 2.1}$$



Where t represents the pretreatment time in minutes, and T is the temperature in degrees Celsius [113]. Table 2.3 presents a summary of selected StEx (autocatalyzed and acid-catalyzed) pretreatment conditions proposed for the pretreatment of SCB and CLM. The glucan and xylan recoveries presented in Table 2.3 are expressed as the sum of glucose and xylose recovered in the aqueous phase (post-pretreatment) and in the enzymatic hydrolysate relative to the glucose and xylose equivalents in the raw material, respectively.

Ewanick and Bura [114] reported the highest combined glucan and xylan recoveries from autocatalyzed StEx pretreated SCB, with xylan and glucan recoveries of 83% and 75% at a pretreatment temperature of 205°C, residence time of 10 min, and an initial moisture content of 80%. However, Carrasco et al., [115] reported significantly lower glucan and xylan recoveries at the same pretreatment conditions, citing xylose loss to degradation during pretreatment as the primary reason for recovering lower xylan yields. The highest glucan recoveries were reported by Amores *et al.,* [116], with the corresponding the glucan and xylan yields being 86.6% and 40%, respectively, at 215 °C and 5 min. Nonetheless, these literature studies suggest that high temperatures (> 200 °C) are required for recovering glucan from StEx pretreated SCB. In contrast, Pal *et al.,* [117] found that low-temperature, long residence time StEx pretreatment conditions favoured high xylan recovery from SCB (primarily in oligomeric form), but the residual solids displayed low enzymatic digestibility.

Only a few studies have considered StEx pretreatment of CLM, with or without acidifying agents. Autocatalyzed StEx pretreatment of CLM in the temperature and residence time ranges of 200 – 210°C and 5 – 15 min, respectively, resulted in glucan and xylan recoveries greater than 80% [9,94]. Ferreira-Leitão *et al.,* [45] found significantly lower xylose degradation during the pretreatment of CLM relative to SCB, with the high ash content of CLM cited as a potential proton neutralizing agent and therefore limiting X-OS hydrolysis to the dehydration susceptible monomeric xylose. This potential neutralization effect of the CLM ash content was also evident from $SO_2$-impregnated StEx pretreated CLM as glucan and xylan recoveries corresponding to 92% and 85%, respectively, demonstrating low xylose degradation during pretreatment [45].

**Table 2.3:** Literature review of steam explosion pretreatment conditions and sugar recoveries from sugarcane bagasse (SCB) and cane leaf matter (CLM). Only studies using an enzyme dosage lower than 20 FPU/g DM were considered.

| Feedstock | Pretreatment conditions | | | | Enzymatic Hydrolysis | | | | | Sugar Recovery | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temp (℃) | Time (min) | Moisture Content (%) | Catalyst Conc. (w/w) | Temp (℃) | pH | Time (h) | Enzyme dosage | Solids loading (% w/w) | % Xylan† | %Glucan† | |
| **Autocatalyzed Steam Explosion** | | | | | | | | | | | | |
| SCB | 215 | 5 | 12,6 | - | 50 | 4.8 | 72 | 15 FPU (NS 50013)/g DM<br>15 IU (NS 50010)/g DM | 5 | 40.3 | 86.8 | Amores *et al.*, [116] |
| SCB | 190 | 5 | 30 | - | 40 | 4.8 | 96 | 15 FPU (1.5 Cellulast)/g DM<br>17 IU (Novozym 188)/g DM | 2 | 47 | 45 | Ferreira – Leitão *et al.*, [45] |
| SCB | 205 | 10 | 80 | - | 50 | 4.8 | 10 | 10 FPU (Spezyme-CP)/g glucan<br>20 CBU (Novozym 188)/g DM | 5 | 83.5 | 75 | Ewanick & Bura, [114] |
| SCB | 205 | 10 | 76 | - | 40 | 4.8 | 72 | 15 FPU (1.5 Cellulast)/g DM<br>17 IU (Novozym 188)/g DM | 2 | 50.4 | 59.8 | Carrasco *et al.*, [115] |
| SCB | 180 | 20 | 70 | - | 50 | 5.0 | 120 | 5 FPU (Cellic® CTec3)/g DM<br>(1% Tween 80) | 10 | 72.1 | 38.3 | Pal et al., [117] |
| CLM | 210 | 5 | 11 | - | 40 | 4.8 | 96 | 15 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 2 | 80 | 82 | Ferreira – Leitão *et al.*, [45] |
| CLM | 200 | 15 | n.a | - | 45 | 4.8 | 72 | 18 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 10 | n.a | 80 | Oliveira *et al.*, [118] |
| **Acid-catalyzed Steam Explosion** | | | | | | | | | | | | |
| SCB | 205 | 15 | 30 | 3% $CO_2$ | 40 | 4.8 | 96 | 15 FPU (1.5 Cellulast)/g DM<br>17 IU (Novozym 188)/g DM | 2 | 51 | 87 | Ferreira – Leitão *et al.*, [45] |
| SCB | 205 | 10 | n.a | 1.1% $SO_2$ | 40 | 4.8 | 96 | 8 FPU (2L Cellulast)/g DM<br>7.8 IU (Novozym 188)/g DM | 5 | 61 | 78 | Martin *et al.*, [119] |
| SCB | 205 | 10 | 79 | 3% $SO_2$ | 50 | 4.8 | 10 | 19 FPU (Spezyme-CP)/g DM<br>38 CBU (Novozym 188)/g DM | 5 | 64.5 | 92 | Ewanick & Bura, [114] |
| SCB | 190 | 5 | 76 | 2% $SO_2$ | 40 | 4.8 | 72 | 15 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 2 | 81.7 | 91.7 | Carrasco *et al.*, [115] |
| SCB | 205 | 10 | 76 | 2% $SO_2$ | 40 | 4.8 | 72 | 15 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 2 | 73.8 | 79.7 | Carrasco *et al.*, [115] |
| CLM | 190 | 5 | 11 | 3% $SO_2$ | 40 | 4.8 | 96 | 16 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 2 | 85 | 92 | Ferreira – Leitão *et al.*, [45] |
| CLM | 210 | 5 | 11 | 3% $CO_2$ | 40 | 4.8 | 96 | 17 FPU (1.5 Cellulast)/g DM<br>23.5 IU (Novozym 188)/g DM | 2 | 60 | 80 | Ferreira – Leitão *et al.*, [45] |

n.a – not available; † - sugar recovery calculated as the sum of monomeric and oligomeric glucose/xylose recovered in the aqueous phase and after enzymatic hydrolysis relative to glucose/xylose equivalents in the raw material before pretreatment.



## 2.4.2 *Ammonia Based Pretreatments*

### 2.4.2.1 *Ammonia Fiber Expansion (AFEX™)*

AFEX™ (trademark of Michigan Biotechnology Institute, Michigan, USA) is an alkaline physiochemical pretreatment in which moist biomass is exposed to subcritical liquid anhydrous ammonia at moderate temperatures (60-140˚C) and high pressure for a period of time (< 30 min) followed by a sudden pressure release [120]. The instantaneous release of the pretreatment pressure prompts the ammonia to flash violently and ultimately disrupting the biomass macro- and ultrastructure. Unlike most low-pH pretreatment technologies, negligible amounts of sugar degradation products are generated after pretreatment as ammonia evaporates [121]. In mature pilot-scale AFEX™ designs, more than 97% the ammonia used in the process can be recovered, recycled and reused. Residual ammonia deposited on the biomass serves as a nitrogen source for downstream microbial fermentation and/or animal feed [120,122]. Detailed mass balances for nitrogen have previously revealed a 300% increase in the total nitrogen content of AFEX™ treated corn stover relative to untreated corn stover [123]. Furthermore, recent work has demonstrated that mature AFEX™ designs can be scaled down to a feed capacity of 100–500 tons/day without significantly affecting processing costs per unit biomass, thereby generating interest in the technology's potential integration to existing industrial sites such as sugar mills for leveraging utilities such as steam and electricity [11].

#### *2.4.2.1.1 Key reactions and mechanisms of AFEX™ pretreatment*

The inherent differences between AFEX™ and other pretreatment technologies lie in certain physical and chemical effects of concentrated ammonia on the plant cell walls. The primary changes imposed by AFEX™ on the plant cell walls include: (1) the cleavage of the lignin carbohydrate cross-links, (2) solubilization and redistribution of hemicellulose/lignin and (3) cellulose decrystallization [120]. During the AFEX™ process, ammonia penetrates the biomass cell wall where in the presence of water it associates to form ammonium ($NH_4^+$) and hydroxyl ($OH^-$) ions that catalyze a series of ammonolytic (amide-forming) and hydrolysis (acid-forming) reactions (Figure 2.9) [83]. These

ammonolysis/hydrolysis reactions are known to cleave various LCC-ester linkages including hemicellulose acetates, *p*-coumarates, ferulates, diferulates, lignin-*p*-coumarates, lignin ferulates, and lignin-diferulates [121,123].

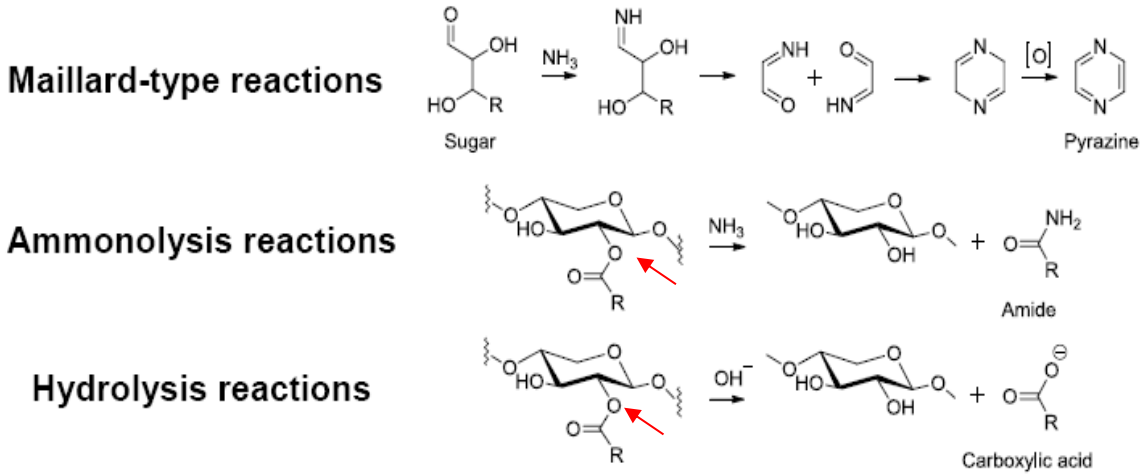



**Figure 2.9:** Schematic description of ammonolysis and hydrolysis reactions of lignin-carbohydrate esters and Maillard-type reactions during AFEX pretreatment. Redrawn from Chundawat *et al.,* [123]

The cleavage of the lignin-carbohydrate crosslinks facilitates the solubilization of hemicellulose oligomers, followed by the removal of lignin and the solubilized hemicellulose polysaccharides to the outer plant cell wall and cell corners [124]. Furthermore, lignin and hemicellulose-derived products from the ammonolysis, hydrolysis, and Maillard reactions (*e.g.* acetamides, phenolic amides, methyl imidazoles, organic acids) are thought to be extracted from the ultra-structure and re-deposited onto the external cell wall [121]. The rapid pressure release at the end of the pretreatment results in the convective transport of ammonium hydroxide and some cell wall components towards the plant cell lumen [121]. Subsequent ammonia vaporization leaves behind outer secondary walls with large pores (> 10nm) [121,124]. The migration of hemicellulose and lignin to the outer surfaces increase the pore volume and subsequently enhance cellulose accessibility to cellulases [120].

Maillard-type reactions involving ammonia, free soluble sugars and high temperatures (usually > 100 °C) have been observed for ammonia-based pretreatments, with imidazoles and pyrazines recently quantified from water extracts of AFEX$^{TM}$-treated corn stover. Although these compounds are



generally produced at significantly lower concentrations relative to the amidation products, they have previously induced toxicity towards cattle in ammoniated animal feed products [125].

#### *2.4.2.1.2 Impact of AFEX™ on sugar recovery from sugarcane residues*

The extent of ester cleavage, hemicellulose/lignin redistribution and the shape, size and special arrangement of the biomass pores depend on the severity of the pretreatment conditions [121]. In particular, the extent of cleavage of LCC-esters has been previously positively correlated to enzymatic hydrolysis yields, indicating that the optimization of AFEX™ pretreatment conditions towards maximal ester cleavage may be an important consideration for overcoming biomass recalcitrance [126]. The key pretreatment variables for AFEX™ are the ammonia-to-dry material (g $NH_3$/g DM) loading ratio, pretreatment temperature, moisture content and the residence time [121,127–129]. A summary of selected sugar yields from selected AFEX™ treated biomass is presented in Table 2.4.

Krishnan *et al.,* [46] evaluated the effect of AFEX™ pretreatment of high-moisture content SCB and CLM on the enzymatic hydrolysis yields by varying the ammonia-to-biomass loading and pretreatment temperature one factor at a time. The moisture content of the SCB (150%, w/w) and CLM (60%, w/w) were not adjusted is in view of avoiding biomass hornification (irreversible loss of fiber water binding ability) and improving downstream hydrolysis yields [10,208]. Within the investigated ranges, both SCB and CLM required high ammonia-to-biomass loadings, with the optimum for AFEX™ conditions for SCB obtained at a pretreatment temperature of 140°C and an ammonia-to-dry bagasse loading of 2 kg $NH_3$/kg DM. In contrast the optimum AFEX™ pretreatment conditions for maximum sugar yields from CLM were obtained at a temperature of 140°C with an ammonia-to-dry leaves loading of 1 kg $NH_3$/kg DM, with the corresponding monomeric glucose and xylose yields being 70% and 60% after 72h enzymatic hydrolysis, respectively. Bals *et al.,* [130] demonstrated that the ammonia loading has the most significant effect on the CAPEX and OPEX of ethanol production from AFEX™. For this reason, the pilot-scale AFEX™ pretreatment unit at Michigan Biotechnology Institute (MBI) is designed to operate at ammonia to biomass loadings lower than 1 kg $NH_3$/kg DM to minimize processing costs and the requirement of make-up ammonia.



**Table 2.4:** Summary of the effect of AFEX™ pretreatment conditions on sugar recoveries from various biomass sources

| Feedstock | Pretreatment conditions | | | | Enzymatic Hydrolysis | | | | | Sugar Recovery | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temp (°C) | Time (min) | Moisture Content (%) | Ammonia loading. (g $NH_3$:g dry DM) | Temp (°C) | pH | Time (h) | Enzyme loading | Solids loading (% w/w) | % Xylose† | %Glucose† | |
| SCB | 120 | 30 | 150 | 1:1 | 50 | 4.8 | 72 | 33mg (Spezyme-CP)/g glucan<br>3.3 mg (Novo 188)/g glucan | 1% glucan | 72 | 55 | Krishnan *et al.*, [46] |
| SCB | 140 | 30 | 150 | 2:1 | 50 | 4.8 | 72 | 33mg (Spezyme-CP)/g glucan<br>3.3 mg (Novo 188)/g glucan | 1% glucan | 76 | 65 | Krishnan *et al.*, [46] |
| CLM | 120 | 30 | 60 | 1:1 | 50 | 4.8 | 72 | 33mg (Spezyme-CP)/g glucan<br>3.3 mg (Novo 188)/g glucan | 1% glucan | 58 | 70 | Krishnan *et al.*, [46] |
| CLM | 140 | 30 | 60 | 1:1 | 50 | 4.8 | 72 | 33mg (Spezyme-CP)/g glucan<br>3.3 mg (Novo 188)/g glucan | 1% glucan | 60 | 70 | Krishnan *et al.*, [46] |
| Upland Switchgrass | 150 | 30 | 200 | 1.5:1 | 50 | 4.8 | 168 | 27 mg (Spezyme-CP + Novo 188 + Multifect Xylanase + Multifect Pectinase)/g glucan | 1% glucan | 78 | 84 | Garlock *et al.*, [131] |
| Lowland Switchgrass | 140 | 30 | 200 | 1.5:1 | 50 | 4.8 | 168 | 27 mg (Spezyme-CP + Novo 188 + Multifect Xylanase + Multifect Pectinase)/g glucan | 1% glucan | 76 | 76 | Garlock *et al.*, [131] |
| Corn stover | 90 | 5 | 60 | 1:1 | 50 | 4.8 | 168 | 15 FPU (Spezyme-CP)/g glucan<br>64 p-NPGU (Novo 188)/g glucan | 1% glucan | 72 | 96 | Teydouri *et al.*, [132] |
| Rice straw | 140 | 30 | 80 | 1:1 | 50 | 4.8 | 168 | 15 FPU (Spezyme-CP)/g glucan<br>64 p-NPGU (Novo 188)/g glucan | 1% glucan | 84 | 85 | Zhong *et al.*, [133] |
| Miscanthus | 160 | 5 | 230 | 2:1 | 50 | 4.8 | 168 | 15 FPU (Spezyme-CP)/g glucan<br>64 p-NPGU (Novo 188)/g glucan | 1% glucan | 70 | 85 | Murnen *et al.*, [134] |
| Miscanthus | 160 | 5 | 230 | 2:1 | 50 | 4.8 | 168 | 15 FPU (Spezyme-CP)/g glucan<br>64 p-NPGU (Novo 188)/g glucan<br>53mg Multifect xylanase/g DM<br>0.35 g Tween 80/g glucan | 1% glucan | 81 | 96 | Murnen *et al.*, [134] |
| DDGS‡ | 70 | 5 | 13 | 0.8:1 | 50 | 4.8 | 168 | 15 FPU (Spezyme-CP)/g glucan<br>64 p-NPGU (Novo 188)/g glucan | 3% glucan | <1% | 97 | Lau *et al.*, [135] |
| Poplar | 180 | 30 | 233 | 2:1 | 50 | 4.8 | 168 | 31mg (Spezyme-CP)/g glucan<br>33 mg (Novo 188)/g glucan | 1% glucan | 35 | 50 | Balan *et al.*, [136] |

† - sugar yield based on the total glucan/xylan available in the dry raw material; ‡ - Dry Distillers and Soluble Grains



Garlock and co-workers [131] reduced ammonia loadings required for AFEX™ pretreatment of wet upland and lowland switchgrass by using a statistical optimization approach. Using a statistical approach to optimize the pretreatment conditions, these authors limited the ammonia-to-biomass loading to 1.5 kg $NH_3$/kg DM for high moisture content (200%, w/w) switchgrass, whilst achieving monomeric glucose and xylose yields greater than 75%.

SCB and CLM recovered from South African sugar mills are typically at moisture contents of approximately 50-60% and 15%, respectively, significantly lower than the moisture levels used by Krishnan *et al.,* [46]. Moreover, it is apparent from Table 2.4 that the AFEX™ pretreatment of high moisture content biomasses generally requires high ammonia loadings to achieve high sugar yields. Hence, these results suggest that there is potential to reduce ammonia loadings from those recommended by Krishnan *et al.,* [46] through the statistical optimization the AFEX™ pretreatment conditions for South African SCB and CLM.

#### 2.4.2.2 *Cellulose $III_I$ forming ammonia pretreatments*

Treatment of native plant biomass cellulose ($CI_\beta$) with anhydrous liquid ammonia is known to produce cellulose $III_I$ ($CIII_I$), a cellulose polymorph that has demonstrated up to five fold increase in cellulose depolymerization rate with improved synergistic effects between endocellulases and exocellulases [79]. Submerging $CI_\beta$ fibres in anhydrous liquid $NH_3$ facilitates the replacement of the OH-O hydrogen bonds between cellulose chains with OH-N bonds, resulting in the formation of a cellulose-$NH_3$ I intermediate. [137–139]. This intermediate complex converts to $CIII_I$ once the ammonia is vaporized/removed, resulting in a cellulose polymorph characterized by a hydrogen bond network with lower intrachain hydrogen bonds and adjusted structural packing of cellulose chains relative to the native $CI_\beta$ [79].

Advanced ammonia pretreatments that include the conversion of $CI_\beta$ to $CIII_I$ have demonstrated high enzymatic digestibility at low enzyme dosages. da Costa Sousa and co-workers [140] pioneered a single-step extractive ammonia (EA) process that used anhydrous liquid ammonia and high temperature conditions to enhance biomass digestibility through the cleavage of lignin-carbohydrate

crosslinks via ammonolysis, extracting more than 40% of lignin and transforming $CI_{\beta}$ to $CIII_{I}$. EA pretreatment of corn stover enabled 60% enzyme dosage reduction (from 18.75 to 7.5 mg protein per g glucan) from high solids loading enzymatic hydrolysis relative to standalone AFEX™. However, the EA process required high ammonia loadings (6 g $NH_3$/g DM) and operating pressures (~8600 kPa), thereby increasing CAPEX and OPEX required for reactor construction and ammonia recovery. Recently, Mittal *et al.,* [141] developed an alternative two stage process where corn stover was pretreated with high temperature (~100 °C) anhydrous ammonia followed by lignin removal using 0.1M NaOH extraction step. This two-step process achieved glucose and xylose yields of 100% and 75%, respectively, using an enzyme dosage of 4 mg protein per g glucan and a solids loading of 1% glucan. However, like the EA pretreatment, this two-stage strategy still required high ammonia loadings and temperatures for the anhydrous ammonia step to fully submerge the low bulk density corn stover and to ensure greater extent of cleavage of ester linkages during the first step of pretreatment. Given these results of $CIII_{I}$-forming ammonia pretreatments for reducing enzyme loading requirements for efficient enzymatic hydrolysis, there lies potential to modify AFEX™ pretreated biomass to produce $CIII_{I}$ in an economically viable fashion, thereby reducing the sensitivity of prospective biorefineries to enzyme costs.

## 2.5 Enzymatic Hydrolysis and Fermentation

Traditionally, pre-treated biomass can be converted to ethanol using five enzymatic hydrolysis/fermentation process configurations, *viz.* Separate Hydrolysis and Fermentation (SHF), Separate Hydrolysis and Co-Fermentation (SHCF), Simultaneous Saccharification and Fermentation (SSF), Simultaneous Saccharification and Co-Fermentation (SSCF), and Consolidated bioprocessing (CBP) (Figure 2.10) [142]. The various process configurations are primarily distinguished by the number of vessels, operating conditions, enzyme production and the co-fermentation capability of the ethanol-producing microorganism (ethanologen).

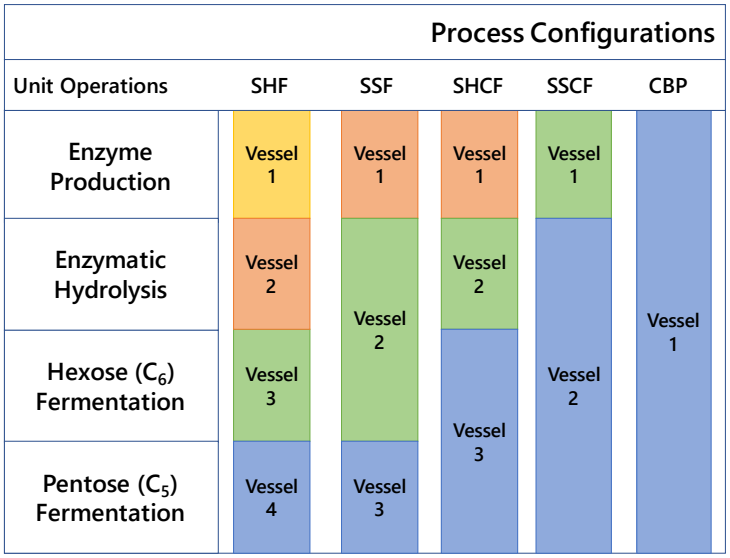



**Figure 2.10:** Process configurations for biofuel production including enzyme production, cellulose hydrolysis, hexose fermentation and pentose fermentation. Redrawn from Lynd *et al.,* [149]

SHF involves four separate vessels for enzyme production, enzymatic hydrolysis, hexose fermentation and pentose fermentation, with each vessel operated at the optimum conditions (*e.g.* temperature, pH, solids loading) for the respective biocatalyst. For example, SHF enables the operation of enzymatic hydrolysis at temperatures and pH's that facilitate maximum cellulase activity (45 – 50°C and 4 – 6 ) and hexose or pentose fermentation in the optimum temperature and pH range for most ethanologens (30 – 37°C and 4 – 7) [143]. For ethanol production systems with an efficient pentose-fermenting ethanologen, SHCF consolidates the hexose and pentose fermentation steps into a single vessel. Despite the ability to operate at the respective hydrolysis and fermentation optimum conditions, the main drawback of SHF/SHCF is the end-product inhibition of the cellulase activity by cellobiose and glucose released during enzymatic hydrolysis. However, latest commercial enzyme preparations such as Cellic® CTec3 consist of enhanced $\beta$-glucosidase activity to minimise enzyme inhibition by cellobiose and higher end-product tolerance, thereby significantly limiting end-product inhibition from SHF processes using end-product tolerant enzyme preparations.

As an alternative strategy to overcome end-product inhibition, SSF combines the enzymatic hydrolysis and fermentation steps, thereby allowing the fermenting microorganisms present in the culture to simultaneously consume the reducing sugars as they are produced [144,145]. Furthermore, SSF processes are easier to operate, and require lower capical costs since enzymatic hydrolysis and



fermentation are integrated into a single vessel. However, SSF operates at sub-optimal conditions for both enzymatic hydrolysis and fermentation and can contribute to ethanol inhibition of many biomass-degrading enzymes, thereby limiting the ethanol yields that can be recovered from the process [144,146]. Like SHCF, the availability of a robust, genetically stable and efficient pentose-fermenting strain allows for the integration of the enzymatic hydrolysis and pentose and hexose fermentation steps into a single vessel (SSCF). In addition to the benefits and disadvantages of the SSF process, further drawbacks of SSCF processes include higher cellulase inhibition by xylo-oligomers, xylose and pretreatment-derived inhibitors [143,147]. The CBP eliminates the need for a dedicated vessel for enzyme production as a single CBP microorganism mediates the production of hydrolytic enzymes and ferments the hydrolyzed reducing sugars into ethanol [148]. To date, CBP is widely considered the ultimate low-cost configuration for enzymatic hydrolysis and fermentation. However, currently available CBP strains have significant limitations to the amounts of hydrolytic enzymes they can produce in addition to having low tolerance to pretreatment-derived inhibitors and ethanol [54,149].

### 2.5.1 *Major enzymatic hydrolysis and fermentation processing bottlenecks in cellulosic biorefineries*

#### 2.5.1.1 *Enzyme dosage and solids effect*

To establish commercially viable cellulosic biorefineries, a major goal for cellulosic ethanol production is to achieve high sugar yields (> 80%) from enzymatic hydrolysis using high solids loadings (> 18%, w/w) and low enzyme dosages (< 10 mg/g glucan). Furthermore, to make ethanol recovery from the downstream distillation step economically feasible, a minimum concentration of 40 $g.L^{-1}$ (4%, w/w) is usually targeted from the fermentation step [92]. Relative to low solids loading enzymatic hydrolysis, higher solids loading processes have the potential to reduce CAPEXs, OPEXs, and process water handling requirements at the biorefinery [147]. Despite these advantages, higher solids loading enzymatic hydrolysis are often limited by the reduction in sugar yields as the initial solids loading is increased.



This phenomenon is often termed the "solids effect", which can be elucidated by various interlinked reasons, including:

I. the lack of free water availability (or lower water activity) in the reactor, thereby presenting a mass transfer limitation to the system by limiting the mass transfer of enzymes to and products away from the substrate binding sites [147],

II. the potential accumulation pretreatment-derived by-products (*i.e.* soluble phenols, aliphatic acids, furan aldehydes) to threshold concentrations that activate enzyme and/or microbial inhibition [150],

III. lower enzyme efficiency due to the higher probability for unproductive enzyme binding to lignin and lignin blockage of the substrate active sites [151,152],

IV. longer hydrolysis times that often bottleneck the entire value chain productivity [147,153],

V. the requirement of high input energy to produce an acceptable shear rate (mixing) due to increased slurry viscosity [153],

VI. cellulase and $\beta$-glucosidase end-product inhibition by the accumulation of XOS, xylose, cellobiose and glucose generated during the hydrolysis reactions [154].

The challenges posed by the "solids effect" typically necessitate the use of high enzyme dosages to achieve high sugar yields from high solids loading enzymatic hydrolysis. Given that the enzyme costs have been previously estimated to account for 15.7% of the total ethanol production costs at enzyme loadings of 20 mg per gram glucan added, it may be necessary to explore processing options that further reduce the required enzyme dosage [92]. Hence, the increasing the solids loading to industrially relevant levels whilst using low enzyme dosages and maintaining high the process yields is one of the major bottlenecks for prospective cellulosic biorefineries. Recent strategies such as designing and optimizing enzyme blends to target inhibitory end-products and maximize enzyme degree of synergy, enzyme recycling (*e.g.* RaBIT process), lignin extraction, and the conversion of the cellulose allomorph to $CIII_I$ have demonstrated enzyme dosage reductions greater than 30% for high solids loading enzymatic hydrolysis and fermentation, thereby presenting potential solutions to unlocking the enzyme dosage-solids loading gridlock [140,155–157].



#### 2.5.1.2 *Enzyme and Microbial inhibition*

High solids loadings also increase the concentrations of pretreatment derived decomposition products in reaction mediums, with enzyme and microbial inhibition being the primary areas affected by the selected pretreatment technology and the subsequent pretreatment conditions adopted [158]. These inhibitory compounds include furan aldehydes (*e.g.* furfural, 5-HMF), phenolic compounds (*e.g.* coniferyl aldehyde, vanillin, phenolic amides, tannic acid, gallic acid), aliphatic carboxylic acids (*e.g.* acetic acid, formic acid, levulinic acid), non-phenolic aromatic acids (*e.g.* cinnamic acid, benzoic acid), quinones (*e.g. p*-benzoquinone), Maillard reaction products, and fermentation metabolites (*e.g.* ethanol, glycerol) [112,119].

AFEX™ has been shown to generate significantly lower concentrations of cell-wall derived inhibitors relative to low-pH pretreatments such as dilute acid pretreatment and StEx, hence enabling efficient enzymatic hydrolysis and fermentation without detoxification, external nutrient supplementation or solids washing [123,135]. However, since AFEX™ does not solubilize hemicelluloses into a separate aqueous phase, the enzyme cocktails employed for AFEX™-treated biomass require hemicellulase and pectinase activities to minimize the inhibition of hydrolytic cellulases by XOS and other high degree of polymerization (DP) oligomers [131,155,159]. Although AFEX™ produces low concentrations of inhibitors, Jin *et al.,* [160] demonstrated that ethanol, fermentation metabolites and decomposition products contributed to the reduction of the maximum specific xylose consumption rate of recombinant *S. cerevisiae* 424A (LNH-ST) in AFEX™ treated corn stover hydrolysate by 31%, 42%, and 13%, respectively. Among the inhibitory compounds, the phenolic amides and aliphatic acids showed the most substantial inhibitory effect.

Unlike AFEX™, StEx generates significant amounts of soluble inhibitors that can synergistically retard enzymatic hydrolysis and inhibit microbial fermentation. For enzymatic hydrolysis, studies by Ximenes *et al.,* [150] and Kumar *et al.,* [154] identified that oligomeric phenolic compounds (including tannic acid), oligomeric (particularly XOSs) and monomeric sugars, and pseudo-lignin moieties are the major enzyme inhibitors from low pH pretreatment technologies. For microbial fermentation, StEx-



generated phenolic compounds (although usually present in lower concentrations) are typically much stronger inhibitors relative to aliphatic acids and furan derivatives [112]. Recently, Martín *et al.,* [161] identified formaldehyde, coniferyl aldehyde, vanillin and to a lesser extent *p*-hydrobenzaldehyde as the major inhibitors during the fermentation of hydrolysates generated from sulphur dioxide impregnated steam exploded softwood. Aliphatic acids such as acetic acid generally cause microbial inhibition through the diffusion of undissociated acids through the yeast plasma membrane, where they release protons and force the cells to expend ATP for transporting the protons out of the cell to prevent acidification [162]. The furan derivatives inhibit yeast glycolysis and alcoholic fermentation, deplete intracellular NAD(P)H and ATP pools and damage intracellular proteins [163]. As a result, high concentrations of 5-HMF and furfural can synergistically act with phenolic compounds, aliphatic acids and fermentation metabolites to inhibit enzyme activity, microbial growth, decrease the volumetric ethanol yield, and/or extend the process lag phase [158].

### 2.5.2 *Processing strategies to mitigate the effect of inhibitors*

Three key strategies to mitigate the effects of inhibitors are to: (1) reducing inhibitor generation through the selection of pretreatment conditions, (2) the discovery/engineering of enzymes and ethanologens with increased resistance to inhibitors, and (3) to remove the inhibitory products through chemical or physical separation methods [164,165]. For StEx, it is inevitable that pretreatment conditions that are typically required to enhance biomass digestibility and reduce enzyme dosage requirements will generate decomposition products that are inhibitory to both the biomass-degrading enzymes and the fermentation microbes. To avoid limiting biomass bioconversion due to the presence of inhibitory compounds, StEx pretreated slurries have previously been separated by means of a solid-liquid separation step to allow for detoxification, recycling of catalysts, or washing the pretreated solids with water to remove soluble inhibitors [146]. The latter approach has been the default processing option for reducing inhibitors in many research works, with the amounts of water required to remove inhibition often not disclosed. Detoxification and washing generally lead to loss in sugars and subsequently increase process complexity, CAPEX and OPEXs due to the need to manage waste



streams, water utilization, and salt disposal [166]. To limit process complexity and its CAPEXs, it is likely that unwashed solids (with lower amounts of soluble inhibitors) or whole slurries (liquid hydrolysate plus solids) will be preferred on an industrial scale [96].

### 2.5.3 *Progress in high solids loading and fermentations*

A summary of selected literature studies demonstrating high ethanol yields, concentrations and productivities from thermochemically pretreated agricultural residues is presented Table 2.5. Among the limited literature studies reporting cellulosic ethanol production from sugarcane residues at industrially relevant conditions, Krishnan *et al.,* [46] evaluated the ethanol production potential from AFEX$^{TM}$ treated SCB and CLM in a SHCF configuration using high enzyme dosage (79 mg protein/g glucan) and high solids loadings (see Table 2.5). AFEX$^{TM}$-treated SCB and CLM achieved ethanol yields of 272 and 250 litres of ethanol per dry tonne and ethanol concentrations lower than the target concentration of 40 g.L$^{-1}$. For both AFEX$^{TM}$-SCB and AFEX$^{TM}$-CLM, xylose conversion was limited to 62.6% and 87%, respectively, with the poor xylose consumption observed for SCB linked to the high ammonia loading pretreatment conditions that are known to produce decomposition products that instigate microbial inhibition of *S. cerevisiae* 424A (LNH-ST). In contrast, Sarks *et al.,* [105] found similar ethanol yields and higher ethanol concentrations for AFEX$^{TM}$-treated corn stover by using lower ammonia loading pretreatment conditions, optimized combinations of commercial enzyme cocktails and lower overall enzyme dosages (~20 mg protein/g glucan).

For low pH pretreatments, cellulosic ethanol production is mostly reported for the pretreated solids (washed or unwashed), with the potential ethanol yields that can be recovered from the pentose-rich aqueous phase (or $C_5$-liquor) often not reported due to the high toxicity of the liquor or the unavailability of an inhibitor-tolerant xylose-fermenting ethanologen. Amores *et al.,* [116] reported high ethanol concentrations (56 g.L$^{-1}$) from StEx-treated SCB under industrially relevant solids loadings of 20% in a SSF configuration. However, neither the fermentability of the $C_5$-liquor nor the ethanol yield (per unit dry untreated biomass) were reported. Similarly, Benjamin *et al.,* [72] reported high ethanol concentrations from unwashed SCB solids derived from dilute acid pretreatment in a SSF



configuration using non-xylose fermenting *S. cerevisiae* MH-1000 and a high enzyme dosage of 61 mg/g glucan. To date, there have been no literature studies demonstrating high ethanol concentrations (> 40 $g.L^{-1}$) from low-pH pretreated SCB or CLM whole slurries with or without detoxification.

Among the newer but less mature pretreatment technologies, Jin *et al.,* [167] demonstrated that combining Extractive Ammonia pretreatment of corn stover with enzyme recycling techniques (*e.g.* RaBIT process) was an effective strategy for lowering enzyme dosages (to 7.5mg/g glucan or 2.85mg/g untreated dry material) whilst achieving ethanol yields, concentrations and productivities of 241 litres per dry ton, 40 $g.L^{-1}$, and 0.57 $g.L^{-1}.h^{-1}$, respectively. In contrast, the combination of low severity pretreatments with mechanical refining (*e.g.* disk milling) and biodetoxification or solids washing has enabled the recovery of high ethanol yields (> 300 litres per ton) and ethanol concentrations (> 85 $g.L^{-1}$) for corn stover using moderate enzyme dosages (20–10 mg /g glucan) [168,169]. Disk milling facilitated high solids loadings (> 25%, w/w) without mass transfer limitations and the subsequent attainment of ethanol concentrations closer to those achieved by 1G corn ethanol or sugarcane ethanol (8 – 12%, w/w) [168]. Nonetheless, although these pretreatment strategies are relatively immature and tested on a few substrates, they provide a basis upon which the optimized 2G ethanol production from StEx and AFEX™ treated SCB and CLM can be compared.

**Table 2.5:** Summary of literature reported ethanol production from agricultural residues with a minimum ethanol concentration of 30 g/L.

| Biomass | Pretreatment | Configuration | Solids Processing Option | Enzyme cocktails & Total Enzyme dosage | Solids Loading (w/w) | Fermentation microorganism | EtOH Yield (L/ton RDM) | EtOH conc. (g/L) | EtOH prod. (g/L/h) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Sugarcane bagasse † | Dilute Acid | SSF | Unwashed solids | 25mg Cellic® CTec2/g pretreated solids<br>36mg Cellic® HTec2/g pretreated solids | 10% | *S. cerevisiae* MH-1000 | n.a | 33 | 0.236 | Benjamin *et al.,* [72] |
| Sugarcane bagasse † | Dilute Acid | SSF | Unwashed solids | 25mg Cellic® CTec2/g pretreated solids<br>36mg Cellic® HTec2/g pretreated solids | 16% | *S. cerevisiae* MH-1000 | n.a | 51 | 0.414 | Benjamin *et al.,* [72] |
| Sugarcane bagasse † | Steam Explosion | PSSF | Washed solids | 20 FPU Novozymes 50013/g WIS<br>20 UI Novozymes 50010/g WIS<br>75 UI Novozymes 50030/g WIS | 20% | *S. cerevisiae* EthanolRed | n.a | 56 | 0,389 | Amores *et al.,* [116] |
| Sugarcane bagasse | AFEX™ | SHCF | No washing, detoxification | 33 mg Spezyme CP/g glucan<br>31 mg Novozyme 188/g glucan<br>15 mg Multifect Xylanase/g glucan | 15% | *S. cerevisiae* 424A (LNH-ST) | 272 | 34 | 0,12 | Krishnan *et al.,* [46] |
| Sugarcane Cane Leaf Matter | AFEX™ | SHCF | No washing, detoxification | 33 mg Spezyme CP/g glucan<br>31 mg Novozyme 188/g glucan<br>15 mg Multifect Xylanase/g glucan | 17% | *S. cerevisiae* 424A (LNH-ST) | 250 | 36 | 0.126 | Krishnan *et al.,* [46] |
| Corn Stover ‡ | AFEX™ | SHCF | No washing, detoxification | 10 mg Cellic® CTec3/g glucan<br>10 mg Cellic® HTec3/g glucan | 20% | *Zymomonas mobilis* 8b | 267 | 55 | 0,65 | Sarks *et al.,* [105] |
| Corn Stover | AFEX™ | SSCF | No washing, detoxification | 24 mg Accellerase 1500/g glucan<br>6 mg Accellerase XY /g glucan<br>6 mg Multifect Pectinase/g glucan | 17.6% | *S. cerevisiae* 424A (LNH-ST) | 243 | 39.9 | 0.23 | Jin et al., [170] |
| Corn Stover ‡ | AFEX™ | RaBIT | No washing, detoxification | 6.9 mg Cellic® CTec3/g glucan<br>6.4 mg Cellic® HTec3/g glucan | 22.1% | *S. cerevisiae* 424A (LNH-ST) | 224 | 40 | 0,57 | Jin *et al.,* [167] |
| Corn Stover | Extractive Ammonia | SHCF | No washing, detoxification | 6.4 mg Cellic® CTec3/g glucan<br>6.8 mg Cellic® HTec3/g glucan | 21.7% | *S. cerevisiae* 424A (LNH-ST) | 218 | 38 | 0,24 | Jin *et al.,* [167] |
| Corn Stover | Extractive Ammonia | RaBIT | No washing, detoxification | 3.9 mg Cellic® CTec3/g glucan<br>3.6 mg Cellic® HTec3/g glucan | 21.7% | *S. cerevisiae* 424A (LNH-ST) | 231 | 40 | 0,55 | Jin *et al.,* [167] |

Symbols: **†** - $C_5$-liquor stream not fermented, no mass balances provided to estimate ethanol yield per ton RDM; ‡ - AFEX™ treated biomass pelletized prior to enzymatic hydrolysis and fermentation

Abbreviations: **RaBIT**: Rapid Bioconversion with Integrated recycle Technology; **RDM**: Raw Dry Material; **EtOH**: Ethanol; **$C_5$-liquor** – hemicellulose rich aqueous phase from pretreatment;

**Table 2.5 (cont.):** Summary of literature reported ethanol production from agricultural residues with a minimum ethanol concentration of 30 g/L

| Biomass | Pretreatment | Configuration | Solids Processing Option | Enzyme cocktails & Total Enzyme dosage | Solids Loading (w/w) | Ethanologen | EtOH Yield (L/ton RDM) | EtOH conc. (g/L) | EtOH prod. (g/L/h) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Corn Stover ϕ | Dry acid pretreatment (DryAP) + disk milling | PSSCF | Disk milling & Biodetoxification | 10 mg Cellic® Ctec2/g glucan in pretreated substrate | 30% | *S. cerevisiae* XH7 | 322 | 85.1 | 0,79 | Liu *et al.,* [169] |
| Corn Stover | Dilute Acid | SHF | Washed solids | 20 mg Cellic® CTec2/g glucan<br>10 mg Cellic® HTec2/g glucan | 10.2% | *S. cerevisiae* 424A (LNH-ST) | 177 | 35 | 0,36 | Uppungudla *et al.,* [171] |
| Corn Stover | Deacetylation + mechanical refining (disk milling) | SHCF | Washed solids | 16 mg Cellic® Ctec3/g glucan<br>4 mg Cellic® HTec3/g glucan | 28% | *Zymomonas mobilis* 13-H-9-2 | 316 | 86 | 0,45 | Chen et al., [168] |
| Corn Stover | Ionic Liquid | SHCF | Washed solids | 11.7 mg Cellic® CTec2/g glucan<br>9.9 mg Cellic® HTec2/g glucan<br>8.4 mg Multifect Pectinase/g glucan | 12.8% | *S. cerevisiae* 424A (LNH-ST) | 260 | 48 | 0,40 | Uppungudla et al., [171] |
| Corn Stover λ | Dilute Alkali + Dilute Acid pretreatment | SHCF | Whole slurry with detoxification | 40 mg Cellic® CTec2/g glucan | 17.5% | *Zymomonas mobilis* 8b | 302 | 63 | 0,29 | Schell et al., [172] |
| Lodgepole pine | SPORL + disk milling | SSCF | $C_5$-liquor concentration + detoxification | 22 FPU Cellic® CTec2/g glucan | 20% | *S. cerevisiae* YRH400 | 285 | 47 | 0.33 | Lan et al., [173] |

Symbols: **ϕ** - Pretreated biomass slurry disk milled, biodetoxified and enzymatic hydrolysis performed in a Helical reactor; **λ** – Sequential two-step pretreatment with furfural removal by $N_2$ gas stripping;

Abbreviations: **SPORL** – Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose; **$C_5$-liquor** – hemicellulose rich aqueous phase from pretreatment;



## 2.6 Animal feeds from sugarcane bagasse and cane leaf matter

### 2.6.1 *Integrating lignocelluloses into livestock production systems*

The potential expansion of the scale of biofuel production from food crops and crop residues has been subject to debate due to its perceived direct and indirect effects on human food, animal feed and land use [11]. Livestock production systems represent the largest anthropic use of land resources, including land dedicated to livestock grazing and cropland dedicated to the production of animal feed crops (*e.g.* green forages and cereal concentrates) [203] . Hence, rapid land allocation to the expansion of biofuel production could have significant ecological (*e.g.* deforestation, loss of productive land) and food security (higher prices of meat and dairy products) impacts if it not managed properly [14].

Integrating existing crop residues or non-edible by-products from arable land production into animal feed diets (particularly ruminants) has been touted as one of the avenues for improving the land-use efficiency of livestock production systems [38]. The advantage of using crop residues as animal feeds is that they do not require “new” land and water allocation since they are derived from the production of primary crops such as cereal grains [174]. In semi-arid regions of Brazil, sugarcane crop residues (such as SCB) are currently being used as sources of roughage in cattle diets, particularly in regions experiencing prolonged periods of drought and low year-round availability of traditional forages and concentrates [175].

However, the potential use of SCB and CLM as animal feeds is limited by their inherent low nutritional value that is associated with their low ruminant digestibility, low palatability, high lignin content, low-level of soluble carbohydrates, and low-level of fermentable nitrogen/protein[176]. As in the case of enzyme hydrolysis, an increase in the nutritional value of lignocelluloses typically requires a pretreatment step to break the lignin seal and disrupt the lignocellulose cell wall network, thereby unlocking the access of energy-rich carbohydrates to ruminant microorganisms [177,178].

There is sufficient literature data demonstrating that 5-10% increases in lignocellulosic biomass digestibility significantly improves the animal performance as measured by its voluntary intake rate,



milk production, or weight gains. Kristjanson and Zerbini [179] showed that increasing the digestibility of sorghum and pearl millet stover through crop breeding or cultivar selection by 1% could increase milk production or beef weight gain efficiency by 6-8%. Similarly, Vogel and Sleper [180] found that genetic engineering and plant breeding of forages to improve their digestibility by 3–5% was associated with up to 24% improvements in livestock productivity. Since energy is the major cost component in livestock diets, enhancing the digestibility of forages (the amount of available nutrients per unit forage) can increase the economic value of the forage and the overall costs associating with ruminant feeding [120,181].

### 2.6.2 *Pretreatment to enhance animal feed value of sugarcane residues*

Traditionally, the dry matter digestibility and crude protein content (nitrogen content) of low-quality forages have been increased using pretreatment processes such as ammoniation [182]. However, high cost of the ammoniation process, the non-recovery of ammonia as well as modest improvements in the ruminant digestibility have limited the use of this process [120].

Steam pretreatment has previously demonstrated significant promise for improving bagasse ruminant *in-vitro* digestibility, with digestibility increases of 23-64% relative to untreated controls reported [177,183]. Furthermore, voluntary dry matter intake of lactating animals was improved by approximately 100% for steam pretreated sugarcane bagasse relative to untreated samples, subsequently supporting the production of 10 litres of milk per day from a steam-pretreated bagasse basal diet [184]. However, animal feed trials have demonstrated that the inclusion of steam-pretreated bagasse elevated levels (>32%) in complete rations significantly reduced the feed palatability and voluntary dry matter intake of beef cattle, thereby limiting its inclusion in cattle diets to intermediate levels [185].

Bench-scale AFEX™ pretreatment was recently shown to increase the *in-vitro* digestibility of 11 different forages and potential energy crops, including sugarcane bagasse [34]. AFEX™ pretreatment of SCB at 1.5 g $NH_3$/g DM for 30 min at 150 °C increased its *in-vitro* digestibility by 68% relative to untreated bagasse. However, as previous described in section 2.4.2.1.2, these high-ammonia loading



and temperature pretreatment conditions are typically associated with inflated CAPEX and OPEX for AFEX™ pretreatment. Moreover, cattle fed basal diets containing forages treated with ammonia recently displayed symptoms of hyperexcitability, possibly due to the formation of imidazole-derived toxins that form during pretreatment through Maillard reactions involving ammonia, reducing sugars and high temperatures (> 100 °C) [120,123,178,186]. A classical symptom of hyperexcitability is the sudden galloping of the cattle in circles [186]. Furthermore, the presence of these compounds beyond threshold concentrations can affect the health of both calve and humans drinking milk from intoxicated ruminants [186]. As a result, the suitability of AFEX™ treated biomass as animal feeds is limited by the potential formation of these Maillard neuro-toxins at pretreatment conditions performed at high temperatures [120]. Recent animal feed trials have demonstrated that low severity AFEX™ pretreated corn stover could substitute for more than 30% of corn grains in Holstein beef steer diets without affecting the livestock productivity or having adverse health effects on the cattle [174]. Hence, adopting lower severity AFEX™ pretreatment conditions for SCB and CLM could simultaneously reduce CAPEX and OPEX associated with pretreatment and minimize the formation of anti-nutritional Maillard products. Carolan *et al.,* [33] estimated that AFEX™ pretreated feeds could be potentially sold for $50-100/ton to the US market and thus improve the overall economics of the cellulose biorefinery.

In South Africa, rations of surplus bagasse pith from sugar mills are typically supplied as animal feed either fresh from the sugar mill or after it has been ensiled [51,187]. In both cases, the pith is supplemented with molasses and other nutritional additives, and pelletized before it is supplied to local animal feed farmers [51]. Therefore, provided AFEX™ and steam explosion sufficiently improve SCB and CLM ruminant digestibility whilst adhere to the local animal feed regulations, there lies potential to integrate animal feed and biofuel production within the same cellulosic ethanol biorefinery (see Figure 2.11) [33,188–190]. Although StEx and AFEX™ have been shown to significantly improve the ruminant digestibility of various agricultural crops, there is no literature reported data providing a side-by-side comparison of using StEx and AFEX™ to simultaneously enhance the

digestibility of SCB and CLM for animal feeds and ethanol production using pretreatment conditions that are relevant to cellulosic biorefineries.

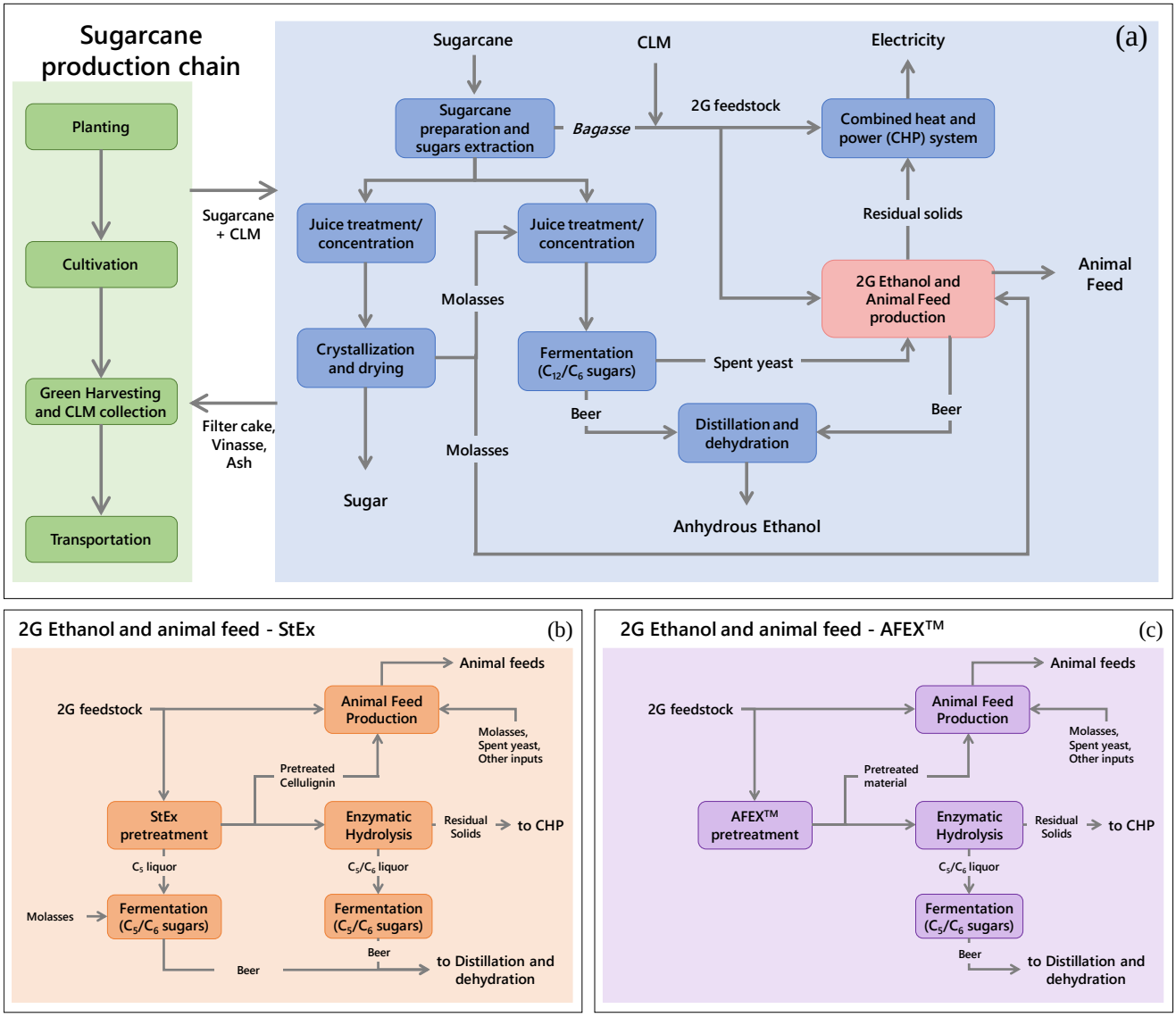



**Figure 2.11:** Using StEx and AFEX™ to produce animal feeds and 2G ethanol feedstocks from sugarcane residues in biorefineries annexed to sugar mills. (a) Integrating pretreatment to sugarcane production chain, (b) 2G ethanol and animal feed production from StEx pretreatment, and (c) 2G ethanol and animal feed production from AFEX™ pretreatment

## 2.7 Anaerobic digestion of sugarcane residues and livestock manure

### 2.7.1 *Anaerobic digestion process*

Anaerobic digestion (AD) is a well-established biologically mediated processes that converts organic matter into a methane-rich biogas in an oxygen-free environment using a consortia of microorganisms [191]. The energy-rich biogas generated from this process can be used for heating, generating electricity, fuel cells, direct vehicle fuel or producing bio-chemicals [192]. The substrates

commonly used in AD include industrial wastewater, municipal sewage waste, animal manure, agricultural waste, waste from food-processing industries, and energy crops [193]. In addition to biogas, the process leaves behind a nutrient rich residue that can be used as fertilizer in agriculture [194].

As shown in Figure 2.12, AD typically consists of four steps, *viz.* hydrolysis, acidogenesis, acetogenesis, and methanogenesis that occur in parallel inside an anaerobic digester vessel. The detailed dynamics of these four steps is thoroughly reviewed in literature and is therefore only briefly discussed in this dissertation [191,195–197]. In the hydrolysis step, large organic polymers, including carbohydrates, proteins and fats, are converted by extracellular enzymes secreted by hydrolytic bacteria to form soluble compounds such as sugars, amino acids and long chain fatty acids (LCFA) [198]. During acidogenesis, the macromolecules from hydrolysis are converted by fermentative bacteria (acidogens) to produce volatile fatty acids (VFA), acetic acid, $H_2$, $CO_2$, alcohols, and ammonia. In the third step, VFAs and alcohols are catabolized by syntrophic bacteria into hydrogen and acetate. The final step involves the conversion of mainly acetate and $H_2/CO_2$ to $CH_4$ and $CO_2$ by strictly anaerobic methanogenic archaea [199]. Approximately 70% of the methane produced is formed from acetate by acetotrophic methanogens, whereas the rest is primarily produced from $H_2/CO_2$ by hydrogentrophic methanogens [200].

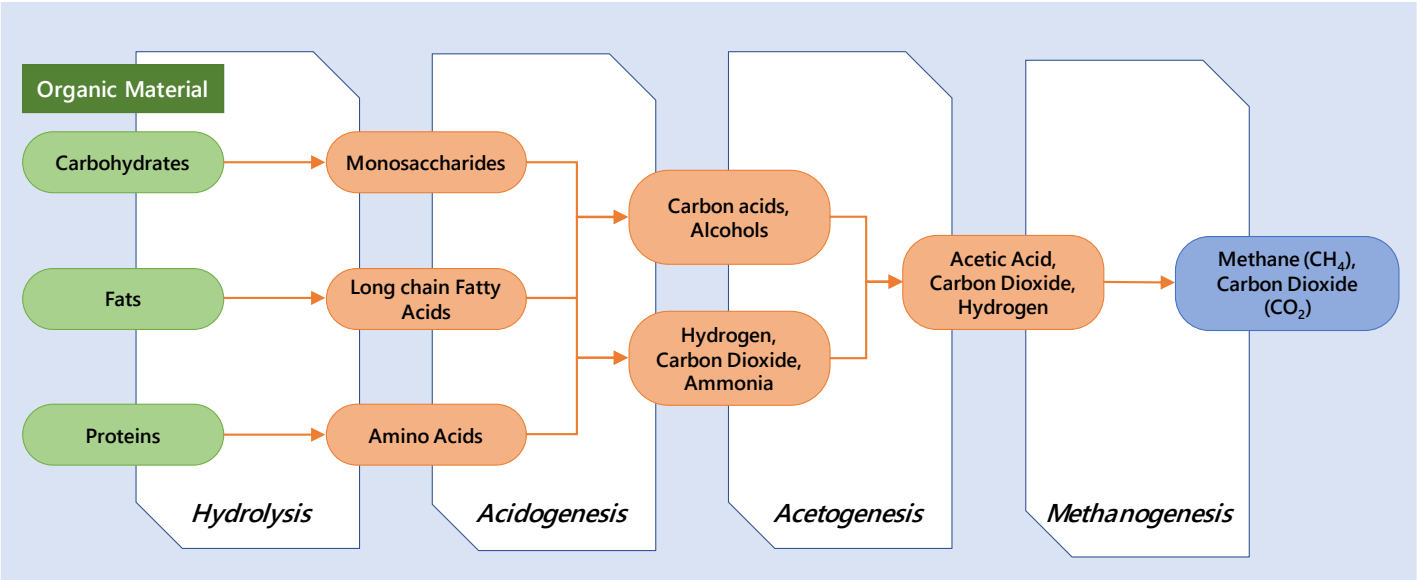



**Figure 2.12:** Schematic representation of the main process steps of anaerobic digestion



The AD microbial community can be easily inhibited by different compounds contained in the inlet feedstocks or released during the digestion process, resulting in an instable AD process with suboptimal methane yields [201,202]. Chen *et al.,* [197] identified ammonia, LCFAs, VFAs, heavy/light metal ions, sulphides, and organics as some of the compounds that contribute to AD instability, with the free ammonia ($NH_3$), LCFAs and VFA accumulation being the most prominent AD inhibitors. Free ammonia typically inhibits anaerobic microorganisms by freely diffusing into the cell, causing proton imbalance and potassium deficiency [197]. LCFA, abundant from the hydrolysis of slaughterhouse waste, vegetable wastes, dairy industrial sludge's, typically adsorb onto the cell membranes of microorganisms, thereby limiting the metabolic transfer of soluble compounds to and from all the AD microorganisms [203]. High VFA concentrations in the inlet substrates or accumulated during the AD process (VFA > 3000 mg/L) contribute to over-acidification, inhibition of the methanogenic community activity, and ultimately result in process failure [191].

Substrates with high carbon to nitrogen (C/N) ratios typically cannot support AD microbiome growth and activity due to nitrogen deficiency, whereas substrates with low C/N ratios lead to lower methane yields due to ammonia inhibition of acetoclastic and hydrogentrophic methanogens and ultimately result in process failure [197]. Furthermore, the extent of ammonia inhibition is aggravated by high pH and temperature AD operating conditions [203]. Hence, the optimum range of C/N ratios for substrates in the range 15:1 to 45:1 is commonly suggested for stable AD [192].

### 2.7.2 *Anaerobic co-digestion of animal manure with lignocelluloses*

AD of animal manures is well-established and serves numerous purposes, including odour management, bioenergy production, elimination of pathogens, reduced water pollution, improved fertilizer value of manure, reduction of GHG emissions from the manures, and economical advantages for the farmers [192,204]. However, the low C/N ratio in animal manures contributes to AD instability due to nutrient imbalance and ammonia toxicity, thus resulting in low biogas production per unit mass of manure. Conversely, lignocelluloses are characterized by high C/N ratios and high volatile solids content but their anaerobic degradability is limited by their low macro- and micronutrient content and



slow degradation rate (hydrolysis being the rate limiting step), resulting in low specific methane yields from mono-substrate AD [191]. Pretreating lignocelluloses using technologies such as alkaline pretreatment or steam explosion, has shown moderate success in improving methane yields from lignocellulosic substrates [198]. Alternatively, co-digesting lignocelluloses with animal manures is widely considered as a promising strategy to harness the synergies between the two substrates by enhancing the digestion nutrient balance (*e.g.* C/N ratio, macro- and micro-nutrients), improving the digestion buffer capacity and potentially mitigating inhibition challenges encountered in mono-digestion [191,192,197].

Table 2.6 and Table 2.7 present selected literature results for the mono-substrate anaerobic digestion of pretreated sugarcane residues and the co-digestion of pretreated lignocellulosic substrates with animal manures, respectively. For non-pretreated SCB and CLM, methane yields in the range 79 – 245 L $CH_4$/kg VS have been reported, with the wide range of methane yields contributed to the different sources of inoculum, different inoculum to solid ratios, the variable nitrogen and soluble sugar contents from the SCB and CLM used in the various studies [193]. De Paoli *et al.,* [205] reported that StEx pretreatment at 200 °C increased the specific methane yields of SCB and CLM from 226 to 258 L $CH_4$/kg VS (14% increase) and CLM from 79 to 181 L $CH_4$/kg VS (129% increase), respectively. In contrast, Risberg *et al.,* [206] investigated the effect of StEx on the potential methane production from wheat straw and did not observe any enhancements to methane yields recovered from StEx-treated wheat straw. However, co-digesting StEx-treated wheat straw with cattle manure increased the specific methane yields by up to 38% due to an improvement in the digestion nutrient balance and dilution of pretreatment derived inhibitors.

Janke and co-workers [207] combined mechanical milling and alkaline pretreatment (using 12g NaOH/g DM) of CLM and reported specific methane yields of 291 L $CH_4$/kg VS, the highest methane yields reported in literature for CLM. The high biodegradability of alkaline pretreated CLM was contributed to the effective cleavage of LCC linkages by concentrated NaOH. However, although the AD process was not inhibited by the high $Na^+$ concentrations, the high $Na^+$ concentrations in the



digestate could potentially limit the applicability of the digestate as fertilizer/soil conditioner due to its potential long-term soil salinization effect. Alternatively, You *et al.,* [208] demonstrated that co-digesting corn stover pretreated using lower alkaline concentrations (6 g NaOH/g DM) with swine manure C/N to achieve a C/N ratio of 25:1 could enhance the methane yields up to 350 L $CH_4$/kg VS.

To date, there are no literature studies evaluating the methane production potential from AFEX™ treated lignocelluloses, neither in mono-digestion nor co-digestion with animal manure. Like the NaOH based alkaline pretreatments, AFEX™ cleaves LCC linkages whilst depositing nitrogen onto the biomass surface to achieve C/N ratio in the range 25 – 35 [35,120,123]. Coincidentally, these C/N ratios are within the recommended optimum range for efficient AD.

**Table 2.6:** Selected literature survey for the effect of pretreating sugarcane residues on the anaerobic mono-substrate digestion efficiency.

| Manure Type | Lignocellulosic substrate | Operation conditions | Lignocellulose Pretreatment | Manure/Residue ratio | Inoculum Source | Methane Yield (L $CH_4$/kg VS) | References |
|---|---|---|---|---|---|---|---|
| - | Sugarcane Bagasse | Mesophilic (± 37.5 °C), C/N = 63 - 89 | No pretreatment | 0/1 | Various sources | 121 – 245 | De Paoli *et al.,* [205] Xu *et al.,* [209] Lima *et al.,* [210] |
| - | Sugarcane Bagasse | Mesophilic (± 37.5 °C), C/N = 64.3 | Steam Explosion (200 °C) | 0/1 | N/A | 258 | De Paoli *et al.,* [205] |
| - | Sugarcane Bagasse | Mesophilic (± 35 °C), C/N = N/A | Hydrothermal pretreatment (200 °C) | 0/1 | Mixture of rumen fluid, sewage sludge, brewery effluent sludge, glycerol sludge | 197 | Costa *et al.,* [211] |
| - | Sugarcane Bagasse | Mesophilic (± 37 °C), C/N = N/A | Hydrothermal pretreatment (180 °C) | 0/1 | Active farm-scale anaerobic digester | 190 | Mustafa *et al.,* [212] |
| - | Sugarcane Bagasse | Mesophilic (± 37 °C), C/N = N/A | 8.5% (w/w) $Ca(OH)_2$ pretreatment | 0/1 | Active farm-scale anaerobic digester | 178 | Mustafa *et al.,* [212] |
| - | Sugarcane CLM | Mesophilic (± 37.5 °C), C/N = 46-54 | No pretreatment | 0/1 | Various sources | 79 – 231 | De Paoli *et al.,* [205] Janke et al., [213] |
| - | Sugarcane CLM | Mesophilic (± 37.5 °C), C/N = 38.6 | Steam Explosion (190 °C) | 0/1 | N/A | 229 | De Paoli *et al.,* [205] |
| - | Sugarcane CLM | Mesophilic (± 37.5 °C), C/N = 38.6 | Steam Explosion (200 °C) | 0/1 | N/A | 181 | De Paoli *et al.,* [205] |
| - | Sugarcane CLM | Mesophilic (± 38 °C), C/N = N/A | Combined mechanical and 12 % (w/w) NaOH pretreatment | 0/1 | Biogas plant treating maize silage and cattle manure | 291 | Janke et al., [207] |
| Cattle Manure | - | Mesophilic (± 37 °C) & Thermophilic (± 54 °C) C/N = 8-32 | No pretreatment | 1/0 | Farm-scale anaerobic digester | 150 - 240 | Valli *et al.,* [214] Lehtomäki *et al.,* [215] |

VS – volatile solids; N/A – not available from the referenced study;

**Table 2.7:** Selected literature survey for the effect of co-digesting lignocelluloses with animal manures on the specific methane yields that can be recovered.

| Manure Type | Lignocellulosic substrate | Operation condition | Lignocellulose Pretreatment | Manure/Residue ratio | Inoculum Source | Methane Yield (L $CH_4$/kg VS) | References |
|---|---|---|---|---|---|---|---|
| Fish Waste | Sugarcane Bagasse | Mesophilic (± 37 °C), C/N = 49 | No pretreatment | 1/1 | Municipal anaerobic digester | 359 | Xu *et al.,* [209] |
| Pig + Dairy Manure (1/1) | Sugarcane CLM | Mesophilic (± 36 °C), C/N = 29 | 6% (w/w) NaOH pretreatment | 1.6/1 | Active lab-scale mesophilic anaerobic digester | 225 | Luo *et al.,* [216] |
| Cattle manure | Palm pressed fiber | Mesophilic (± 37 °C), C/N = 46.6 | No pretreatment | 1/3 | Active anaerobic digester using swine and chicken manure | 346 | Bah *et al.,* [217] |
| Cattle manure | Corn Stover | Mesophilic (± 35 °C), C/N = 32 - 45 | NaOH pretreatment | 1/1 | Sludge from mesophilic anaerobic digester | 194 | Li *et al.,* [218] |
| Cattle manure | Cotton Stalk | Mesophilic (± 35 °C), C/N = 25 | 0,9% (w/w) $H_2SO_4$ pretreatment | 1/1 | Wastewater treatment plant | 267 | Cheng *et al.,* [219] |
| Cattle manure | Cotton Stalk | Mesophilic (± 35 °C), C/N = 25 | 6% (w/w) NaOH pretreatment | 1/1 | Wastewater treatment plant | 296 | Cheng *et al.,* [219] |
| Cattle manure | Salix | Mesophilic (± 37 °C), C/N = 23 - 39 | Steam Explosion | 53/47 | Sludge from mesophilic anaerobic digester | 235 | Estevez *et al.,* [220] |
| Cattle manure | Wheat Straw | Mesophilic (± 37 °C), C/N = 11-75 | Steam Explosion | 75/25<br>26/74<br>100/0 | Active mesophilic anaerobic digester using household waste and grass silage | 130 - 210 | Risberg *et al.,* [206] |
| Dairy Manure | Switchgrass | Mesophilic (± 37 °C), C/N = 29.4 | No pretreatment | 1/1 | Sludge from lab-scale digester | 158.6 | Zheng et al., [221] |
| Cattle Manure | Rice Straw | Mesophilic (± 37 °C), C/N = 20-30 | No pretreatment | 1/1 | Sludge from anaerobic digester treating pig manure | 383 | Li *et al.*, [222] |
| Swine Manure | Corn Stover | Mesophilic (± 35 °C), C/N = 25 | 6% (w/w) NaOH pretreatment | N/A | Sludge from anaerobic digester of corn stover and swine manure | 350 | You *et al.,* [208] |

VS – volatile solids;



### 2.7.3 *Anaerobic digestion in integrated biofuel-livestock production systems*

In addition to reducing the amount of human food competing components in livestock diets, the adoption of intensified livestock production practices is another strategy for improving the land use efficiency for the livestock production sector whilst allowing the sustainable expansion of biofuel production [5,36,38,204,223]. However, intensive systems generate surplus manure that significantly contributes to manure disposal costs, groundwater contamination and the greenhouse gas (GHG) footprint of livestock farming [192].

Integrating anaerobic digestion to sugarcane residue-based biofuel-livestock production systems presents a potential strategy for producing farm-based or centralized bioenergy and reducing waste generated by livestock effluents from intensive livestock farming. Anaerobic co-digestion of sugarcane residues with livestock manure can improve the overall biogas production capacity whilst facilitating the application of the nutrient rich digestates to the sugarcane cultivation fields and the storage stable carbon in the soil (see Figure 2.13) [224]. Moreover, surplus digestate can be sold to nearby farms as partial mineral fertilizer replacement or as animal bedding to generate additional income [225,226]. Since prospective sugarcane based cellulosic biorefineries are expected to have an anaerobic digestion-based wastewater treatment circuit, further integration benefits can be realized when manure from intensive animal farms near the biorefinery is transported and co-digested with sugarcane residues at the sugar biorefinery.

Lauer *et al.,* [227] recently studied the economic impact of integrating anaerobic digestion to intensive livestock farms in Idaho (USA) and reported that the biogas yield per animal represents the most significant effect on the overall process economic viability. Furthermore, these authors demonstrated that a minimum of 3000 cattle head per farm would be required to enable centralised biogas production to be profitable whilst reducing the environmental impacts associated with intensive livestock farms. However, with lower methane yields expected from the mono-digestion of livestock manure, co-digesting livestock manure from intensive systems with high carbon

lignocelluloses such as sugarcane residues can increase the overall biogas yields per animal and therefore potentially reduce the number of animals required per farm to be economically viable.

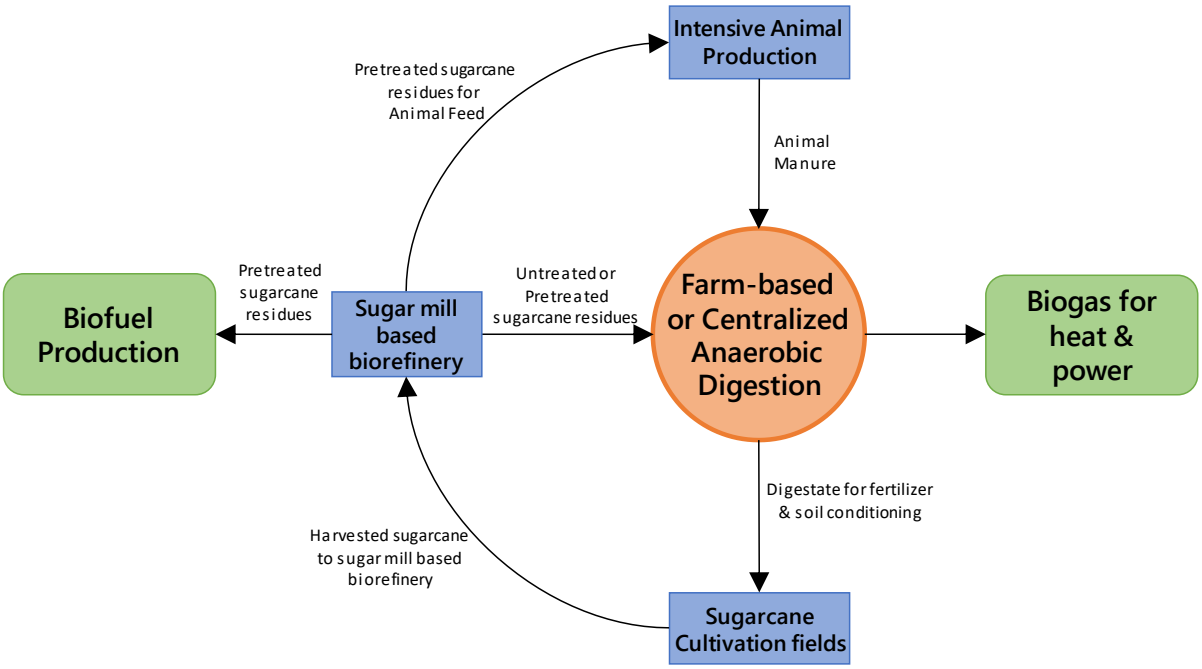



**Figure 2.13:** Incorporating anaerobic digestion into integrated biofuel-livestock production systems produce energy and recycle nutrients. Modified from Holm-Nielsen *et al.,* [180]

## 2.8 Conclusions from literature review

Steam explosion and AFEX™ are among the leading thermochemical pretreatment technologies that are considered for integration into existing industrial sites such as sugar mills or autonomous 1G ethanol distilleries. The following five key gaps were identified from the literature review pertaining to the potential use of these two pretreatment technologies for integrated biofuel, animal feed and/or biogas production:

### 2.8.1 *Side-by-side comparison of ethanol yields from StEx and AFEX™*

To understand the economic and environmental impacts of StEx and AFEX™ pretreatment, a systematic evaluation of the effect of these two pretreatment technologies on the overall process ethanol yield, final ethanol concentration and overall process rate (productivity) is required to determine key process bottlenecks associated with these pretreatment technologies. There are no literature studies demonstrating a side-by-side and systematic comparison of the downstream



impacts of StEx and AFEX™ pretreatment for biofuel production for any biomass. Furthermore, whereas process bottlenecks from StEx and AFEX™-treated biomass are typically discussed in literature, the extent to which they limit ethanol yields under industrially relevant enzyme and solids loadings is not discussed in literature.

### 2.8.2 *Using StEx and AFEX™ to enhance the animal feed value of sugarcane residues*

Both AFEX™ and StEx have demonstrated significant promise for enhancing the animal feed value of agricultural grasses such as corn stover and wheat straw. However, little effort has been done to compare the potential for StEx and AFEX™ to simultaneously generate enhanced animal feeds and biofuel feedstock from sugarcane residues for sugarcane based biorefineries. Given that livestock production represents the largest anthropic use of agricultural land, integrating animal feed and biofuel production could potentially facilitate increase the agricultural output per hectare without increasing the area of sugarcane cultivated.

### 2.8.3 *Anaerobic mono-digestion and co-digestion of StEx and AFEX™-treated sugarcane residues*

Anaerobic digestion is well-known technology that has been demonstrated as a strategy for managing organic waste from various sectors. However, there are no literature studies describing the potential methane yields that can be recovered from AFEX™ treated sugarcane residues, even though AFEX™ significantly alters the structure of biomass and fixes biodegradable nitrogen onto the biomass. In addition, there is no literature data describing the potential co-digestion of StEx or AFEX™-treated sugarcane residues with livestock manure. The assessment of the co-digestion potential of these pretreated substrates would deepen insights into the potential use of sugarcane residues in intensive animal farms to co-produce bioenergy in the form of biogas, and nutrient rich digestates.



### 2.8.4 *Upgrading AFEX™ or StEx pretreated biomass via Cellulose $III_I$*

Cellulose $III_I$ ($CIII_I$) is a cellulose polymorph that has demonstrated up to five-fold increase in cellulose depolymerization rate with improved synergistic effects between endocellulases and exocellulases. Previous pretreatment technologies (*e.g.* extractive ammonia) that activate $CIII_I$ have shown more than 60% reduction in enzyme dosage from industrially relevant ethanol production. However, these technologies have previously relied on high ammonia loadings and high operating pressures to simultaneously activate $CIII_I$ and achieve significant cleavage of lignin-carbohydrate complexes. Although liquid ammonia is known to transform native cellulose to $CIII_I$ even at room temperature, there is no literature data exploring the potential use of a room temperature $CIII_I$-activating process to upgrade the allomorph of pretreated biomass in view of lowering enzyme dosage requirements for ethanol production of sugarcane residues.

### 2.8.5 *Evaluating the fermentability of steam explosion whole slurries using hardened ethanologens*

Microbial inhibition due to stresses imposed by the toxicity of pretreatment derived compounds is one of the key areas limiting ethanol yields from StEx pretreatment whole slurries. In particular, most recombinant ethanologens engineered to metabolise xylose typically show low inhibitor tolerance phenotypes during whole slurry fermentations. Recently, several laboratory and industrial ethanologens have been developed for glucose and xylose fermentation in lignocellulosic hydrolysates. However, there haven't been any studies evaluating and comparing the performance of industrial xylose-fermenting *S. cerevisiae* strains in non-detoxified and inhibitor-laden StEx whole slurry's relative to fermentable AFEX™ treated biomass.

The research contributions addressing these five key areas are discussed in **CHAPTER 3** and their relevance for prospective sugarcane residue-based bioenergy-livestock production systems will be supported by experimental results and discussion presented in **CHAPTERS 4,5,6,7** and **8**.



# CHAPTER THREE: RESEARCH OBJECTIVES

A critical literature survey highlighted that both sugarcane bagasse and cane leaf matter shown significant promise as primary or supplementary feedstock to sugarcane based biorefineries. Moreover, the co-production of animal feeds could provide a more sustainable approach for increasing land use efficiency and mitigating indirect land use change impacts for expanding biofuel markets. This chapter extends on the principal research aims introduced in **Chapter 1** and the research gaps presented in **Chapter 2**, while highlighting the novel research contributions corresponding each research objective.

## 3.1 Contribution 1: Exploring the ethanol production potential from AFEX™ and StEx-treated sugarcane residues

### 3.1.1 *Statement of Novelty*

A major fundamental difference between AFEX™ and StEx pretreatment mechanisms is that the latter pretreatment typically generates significant amounts of hemicellulose- and cellulose-derived degradation compounds that inhibit enzymatic hydrolysis and fermentation. Furthermore, whereas AFEX™ will disrupt chemical bonds in lignocellulosic components, it will not solubilise a significant portion of the biomass, while StEx prompts significant hemicellulose solubilization together with minor portions of cellulose and lignin. To avoid limiting biomass conversion due to the presence of inhibitory compounds, most literature studies typically adopt a default processing strategy of separating the StEx pretreatment slurry by means of a solid-liquid step, followed by washing the residual solid with water to remove the inhibitors. However, on an industrial scale, it is likely that either unwashed solids or whole slurries (liquid plus solids) will be preferred downstream processing strategies in view of minimizing water consumption and the corresponding water recovery costs. Yet, there are no literature studies reporting ethanol yields or ethanol concentrations that can be

recovered from StEx-treated SCB or CLM whole slurries without detoxification under industrially relevant solids loadings. Furthermore, there is limited literature data to elucidate the extent of microbial inhibition of the pentose-rich liquor (or $C_5$-liquor) stream generated from StEx pretreatment on the overall process yield, ethanol concentration and productivity.

This contribution presents, for the first time, a systematic evaluation and side-by-side comparison of the pervasive impacts of StEx and AFEX™ pretreatment of SCB and CLM on the overall process yield, final ethanol concentration and productivity for all processing streams. To understand the benefits of solid-liquid separation and washing to mitigate enzymatic and microbial inhibition effects, StEx pretreatment slurries will be processed via three processing options, *i.e.* whole slurry, unwashed solids with separate $C_5$-liquor fermentation, and washed solids with separate $C_5$-liquor fermentation. Moreover, this contribution identifies key process bottlenecks and potential integration strategies for maximising ethanol yields per unit sugarcane cultivation area for sugarcane based biorefineries. Ultimately, this contribution provides essential data and insights that will enable later economic and environmental impact evaluations of the various processing options for future StEx or AFEX™-based sugarcane residue ethanol biorefineries. The integration of AFEX™ and StEx into sugar mills or 1G ethanol distilleries is summarised in Figure 3.1 below.

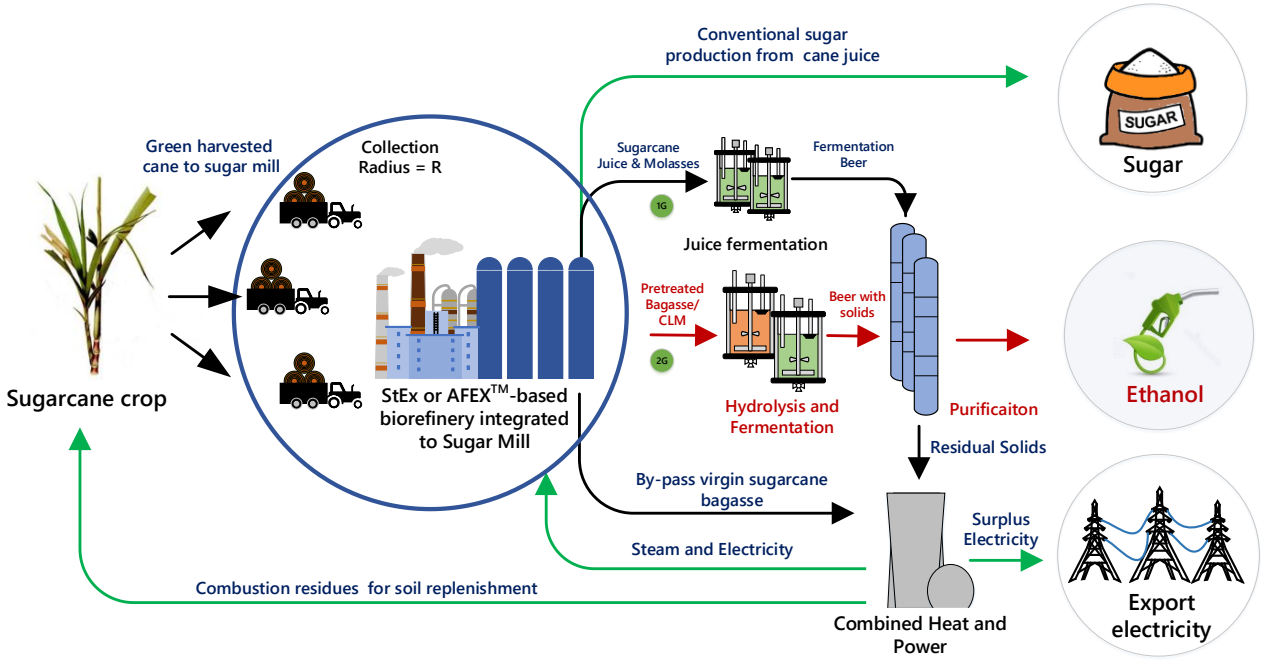



**Figure 3.1:** Integrating AFEX™ and StEx into existing sugar mills or 1G sugarcane ethanol distilleries for 2G ethanol production from sugarcane residues



### 3.1.2 *Objectives*

The aim of this contribution was to evaluate and compare the potential ethanol yields that can be recovered from StEx and AFEX™-treated SCB and CLM at industrially-relevant conditions. To achieve this, the objectives for this contribution were:

I. To study a wide range of StEx and AFEX™ pretreatment conditions for SCB and CLM, followed by the selection of conditions that facilitate high sugar recovery at moderate enzyme loading with limited pretreatment catalyst loading.

II. To maximise the synergy of the latest commercial enzyme cocktails for each substrate by conducting optimization studies to determine the best combinations of Cellic® CTec3, Cellic® HTec3, and Pectinex Ultra-SP that enable maximum sugar yields from each pretreated substrate.

III. To establish the effect of adding a solid-liquid separation and/or washing steps on enzymatic hydrolysis efficiency by performing high solids loading enzymatic hydrolysis experiments at varying enzyme loadings using the optimized combinations from *Objective II*.

IV. To determine the extent of microbial inhibition due to AFEX™- and StEx pretreatment-derived inhibitors by evaluating the glucose and xylose fermentability from all carbohydrate streams from StEx and AFEX™ pretreatment using *Saccharomyces cerevisiae* 424A (LNH-ST) as the ethanologen.

V. To develop carbohydrate and ethanol mass balances for each pretreated substrate and subsequently estimate the potential ethanol yields per unit land for sugarcane biorefineries based on either AFEX™ or StEx for 2G ethanol production.

VI. To perform a sensitivity analysis to reveal the major processing bottlenecks that had the greatest effect on ethanol yields per unit sugarcane cultivation area.

The study performed to address these objectives is detailed in **CHAPTER 4**.



## 3.2 Contribution 2: Using steam explosion and AFEX™ to produce animal feeds and biofuel feedstocks for biorefineries based of sugarcane residues

### 3.2.1 *Statement of Novelty*

Livestock production is the greatest user of land resources, with land dedicated to pasture and livestock forage cultivation accounting for more than 70% of global agricultural land use. Crop residues play important role in livestock production in developing and transition countries. Hence, increasing amount of nutrients that can be recovered per unit crop residue can have significant impact on feed resources, food security and natural resource use efficiency for developing economies [174]. Both StEx and AFEX™ have demonstrated significant promise in enhancing the digestibility of agricultural crops such as corn stover and wheat straw. Furthermore, there is sufficient literature evidence that increases in crop residue digestibility significantly improve ruminant (cattle) performance (*i.e.* higher milk production, beef weight gains, and voluntary intake) and therefore increase the economic value of the treated crop residues [179–181]. Literature estimations suggest that treated AFEX™ biomass could be sold to the US market at approximately $50-100/dry ton [19].

Given the synergies between the sugarcane production chains for biofuels and livestock production, this contribution presents a biorefinery concept whereby biomass pretreatment technologies are integrated into existing industrial sites (*e.g.* sugar/ethanol mills) to simultaneously produce conversion-ready biofuel feedstocks and highly digestible ruminant animal feeds from sugarcane residues. For the first time, we compare the nutritional composition, *in-vitro* true digestibility (IVTD), metabolizable energy (ME) and nitrogenous compounds of pilot-scale StEx and AFEX™-treated SCB and CLM. Moreover, we evaluate and compare ethanol production from the same feedstocks under industrially relevant conditions of high solids loading and moderate enzyme dosages. Ultimately, the results of this work present an example of an integrated system that can potentially take advantage of the synergies that exist between food and bioenergy production to promote

increased land use efficiency, while avoiding the potential for indirect land use change. The integrated animal feed and biofuel feedstock production from sugarcane residues based biorefineries is summarised in Figure 3.2 below.

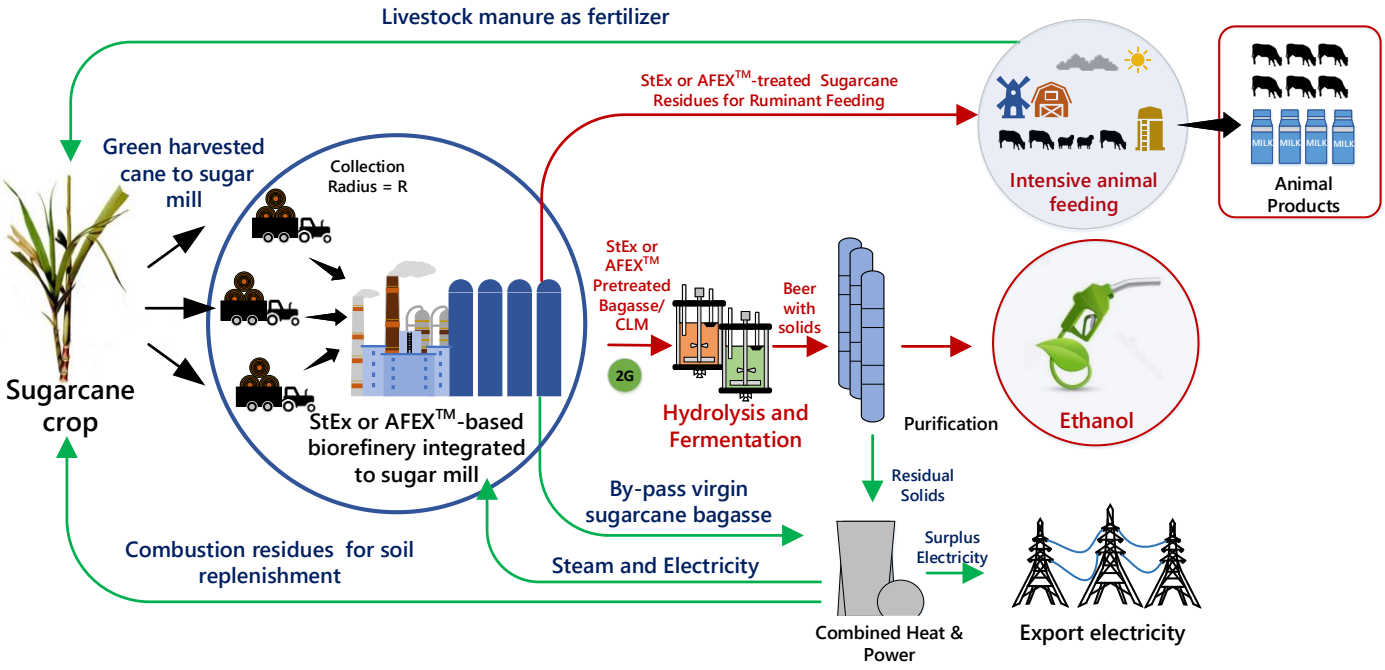



**Figure 3.2:** The co-production of AFEX™ or StEx-treated animal feeds and biofuel feedstocks from sugarcane residues in a sugarcane biorefinery

### 3.2.2 *Objectives*

The aim for this contribution was to evaluate and compare the animal feed value and ethanol production potential of pilot-scale AFEX™ and StEx treated SCB and CLM. To achieve this, the following objectives were set for this contribution:

I. To conduct pilot-scale StEx and AFEX™ pretreatment of SCB and CLM and compare the changes in nutritional composition of the pretreated residues relative to untreated controls.

II. To evaluate and compare the *in-vitro* true digestibility and metabolizable energy of AFEX™ and StEx-treated SCB and CLM relative to untreated controls.

III. To quantify the major AFEX™ and StEx generated cell wall decomposition products and nitrogenous compounds using gas chromatography and mass spectroscopy based analytical methods.

IV. To evaluate and compare the ethanol yields from pilot-scale pretreated AFEX™ and StEx sugarcane residues under industrially relevant conditions.



The study performed to address these objectives is detailed in **CHAPTER 5**.

## 3.3 Contribution 3: Incorporating anaerobic co-digestion of livestock manure with steam exploded or AFEX™ pretreated sugarcane residues into sugarcane-based bioenergy-food systems

### 3.3.1 *Statement of Novelty*

Three key strategies to curb the adverse environmental effects of the livestock sector include: (1) reducing livestock feed components that compete with direct human food crop production (e.g. Contribution 2), (2) decreasing the share of animal products in human consumption diets, and (3) increasing livestock production efficiency through intensification of livestock production [38]. For the latter strategy, the prospective integration of biofuel and intensive livestock production systems will be accompanied by the production of surplus animal manure, which represents a significant pollution risk with potential negative environmental impacts [204]. Anaerobic digestion is a well-know and mature technology that has previously been used as a waste management and methane production strategy for animal manure. However, the digestion of manure alone can lead to unstable anaerobic digestion due to nutrient imbalance and ammonia inhibition, thereby resulting in low biogas production per unit mass of manure.

In this contribution, we explore the potential integration of anaerobic co-digestion of pretreated sugarcane residues with livestock manure from intensified animal feed operations for reducing the environmental footprint of livestock production and for producing farm-owned or biorefinery-based bioenergy (Figure 3.3). For the first time, we investigated and compared the effect of StEx and AFEX™ pretreatment on the efficiency of anaerobic co-digestion as measured by the cumulative methane yields, substrate biodegradation rates, and gross energy conversion efficiency compared with untreated controls. Ultimately, the results from this contribution will provide insights into the incorporation of anaerobic co-digestion of sugarcane residues with livestock manure into the

bioenergy-livestock production nexus for providing a more sustainable bioenergy-livestock nexus for sugarcane and livestock dense regions.

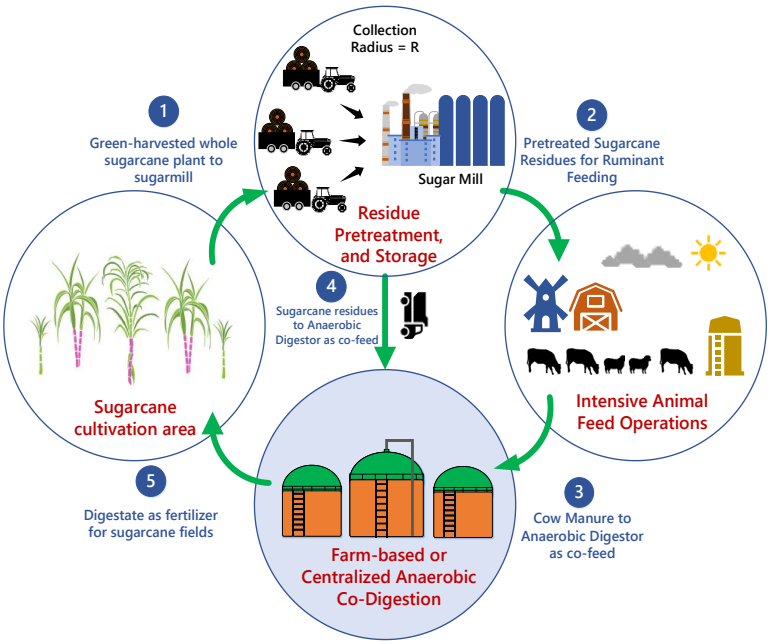



**Figure 3.3:** Using AFEX™ or StEx-treated sugarcane residues as anaerobic digestion feeds in the livestock-biofuel production nexus

### 3.3.2 *Objectives*

The aim of this contribution was to experimentally assess the potential use of untreated, StEx- or AFEX™-treated of sugarcane residues as co-substrates with dairy cow manure (DCM) in batch anaerobic co-digestion to produce methane rich biogas and to evaluate the potential fertilizer value of the solid digestate. To achieve this, the following objectives were defined for this contribution:

I. To identify the structural and functional group changes imposed by StEx and AFEX™ on SCB and CLM relative to untreated controls.
II. To evaluate and compare the impact of the two pretreatment technologies in mono- and co-digestion in terms of cumulative methane yield, methane content, total volatile fatty acid production.
III. To quantify the effect of co-digesting sugarcane residues with livestock manure on the rate of substrate biodegradation relative to mono-digestion using a kinetic analysis
IV. To evaluate the effect of biomass to livestock manure ratio on the efficiency of anaerobic digestion
V. To conduct an energy conversion assessment for mono- or co-digestion substrates yielding specific methane yields greater than the mono-digestion of DCM.

VI. To quantify the macro-nutrient value of the solid digestates for mono- or co-digestion substrates yielding specific methane yields greater than the mono-digestion of DCM.

The study performed to address this objective is detailed in **CHAPTER 6**.

## 3.4 Contribution 4: CIII$_l$-activation of AFEX$^{TM}$ and StEx-treated sugarcane residue pellets for low enzyme loading ethanol production from centralized biorefineries

### 3.4.1 *Statement of Novelty*

To supply a national bioeconomy, biomass supply logistics will need to confront and manage unfavourable biomass characteristics, *i.e.* low bulk density, geographical dispersion, and variable moisture content and chemical composition [228]. It is well documented that biomass transportation and storage costs limit the size of prospective biorefineries, preventing them from achieving the economies of scale necessary to significantly reduce biofuel production prices [7,229,230]. Moreover, multiple literature analyses have demonstrated that current conventional biomass supply systems (*e.g.* agricultural residue baling) will not be able to facilitate large scale biorefineries (5000 – 20 000 dry tons of biomass per day) required to reach national energy and GHG reduction targets outside of highly productive regions and biomass dense regions [33,228,229,231–233]. Hence, recent research efforts have been dedicated to developing uniform feedstock supply systems to produce commodity-type and infrastructure compatible bulk solid lignocellulosic biomass [232,234]. StEx and AFEX$^{TM}$ have previously demonstrated significant promise in "activating" lignin to allow easier binding during pelletization, thereby facilitating the production of uniform, dense, durable, and easier to handle biomass pellets from agricultural residues such as corn stover. Hence, integrating StEx- and AFEX$^{TM}$ to existing sugar/ethanol mills to produce SCB and CLM pellets that are dense, mechanically stable, and conversion-ready is a potential strategy for enabling the mass mobilization of sugarcane residues in decentralized uniform feedstock supply systems.



In addition to feedstock supply systems, enzyme related costs remains a major processing bottleneck for prospective 2G biorefineries, with enzyme costs previously estimated to account for 15.7% of the total ethanol production costs even at enzyme loadings of 20 mg protein per gram glucan [92]. As will be demonstrated in Contribution 1, StEx and AFEX$^{TM}$ require enzyme dosages higher than 20 mg protein per gram glucan to facilitate high ethanol yields, suggesting that even if depots can produce dense and durable StEx or AFEX$^{TM}$-treated biomass pellets, further biomass pretreatment may be necessary to lower the enzyme cost contribution to ethanol production. Anhydrous liquid ammonia is known to facilitate the transformation of crystalline allomorph of plant-derived cellulose (cellulose $CI_{\beta}$) to the more digestible allomorph (cellulose $CIII_{I}$) even room temperature. Pretreatment technologies such as Extractive Ammonia have already demonstrated significant enzyme dosage reductions (from 18 mg/g glucan to 7.5 mg/g glucan) for $CIII_{I}$-activated corn stover. To our knowledge, there are no literature studies considering the use of a room temperature $CIII_{I}$-activation process for upgrading AFEX$^{TM}$ or StEx pretreated biomass in view of achieving high ethanol yields whilst using enzyme dosages lower than 10 mg protein per gram glucan (~4 mg per gram dry biomass).

In this contribution, we investigate for the first time, a uniform feedstock supply system whereby StEx or AFEX$^{TM}$ are integrated into existing sugar/ethanol mills to form pre-processing depots that convert low bulk density sugarcane residues into dense, durable and conversion-ready biomass pellets. Once transported to large-scale biorefineries, these pellets would be upgraded via a room temperature $CIII_{I}$-activation step to enable lower enzyme loading requirements for ethanol production. This uniform feedstock supply system is illustrated in Figure 3.4 below.

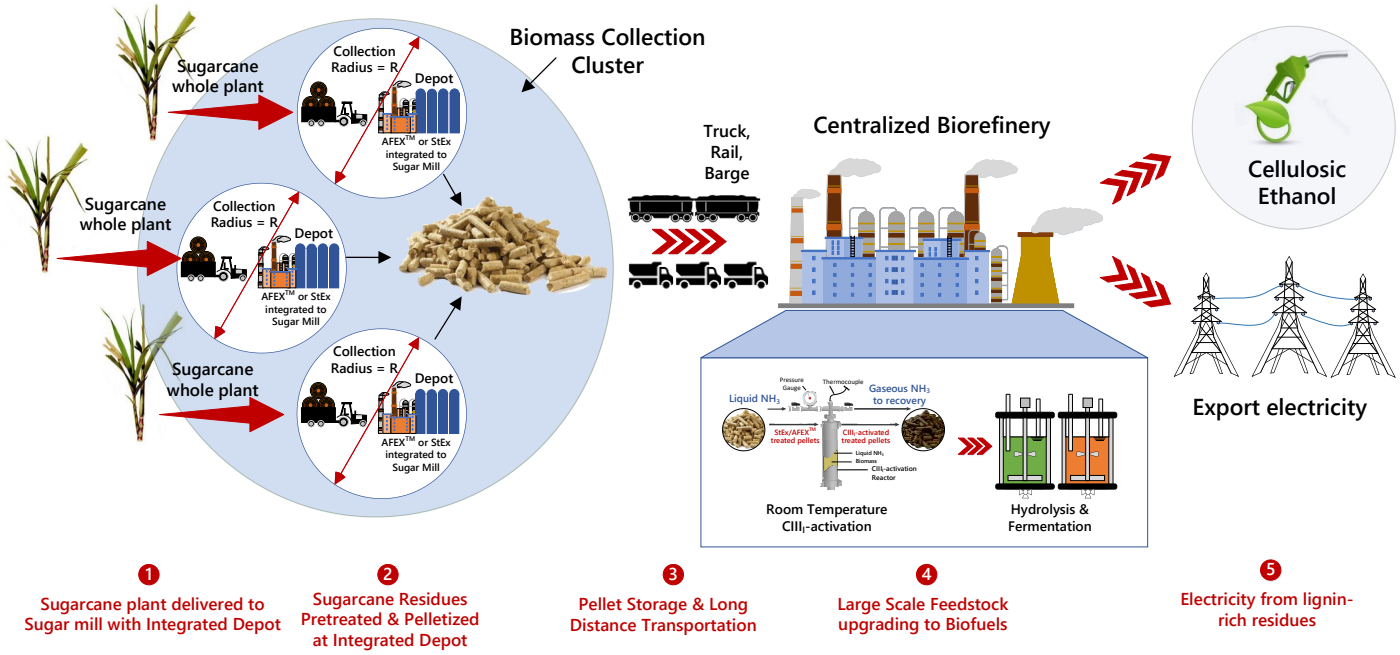



**Figure 3.4:** Illustration of uniform-feedstock supply system that integrates pre-processing depots into existing sugar mills to produce uniform feedstocks that can be transported to centralized biorefineries and upgraded via a CIII$_l$-activation step prior to bioconversion to ethanol

### 3.4.2 *Objectives/Aims*

The aim of this contribution was to evaluate the potential for StEx and AFEX$^{TM}$ to produce dense and mechanically durable sugarcane residue pellets that can be upgraded via a room temperature CIII$_l$-activation process to reduce enzyme dosage requirements for efficient ethanol production. To achieve this, the following objectives were defined for this contribution:

I. To produce pellets from pilot-scale StEx and AFEX$^{TM}$ treated SCB and CLM, quantify their physical and mechanical properties, and compare their properties to those reported for compacted SCB piles, CLM bales, and corn grains.

II. To evaluate the minimum enzyme dosages required from StEx and AFEX$^{TM}$-treated SCB and CLM to achieve minimum combined sugar yield and ethanol concentrations of 75% and 40 g.L$^{-1}$, respectively.

III. To assess the potential for using a room temperature CIII$_l$-activation process to upgrade StEx/AFEX$^{TM}$ pellets in view of reducing the enzyme dosages required to achieve combined sugar yields and ethanol concentrations of 75% and 40 g.L$^{-1}$ respectively.

IV. To perform an energy conversion assessment to evaluate the potential recovery of the inlet feedstock heat of combustion energy in ethanol and electricity equivalent energy for the low enzyme dosage StEx/AFEX$^{TM}$ coupled with CIII$_l$-activation scenario.

The study performed to address this objective is detailed in **CHAPTER 7**.



## 3.5 Contribution 5: Evaluating the fermentability of steam exploded and non-detoxified sugarcane bagasse whole slurry using industrial xylose-fermenting *Saccharomyces cerevisiae* strains

### 3.5.1 *Statement of Novelty*

Feedstock or raw material costs represent the largest contribution for 2G biorefineries, hence maximising ethanol yields through the efficient conversion of all the highly functionalised carbohydrates in the feedstock is of paramount economic importance [4]. For StEx pretreatment based biorefineries, performing enzymatic hydrolysis and fermentation using the whole slurry is one of the strategies suggested for reducing CAPEX, OPEX and process water consumption and recovery by avoiding processing steps associated with solid/liquid separation, washing, and detoxification. However, attaining high fermentation yields from whole slurries in the presence of pretreatment derived inhibitory compounds (*e.g.* organic acids, furan derivatives, phenolic compounds), fermentation metabolites (*e.g.* ethanol, glycerol) and insoluble solids is challenging without the availability of inhibitor tolerant ethanologens. In particular, the xylose-utilization capability of several recombinant yeast strains is severely limited by the synergistic action of these microbial stresses.

To this end, the development of sufficiently hardened xylose-fermenting mutant strains with high tolerance to pretreatment-derived inhibitors and fermentation metabolites has been extensively studied, with research efforts focused on improving fermentation yields and ethanol concentrations from lignocellulosic hydrolysates. Through metabolic engineering, several recombinant xylose-fermenting ethanologens have been reported in literature. However, the performance of these strains has been predominantly demonstrated in synthetic media supplemented with selected inhibitory compounds to simulate hydrolysate microbial stresses. As a result, most of these studies negate the potential synergistic impact of inhibitors and fermentation metabolites on the ethanologens' ability to efficiently convert both hexose and pentose sugars to ethanol.

There is a lack of literature data describing the performance of some of the current industrial recombinant xylose-fermenting yeast strains in the presence of pretreatment derived inhibitors, primarily due to proprietary issues and/or the inability of some of these strains to efficiently convert pentose sugars to ethanol in non-detoxified whole slurry hydrolysates. In this contribution, we screened four selected industrial yeast strains with high inhibitor tolerance in terms of their ability to efficiently metabolize both glucose and xylose and produce high ethanol titres from the fermentation of StEx whole slurry hydrolysates. For the first time, we evaluate and compare the performance of three variants of *S. cerevisiae* CelluX™ 1 (trademark of Leaf technologies, France) genetically engineered for efficient fermentation of non-detoxified spent sulphite liquor (Brandt *et al.*, 2018, manuscript prepared for submission), and the fourth-generation strain of CelluX™ 1, *viz. S. cerevisiae* CelluX™ 4 (Figure 3.5). Ultimately, this contribution provides insights into the potential ethanol yields that can be recovered from steam exploded sugarcane residues using sufficiently hardened (inhibitor and fermentation metabolite tolerant) ethanologens.

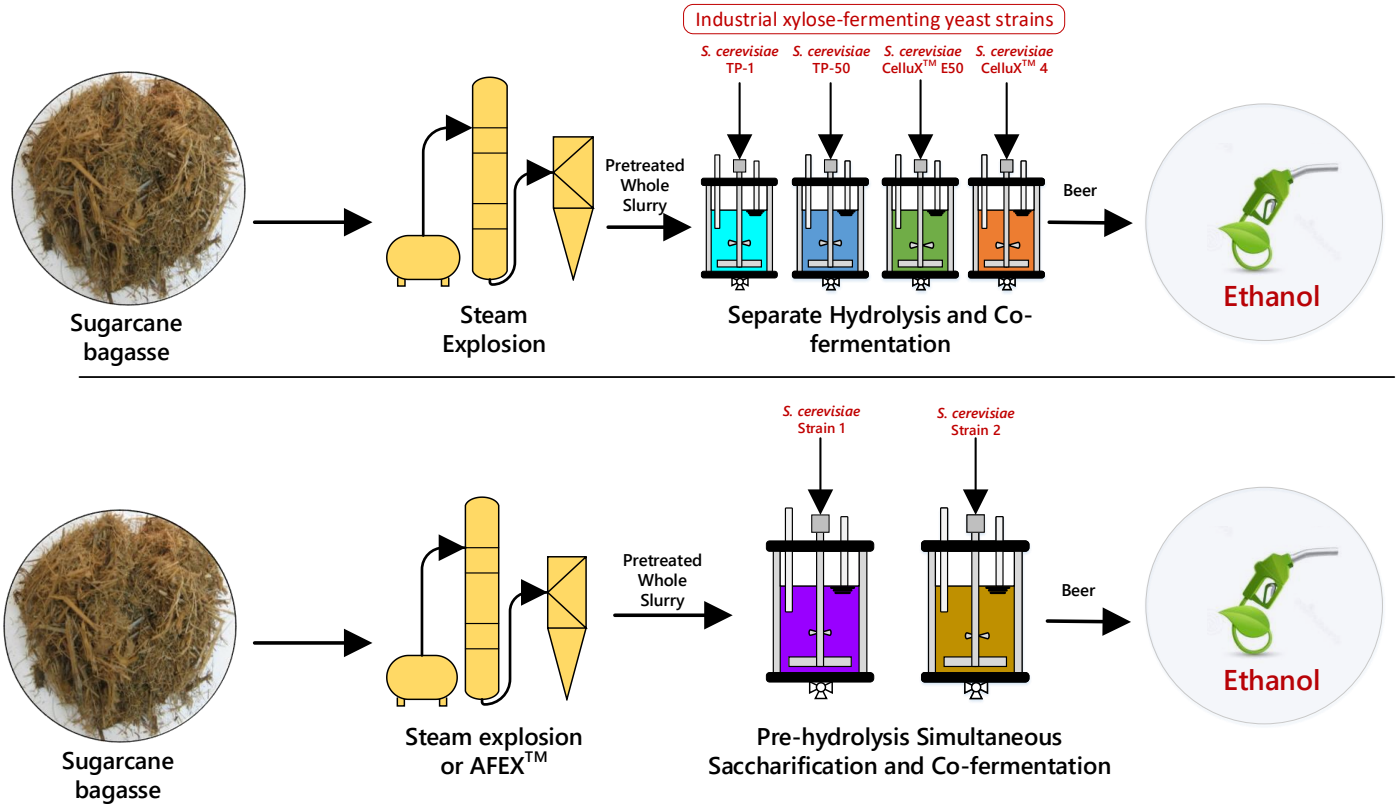



**Figure 3.5:** Schematic illustration of the evaluation of the fermentability of StEx-treated and non-detoxified sugarcane bagasse whole slurry's using four industrial xylose-fermenting yeast strains



### 3.5.2 *Objectives/Aims*

The aim of this contribution was to evaluate the potential de-bottlenecking of the fermentation of steam exploded SCB whole slurries without detoxification using inhibitor tolerant industrial xylose-fermenting yeasts. To achieve this, the following objectives were defined for this contribution:

I. To generate and characterise the composition the StEx-treated whole slurry hydrolysates.
II. To evaluate the performance of four selected industrial yeast strains for the fermentation of StEx-treated SCB whole slurry hydrolysates without detoxification.
III. To select two of the best performing strains from *Objective II* and evaluate their ethanol production capabilities for StEx-treated SCB whole slurries under moderate enzyme loading and high solids loading PSSF conditions.
IV. To study the reduction of targeted furan aldehydes and phenolic compounds before and after PSSF of StEx-treated SCB whole slurry hydrolysates.
V. To compare the performance of the two selected yeast strains for PSSF of high inhibitor containing StEx-treated SCB whole slurry to low inhibitor containing AFEX™ treated SCB.

The study performed to address this objective is detailed in **CHAPTER 8**.

# CHAPTER FOUR:

# CONTRIBUTION 1

**Chapter published in**: Biotechnology for Biofuels (11:127): 1 – 21, ISI 5-year Impact factor = 6.732

**Article title:** Ethanol production potential from AFEX™ ad steam exploded sugarcane residues for sugarcane biorefineries

**Authors: Thapelo Mokomele**, Leonardo da Costa Sousa, Venkatesh Balan, Eugéne van Rensburg, Bruce E. Dale, and Johann F. Görgens

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## Objective of dissertation and summary of findings in chapter

This chapter addresses the research objectives highlighted in **Contribution 1**. This study conducted a side-by-side and systematic comparison of the pervasive impacts of StEx and AFEX™-treatment of sugarcane residues on the downstream overall ethanol yield per Mg untreated biomass basis. Furthermore, for StEx treated SCB and CLM, various process configurations for mitigating the impact of pretreatment derived inhibitors on the fermentability of StEx pretreated sugarcane residues were evaluated and compared to determine the extent of ethanol yield reduction because of enzyme and microbial inhibition.

The results showed that AFEX™ pretreatment of SCB and CLM facilitated the production of ethanol yields of 256 and 249 kg per Mg RDM for SCB and CLM, respectively, the highest ethanol yields from sugarcane residues reported in literature. Lower ethanol yields were recovered from StEx pretreatment irrespective of the processing configuration, with sugar loss during pretreatment, enzyme inhibition and microbial inhibition during the fermentation limiting the ethanol yields from StEx treated SCB and CLM to the range 162 to 203 kg per Mg RDM. The identification of auxiliary hydrolytic enzymes, adequate process integration and the use of inhibitor-tolerant ethanologens were identified as key areas for improving ethanol yields from both pretreatment technologies.

# Candidate declaration

With regards to **Chapter 4**, pages 90 – 146 of this dissertation, the nature and scope of my contributions were as follows:

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental planning | 70 |
| Executing experiments | 100 |
| Intepretation of experiments | 70 |
| Writing of chapter | 100 |

The following co-authors have contributed to **Chapter 4**, pages 90 – 146 of this dissertation:

| Name | e-mail address | Name of contribution | Extent of contribution (%) |
|---|---|---|---|
| Leonardo da Costa Sousa | sousaleo@egr.msu.edu | ✓Experimental planning<br>✓Intepretation of experiments<br>✓Review of chapter | 15<br>10<br>30 |
| Venkatesh Balan | vbalan@uh.edu | ✓Experimental planning<br>✓Intepretation of experiments<br>✓Review of chapter | 5<br>10<br>10 |
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| Bruce E. Dale | bdale@egr.msu.edu | ✓Review of chapter<br>✓Co-ordination of collaboration | 20<br>50 |
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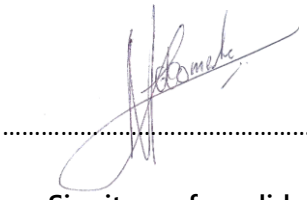

..... October 2018

**Signiture of candidate** **Date**

## Declaration by co-authors

All authors read and approved the final manuscript and hereby confirm that:

I. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to **Chapter 4**, page numbers 90-146 in the dissertation,

II. no other authors contributed to **Chapter 4**, page numbers 90-146 in the dissertation beside those specified above, and

III. potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in **Chapter 4**, page numbers 90 – 146 of the dissertation.



# Ethanol production potential from AFEX™ and steam exploded sugarcane residues for sugarcane biorefineries

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## Abstract

**Background:** Expanding biofuel markets are challenged by the need to meet future biofuel demands and mitigate greenhouse gas emissions, while using domestically available feedstock sustainably. In the context of the sugar industry, exploiting under-utilized cane leaf matter (CLM) in addition to surplus sugarcane bagasse as supplementary feedstock for second generation ethanol production has the potential to improve bioenergy yields per unit land. In this study, the ethanol yields and processing bottlenecks of ammonia fiber expansion (AFEX™) and steam explosion (StEx) treated from sugarcane bagasse and CLM were experimentally measured and compared for the first time.

**Results:** Ethanol yields between 249 and 256 kg per Mg raw dry biomass (RDM), were obtained with AFEX™ pretreated sugarcane bagasse and CLM after high solids loading enzymatic hydrolysis and fermentation. In contrast, StEx pretreated sugarcane bagasse and CLM resulted in substantially lower ethanol yields that ranged between 162 and 203 kg per Mg RDM. The ethanol yields from StEx-treated sugarcane residues were limited by the aggregated effect of sugar degradation during pretreatment, enzyme inhibition during enzymatic hydrolysis and microbial inhibition of *S. cerevisiae* 424A (LNH-ST) during fermentation. However, relatively high enzyme



dosages (> 20 mg/g glucan) were required irrespective of pretreatment method to reach 75% carbohydrate conversion, even when optimal combinations of Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP were used. Ethanol yields per hectare sugarcane cultivation area were estimated at 4496 and 3416 L/ha for biorefineries using AFEX™- or StEx-treated sugarcane residues, respectively.

**Conclusions:** AFEX™ proved to be a more effective pretreatment method for sugarcane residues relative to StEx due to the higher fermentable sugar recovery and enzymatic hydrolysate fermentability after high solids loading enzymatic hydrolysis and fermentation by *S. cerevisiae* 424A (LNH-ST). The identification of auxiliary enzyme activities, adequate process integration and the use of robust xylose-fermenting ethanologens were identified as opportunities to further improve ethanol yields from AFEX™- and StEx-treated sugarcane residues.

## 4.1 Background

Sustainably-produced liquid biofuels are key to a projected future where biomass-derived biofuels will partially displace petroleum-based transportation fuels [235]. The progressive transition toward indigenous cellulosic second-generation (2G) biofuel production from first-generation (1G) which uses food resources can potentially facilitate environmental, economic, and socio-economic benefits in both developing and developed countries [20,233]. While 2G biofuel technology is steadily entering the commercial deployment phase, major impediments to its commercial appeal remain, specifically related to the feedstock supply chain, land availability for expansion, technology maturity and overall economic feasibility [20,236,237].

Sugarcane is a major agricultural crop widely considered as one of the leading candidates for bio-energy, with Brazil producing 651 million tons of sugarcane during the 2016-2017 harvest season [238]. First generation ethanol produced from sugarcane (from extractable sugars) is a commercial process with an industrial maturity of greater than 40 years [239]. However, with a growing world population and biofuel demand, expanding biofuel production beyond existing farmlands is challenged by land conservation concerns, especially in countries with limited capacity for sugarcane



cultivation area expansion [15,37,240]. Consequently, there is substantial interest in crop variety selection and the utilisation of the whole sugarcane plant for biofuel production as sustainable approaches to increasing sugarcane ethanol yields per unit land [72].

The sugarcane processing industry typically generates approximately 140 kg dry weight bagasse (fibrous residue after juice extraction) and an equal amount (dry weight) of cane leaf matter (green leaves, tops and trash) per ton of wet harvested cane [22]. Presently, bagasse is burned in inefficient mill boilers to produce heat and electricity for sugar milling operations, with surplus energy exported to the grid [23,24]. Improvements in the sugar mill operation energy efficiency and investment in more energy efficient power cogeneration technology would liberate surplus bagasse for future biorefinery applications [23,25]. Moreover, it has previously been common practice to burn sugarcane cane leaf matter (CLM) on the stalk prior to harvesting to facilitate easier and cheaper sugarcane stalk collection and transportation [23,26,27]. As a result of environmental regulations coupled with an industry-wide effort to phase out CLM burning, the utilisation of this biomass as substrate for bioconversion to bioethanol, electricity and/or other value-added products in a biorefinery setting provides an alternative, potentially greener and more sustainable approach [28]. Whereas the requirements for sustainable agriculture prevent the complete removal of CLM from the field due to reduced soil fertility over a period of years, some studies suggest that 50% of the sugarcane harvest residues can be removed from the field, with the remainder ploughed back in to soil without significantly affecting nutrient cycling, soil biodiversity, soil carbon sequestration and pest control [28,48,98,241]. Therefore, depending on the amount of CLM that can be recovered from the field and proximity to the sugar mill, these residues can either be baled or transported together with the sugarcane stalk to the sugar mill to supply either 2G biofuel production or energy cogeneration [242]. The availability of these residues as either supplementary feedstock to sugarcane juice in integrated 1G-2G biorefineries or as sole feedstock in standalone 2G biorefineries annexed to sugar mills, has the potential to enhance the ethanol yield per unit land without expanding the cultivation area, whilst maximizing environmental benefits and minimizing capital and production costs [9,25,98].



In addition to energy integration benefits, these 2G sugarcane residue biorefineries integrated to sugar mills or 1G biorefineries present an attractive opportunity for sharing of existing feedstock supply, handling infrastructure and logistical systems that currently represent a significant hurdle for the nascent 2G biofuel production industry [64].

To compete with traditional petroleum refineries, high biomass-to-biofuel yields with low enzyme loadings are required for the biochemical processing of recalcitrant sugarcane residues [243,244]. Although there are numerous pretreatment technologies with different biomass deconstruction chemistries, most pretreatments present various economic and environmental challenges concerning costly chemical use and recovery, excess water use, feedstock handling, energy requirements and downstream solids processing [25]. Among the leading thermochemical pretreatment options, steam explosion (StEx) and ammonia fiber expansion (AFEX™) are two well-studied and scalable technologies (demonstrated at pilot scale) that are being considered for overcoming biomass recalcitrance, given their different biomass deconstruction patterns (acidic vs alkaline) and potential for integration into existing sugarcane mills [29,30].

Autocatalyzed StEx is a well-known thermochemical pretreatment approach that uses high temperature saturated steam and intrinsic biomass-derived organic acids (*e.g.* acetic acid) to enhance cellulose digestibility. During the pretreatment process there is selective fractionation of hemicellulose, partial cleavage of lignin-carbohydrate complex ester linkages, and increased substrate accessibility toward hydrolytic enzymes [102,104,106,245]. Advantages of StEx pretreatment for integration in sugar mill operations include the use of water as a green solvent, relatively low capital investment, moderate energy requirements, and the ability to use high-moisture content biomass (such as bagasse) [106,110]. However, due to pretreatment severities required for obtaining high cellulose digestibility, StEx generates hemicellulose and cellulose-derived degradation products that are inhibitory to downstream enzymatic hydrolysis and fermentation [163]. To avoid limiting biomass-to-biofuel yields due to the presence of inhibitory compounds, the pretreatment slurry has been previously separated by means of a solid-liquid separation step followed by washing the residual solid



with water to remove soluble sugars and inhibitors [101]. However, during commercial application, it is likely that either unwashed (pressed) solids or whole slurries (hydrolysate liquor plus solids) will be preferred in view of minimizing process water consumption and downstream water recovery costs [96,104,146]. Therefore, detailed carbohydrate-to-biofuel yields are necessary to understand the benefits of washing/separating the pretreatment slurry to mitigate the impact of pretreatment-derived inhibitors on enzymatic hydrolysis and microbial fermentation.

In comparison, an alkaline pretreatment process, AFEX™ (trademark of MBI International, Lansing, Michigan) treats moist biomass with anhydrous ammonia at moderate temperatures and pressures, followed by the rapid release of pressure and recovery of vaporized ammonia [121]. AFEX™ is a "dry-to-dry" process that eliminates the requirements for wastewater recovery and solid-liquid separations. Recent advances in renewable hydrogen production and the subsequent production of ammonia from renewable hydrogen provide enthusiasm for the future use of ammonia as a green solvent [246]. AFEX™ pretreatment enhances biomass enzymatic digestibility through the cleavage of lignin-carbohydrate complex ester linkages, cellulose de-crystallization, de-acetylation, lignin/hemicellulose redistribution towards the outer plant cell wall, and increased enzyme-accessible area. Furthermore, AFEX™ preserves the native plant nutrients and generates minimal inhibitory degradation products, resulting in a fermentable enzymatic hydrolysate that does not require detoxification or significant external nutrient supplementation [122]. However, ammonia recovery operations and make-up ammonia increase the capital and operating costs for AFEX™. Therefore, optimizing pretreatment conditions at low ammonia to biomass loading has been proposed as a potential strategy to reducing ammonia recovery costs [130].

In this study, the potential ethanol yields that can be recovered from StEx and AFEX™-treated sugarcane bagasse and CLM at industrially-relevant conditions were explored and compared for the first time. A wide range of StEx and AFEX™ pretreatment conditions were evaluated for sugarcane bagasse and CLM, followed by selecting conditions that facilitate high sugar recovery at moderate enzyme loading with limited pretreatment catalyst loading. To establish the effect of solids separation



and/or washing, high solids loading enzymatic hydrolysis experiments were performed at varying enzyme loadings using optimized combinations of Cellic® CTec3, Cellic® HTec3, and Pectinex-Ultra-SP. Further, the fermentability of all carbohydrate fractions from both AFEX™ and StEx were evaluated to determine the extent of microbial inhibition due to AFEX™- and StEx pretreatment-derived inhibitors. From carbohydrate and ethanol mass balances, the potential ethanol yields per unit land for sugarcane biorefineries based on either AFEX™ or StEx for 2G ethanol production were estimated. Ultimately, this work provides data and insights that will enable subsequent economic evaluations of the various processing options for future StEx or AFEX™-based sugarcane residue ethanol biorefineries.



## 4.2 Materials and methods

### 4.2.1 *Biomass collection and preparation*

Sugarcane bagasse (at 50-60% w/w moisture content) and manually harvested cane leaf matter (including green leaves, tops and trash) were collected from two sugarcane mills located in Malelane (TSB Sugar, Mpumalanga) and Mount Edgecombe (SASRI, Kwazulu Natal), South Africa. To prevent biomass spoilage, the bagasse and CLM were air-dried in separate greenhouses until the equilibrium moisture content was approximately 7% (w/w). The bagasse was milled using a laboratory toothed disk mill (Condux LV15M, Netzch-Condux GmbH, Germany) and passed through a 20 mm screen. The size-reduced bagasse samples were sieved in a stacked-sieve system to remove mineral impurities (*e.g.* sand), bagasse pith and fines that are smaller than 600 µm x 600 µm. De-pithing bagasse is common practice in South African sugar mills to facilitate the use of longer bagasse fibres as fuel for steam/energy production, with the bagasse pith is typically used as a molasses-carrier in animal feed products [51]. The bagasse from two sources was thoroughly mixed and stored in vacuum-sealed bags at room temperature until use.

Air-dried CLM was hammer-milled (Massey-Ferguson, USA) and passed through a hexagonal screen with a 20 mm diameter to attain particles with an approximate length ranging between 50 to 70 mm. The milled CLM samples were sieved to remove mineral impurities and fines smaller than 600 µm x 600 µm. The CLM from both sources was well mixed to achieve a representative sample of South African post-harvest CLM and stored in vacuum-sealed bags at room temperature until use.

### 4.2.2 *Composition analysis*

The composition of the raw biomass samples was determined according to National Renewable Energy Laboratory (NREL) protocols NREL/TP-510-42618 and NREL/TP-510-42620. The higher heating value (HHV) was measured using a bomb calorimeter (Cal2k Eco Calorimeter) based on ASTM standard D5865-11a. Statistical significance between experimental values was determined through the application of a one-way ANOVA in combination with Tukey's HSD post hoc test for



multiple comparisons (Minitab Inc., State College, PA, USA). A *P*-value less than 0.05 was considered statistically significant.

### 4.2.3 *Steam explosion*

Steam explosion (StEx) was performed in an automated batch pilot-scale unit (IAP GmBH, Graz, Austria) equipped with a 19 L reaction vessel, a 100 L expansion vessel and a 40 bar rated steam boiler [247]. In preparation for StEx pretreatment, untreated sugarcane bagasse or CLM was pre-soaked in reverse-osmosis water overnight at a solid-to-water ratio of 1:2 to ensure maximum water absorption into the biomass. The water-impregnated material was subsequently dewatered in a gravity drain spin dryer (AEG SV4028, Germany) to a moisture content akin to industrial bagasse (65-75%). The StEx reaction vessel, preheated to 185 °C, was top-loaded with 500 g (dry basis) of water-impregnated bagasse or CLM and was directly heated to the desired temperature using 30 bar (absolute) saturated steam. After the required pretreatment time had elapsed, the reactor contents were discharged into the expansion vessel maintained at atmospheric pressure. Each pretreatment was performed in duplicate. Three 100 gram samples of the pretreatment slurry were characterized in terms of the total solids (TS), water soluble solids (WSS), water insoluble solids (WIS), and pH. The remaining slurry was separated into a solid (pressed solids) and a liquid fraction (pretreatment $C_5$-liquor) using a pneumatic piston press (Eurotool TY5001, South Africa). The pressed (unwashed) solids with an approximate moisture content of 65% (w/w) were air dried at 35 °C to a moisture content of 15% (w/w). The combined sugar yield for StEx was calculated from the soluble monomeric and oligomeric sugars (glucose + xylose) in the pretreatment liquor and the soluble monomeric sugars (glucose + xylose) released after low solids loading enzymatic hydrolysis (described below) of washed solids.

The bagasse and the CLM were pretreated at temperatures and residence times ranging from 185 to 215 °C and 10 to 15min, respectively (Table S4.1, Supplementary Information). For each biomass material, three pretreatment conditions were considered based on previous work and preliminary data from unpublished work by Hamann *et al.,* (2018) [45,115,116,118,119]. First, low

severity pretreatment conditions leading to high hemicellulose solubilization and recovery in the pretreatment liquor with low degradation product generation were evaluated. Secondly, high severity pretreatment conditions facilitating high cellulose digestibility in the pretreated fibres were evaluated. Lastly, intermediate severity pretreatment conditions resulting in high combined sugar recovery from both the pretreatment liquor and enzymatic hydrolysis steps were evaluated.

## 4.2.4 *AFEX™ pretreatment*

### 4.3.4.1 *High-throughput batch AFEX™*

High-throughput AFEX™ pretreatment was performed in 22 mL pressure vessels (Parr Instrument Company, Moline, IL, USA) [248]. To facilitate the high-throughput pretreatments, untreated sugarcane bagasse and CLM samples were milled and passed through a 5 mm screen using a Wiley Mill. AFEX™ conditions for evaluating the effect of pretreatment conditions were selected using a central composite statistical design (CCD) (Table S4.2, Supplementary Information). Experimental data were taken within ammonia loading, water loading, and pretreatment temperature ranges between 0.5 and 1.5 g $NH_3$/g dry biomass, 0.4 and 0.8 g $H_2O$/g dry biomass, and 100 and 140°C, respectively. A minimum of 40 experimental data points was generated for statistical analysis using Minitab software (Minitab Inc., State College, PA, USA) for sugarcane bagasse and CLM each, including duplicates and five centre point replicates. The combined sugar yield (monomeric glucose + xylose) from low solids loading enzymatic hydrolysis (see below) was used as the metric of pretreatment efficacy. A full quadratic model was used to fit the experimental data containing all three pretreatment variables, including their main, interaction and quadratic effects. The models were refined to include parameters deemed significant by ANOVA and influence of the model predictive ability ($p < 0.05$ and $R^2_{predicted}$). The regression models were validated and used to predict the effect of the pretreatment conditions on the sugar yield within the experimental boundaries.

### 4.3.4.2 *Pre-pilot scale AFEX™*

Pre-pilot scale AFEX™ pretreatment was performed in a 3.8 L high-pressure reaction vessel (Parr) equipped with temperature and pressure sensors, as described previously [249]. Sugarcane

bagasse was treated with AFEX™ at 0.6 g $H_2O$/g dry biomass, and 1.0 g $NH_3$/g dry biomass, 140 ± 2 °C, and 60 min. AFEX™-treatment of CLM was performed at 0.7 g $H_2O$/g dry biomass, and 1.0 g $NH_3$/g dry biomass, 135 ± 2 °C, and 30 min. Each pretreatment was performed in duplicate. Pretreated samples were stored in sealed bags at 4 °C prior to enzymatic hydrolysis at low and high solids loading.

### 4.2.5 *Enzymes*

Commercial fungal enzyme preparations Cellic® CTec2 and Cellic® HTec2 were used to determine the effect of StEx pretreatment conditions and were generously donated by Novozymes (Copenhagen, Denmark). Commercially relevant Cellic® CTec3, Cellic® HTec3, and Pectinex Ultra-SP were used in subsequent studies with AFEX™ pretreatment optimization, enzyme mixture optimization and high solids loading enzymatic hydrolysis. These preparations were also generously donated by Novozymes Inc. (Franklinton, NC, USA). The protein concentration of the enzyme preparations was estimated using Kjeldahl nitrogen analysis (AOAC Method 2001.11, Dairy One Corporative Inc., Ithaca, NY, USA).

### 4.2.6 *Low solids loading enzymatic hydrolysis*

Low solids loading enzymatic hydrolysis was used to determine the impact of AFEX™ and StEx pretreatments on the sugar release from the pretreated solids. After StEx pretreatment, enzymatic hydrolysis was performed at a solids loading of 2% (w/v) WIS in 100 mL shake flasks at a total enzyme dosage of 33 mg protein per gram glucan and incubated at 50 °C, and pH 4.8 for 72 h on an orbital shaker (Lasec SA, Cape Town, South Africa) adjusted to 150 rpm. A fixed enzyme cocktail mixture consisting of 22 mg CTec2/g glucan and 11 mg HTec2/g glucan was used. The reaction mixture was supplemented with 50 mM citrate buffer and 0.02 % (w/v) sodium azide (Sigma Aldrich, South Africa) to maintain the hydrolysis pH and to prevent microbial contamination, respectively.

During the optimization of AFEX™ pretreatment, enzymatic hydrolysis was performed in 20mL screw-cap scintillation vials at 1% (w/v) glucan loading using 15 mg protein per gram of glucan, incubated at 50 °C, pH 4.8 for 72 h in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA). A



standard enzyme cocktail mixture consisting of 10 mg CTec3/g glucan and 5 mg HTec3/g glucan was used. After enzymatic hydrolysis, samples of the hydrolysate were withdrawn, incubated at 95 °C for 20 min (Thermomixer® R, Eppendorf, Westbury, USA) to denature the enzymes, and prepared for HPLC analysis.

### 4.2.7 *Enzyme mixture optimization*

A second-degree simplex lattice mixture design was carried out to determine optimal combinations of commercial enzymes Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP for the release of sugars from optimally-pretreated AFEX™ and StEx sugarcane bagasse and CLM. The total enzyme dosage was fixed at 15 mg total protein/g glucan and the ratio of the enzymes ranged from 0 to 1. A total of 40 experiments were generated in Minitab software for each pretreated substrate, including replicates (Minitab Inc.). The monomeric combined sugar yield (glucose + xylose) from low solids loading enzymatic hydrolysis was used to evaluate the effect of the different enzyme mixtures. Refined cubic regression models were generated, validated and used to predict the optimum enzyme combinations based on the combined sugar yield.

### 4.2.8 *High solids loading enzymatic hydrolysis*

High solids loading enzymatic hydrolysis was performed in 250 mL baffled Erlenmeyer flasks with a 100 mL working volume, incubated at 50 °C, and pH 5.0 on an orbital shaker adjusted to 250 rpm (New Brunswick Scientific, NJ, USA). Enzymatic hydrolysis was performed at 10% (w/w) carbohydrate loading, defined as the sum of the insoluble glucan and xylan, soluble xyloligosaccharides (X-OS) and glucoligosaccharides (G-OS), and soluble monomeric glucose and xylose in the pretreated material. The enzymatic hydrolysis mixtures were supplemented with 50 mM phosphate buffer and 50 mg/L chloramphenicol to maintain the hydrolysis pH and prevent bacterial contamination, respectively. Optimized ratios of Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP were used for the various pretreated feedstocks at enzyme dosages that ranged between 7.5 and 45 mg enzyme/g glucan.



The hydrolysis was carried out using a fed-batch strategy in which half the biomass added at *t* = 0 h, and the remainder added at *t* = 3 h. After a 96 h hydrolysis period, the slurry was centrifuged at 10,000 *x* g for 30 min to separate the unhydrolyzed solids from the hydrolysate. Samples of the hydrolysate were removed and analysed for monomeric and oligomeric sugar content. The unhydrolyzed solids were washed with 100 mL distilled water, centrifuged for a further 30 min at 10,000 *x* g. The supernatant was analysed for sugar content for mass balance closure. In preparation for fermentation, the hydrolysate was supplemented with 0.25% (w/w) corn steep liquor, and the pH adjusted to 5.5 before being filter sterilized through a 0.22 µm filter and refrigerated at 4 °C until use.

### 4.2.9 *StEx $C_5$-liquor post hydrolysis treatment*

Post-hydrolysis treatment with dilute sulphuric acid was performed to recover the oligomeric sugars in the StEx pretreatment hemicellulose-rich liquor (referred to as $C_5$-liquor) in monomeric form. The hydrolysis was performed in 100 mL glass pressure tubes with Teflon caps and *o*-ring seals (Ace Glass, New Jersey, USA). About 80 mL of $C_5$-liquor was added to the pressure tubes followed by the addition of 72% $H_2SO_4$ to achieve acid loadings of 1.0 % (w/w) and 0.75% (w/w) for bagasse and CLM, respectively. The pressure tubes were autoclaved at 121 °C for 60 min and subsequently cooled in ice. After cooling, the liquor pH was adjusted to pH 5.0 using a 30% (v/v) ammonium hydroxide solution, supplemented with 0.25 % (w/w) corn steep liquor, then re-adjusted to pH 5.5. The pH adjusted $C_5$-liquor was filter sterilized through a 0.22 µm filter and stored at 4 °C until use. Triplicate samples were prepared for each $C_5$-liquor sample evaluated.

### 4.2.10 *Fermentation*

The genetically modified, xylose-fermenting *Saccharomyces cerevisiae* strain 424A (LNH-ST), kindly provided by Prof. Nancy W.Y. Ho, Purdue University, was used to ferment AFEX™ and StEx enzymatic hydrolysates and the StEx $C_5$-liquor. The seed culture of this strain was prepared in 250 mL Erlenmeyer flasks containing YPDX medium that consisted of (per litre) 75 g glucose, 25 g xylose, 10 g yeast extract, 20 g tryptone. A frozen glycerol stock was used for seed culture inoculation at an initial optical density of 0.1. The seed culture was cultivated at 30 °C and 150 rpm for 18 h to an approximate

optical density ($OD_{600}$) of 12. The culture was subsequently harvested and used as inoculum for AFEX™, StEx (washed solids) and StEx (pressed or unwashed solids) enzymatic hydrolysate fermentations. In experiments where the whole slurry after StEx pretreatment or StEx $C_5$-liquor was fermented, the yeast was pre-conditioned in an additional cultivation step prior to inoculating the growth medium. Pre-conditioning was carried out by inoculating 75 mL YPDX media and 25 mL of $C_5$-liquor in a 250 mL Erlenmeyer flask using the seed culture described above. After inoculating the pre-conditioning medium to an initial $OD_{600}$ of 2, cultures were incubated in a rotary incubator adjusted to 30 °C and 150 rpm for 18 h. The pre-conditioned seed culture medium was centrifuged at 4,000 rpm for 15 min and the yeast pellets were used as inoculum for StEx whole slurry or $C_5$-liquor fermentation.

Enzymatic hydrolyses and $C_5$-liquor fermentations were performed in 125 mL Erlenmeyer flasks with 50 mL working volume at pH 5.5, 30 °C, and 150 rpm for 120 h. A rubber stopper with a hypodermic needle piercing was used to cap the flask and maintain predominantly anaerobic conditions. The fermentation flasks were inoculated at $OD_{600}$ of 2, which corresponded to a yeast biomass concentration of 0.96 g CDW/L. Samples were withdrawn at frequent intervals and after centrifugation, the cell-free supernatants were prepared for HPLC analysis. The ethanol metabolic yield was calculated from the glucose and xylose consumed relative to the theoretical ethanol yield of 0.51 g ethanol per gram glucose or xylose consumed. The overall process ethanol yield was determined based on the sugar yield from enzymatic hydrolysis and the sugar consumption and metabolic yield during fermentation. Monomeric sugars (glucose, xylose, arabinose), pretreatment products (acetic acid, formic acid) and fermentation products (lactate, xylitol, glycerol and ethanol) were determined by HPLC system equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as described previously [247]. The column temperature was maintained at 50 °C, with sulphuric acid (5 mM) used as the mobile phase at a flowrate of 0.6 mL/min.

### 4.2.11 *Process configurations*

Four process configurations were evaluated for the pretreated materials using a separate hydrolysis and fermentation (SHF) flow scheme (Figure 4.1).

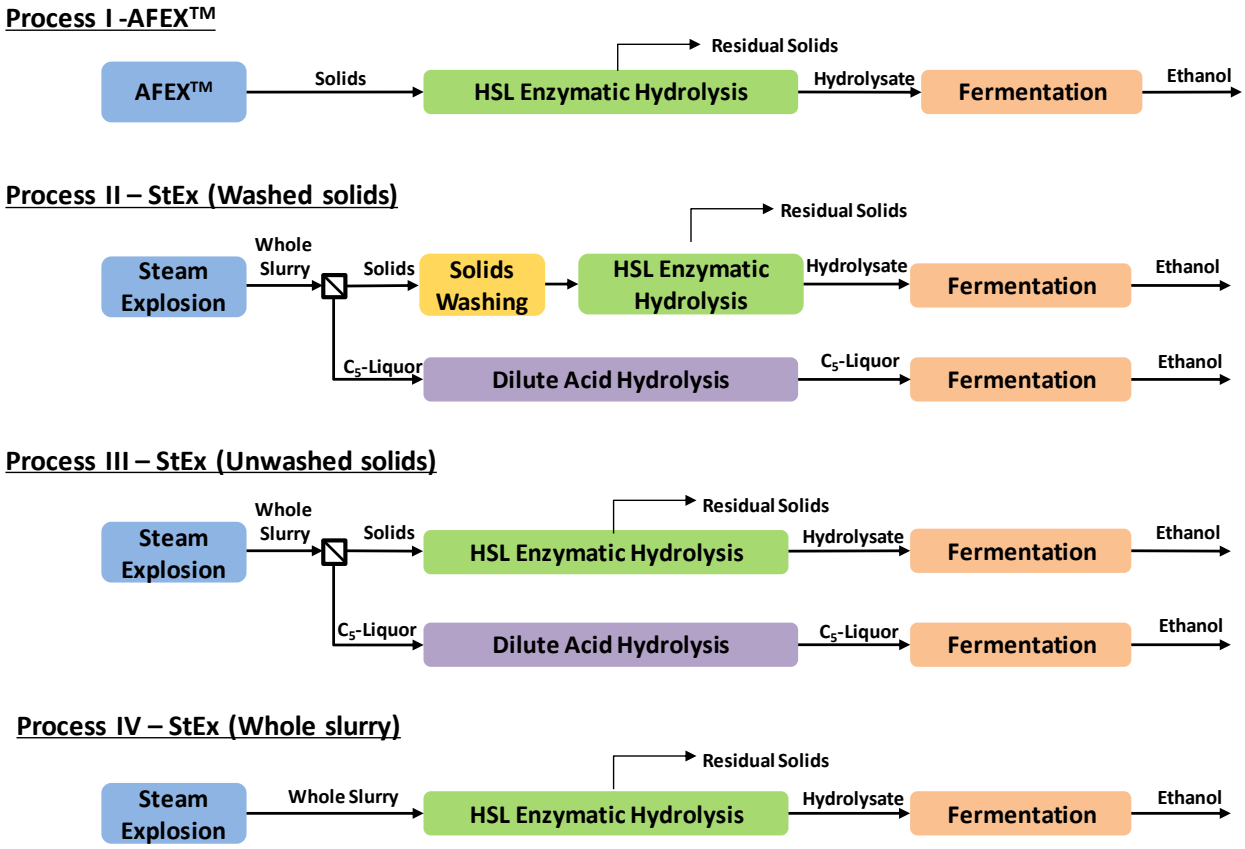



**Figure 4.1:** Process flowsheets studied for the conversion of sugarcane residues to ethanol. **Process I** - AFEX™ pretreatment with high solids loading separate enzymatic hydrolysis and fermentation (SHF) of the solids, **Process II** – Steam explosion followed by solids washing and high solids loading SHF with separate fermentation of the $C_5$-rich liquor, **Process III** – steam explosion followed by high solids loading SHF of unwashed solids and separate fermentation of the $C_5$-rich liquor, **Process IV** – steam explosion followed by high solids loading SHF of the whole slurry. HSL – High Solids Loading

In Process I, AFEX™-treated bagasse or CLM underwent high solids loading enzymatic hydrolysis, followed by the removal of undigested solids and fermentation of the enzymatic hydrolysate. To determine the extent of enzymatic and microbial inhibition due to the presence of StEx-derived degradation products, the StEx pretreated slurry was processed in three ways, referred to as Processes II, III and IV. In Process II, StEx pretreatment was followed by solid-liquid separation to recover the $C_5$-rich liquor and the solid fraction. The solid fraction was washed in three stages with distilled water heated to 50 °C, using a total of 10 L water per kg pressed solids to remove soluble



sugars and pretreatment-generated organic acids, furan derivatives and water soluble phenolic compounds [112]. After washing, the solids were subjected to high solids loading enzymatic hydrolysis followed by separate fermentation of the enzymatic hydrolysate and the acid-hydrolysed $C_5$-liquor. Lignin-rich residual solids were recovered after enzymatic hydrolysis. Process III was performed under identical conditions as Process II, except that the solids washing step was excluded. Finally, in Process IV, eliminating the solid/liquid separation and washing steps after StEx pretreatment was also evaluated, resulting in a one-stream, whole slurry configuration. Monomeric, oligomeric and polymeric sugars and ethanol concentrations before and after each process unit operation were determined and mass balances were calculated as previously described [122].

## 4.3 Results and discussion

### 4.3.1 *Biomass composition and energy value*

The composition and calorific value of sugarcane bagasse and CLM are presented in Table 4.1 and was similar to that previously reported for South African industrial sugarcane residues [250].

**Table 4.1:** Chemical composition and energy value of sugarcane bagasse, cane leaf matter and a bagasse-CLM mixture

| **Biomass Component** † | **Bagasse** | **Cane Leaf Matter** | **Bagasse + CLM Mixture (1:1 w/w)** |
|---|---|---|---|
| Glucan (kg/100kg DM) | 39.50 ± 0.41 $^{A}$ | 37.45 ± 0.6 $^{B}$ | 38.11 ± 0.14 $^{B}$ |
| Xylan (kg/100kg DM) | 25.21 ± 0.13 $^{A}$ | 24.81 ± 0.4 $^{A}$ | 24.21 ± 0.2 $^{B}$ |
| Arabinan (kg/100kg DM) | 1.23 ± 0.38 $^{B}$ | 2.73 ± 0.1 $^{A}$ | 1.48 ± 0.24 $^{B}$ |
| Acetyl (kg/100kg DM) | 3.43 ± 0.04 $^{B}$ | 2.21 ± 0.06 $^{C}$ | 4.32 ± 0.18 $^{A}$ |
| Lignin (kg/100kg DM) | 19.35 ± 0.06 $^{A}$ | 16.17 ± 0.81 $^{B}$ | 19.5 ± 0.59 $^{A}$ |
| Ash (kg/100kg DM) | 2.89 ± 0.65 $^{C}$ | 7.34 ± 0.21 $^{A}$ | 5.21 ± 0.71 $^{B}$ |
| Extractives (kg/100kg DM) | 6.02 ± 0.42 $^{C}$ | 12.07 ± 1.54 $^{A}$ | 10.32 ± 0.39 $^{B}$ |
| **Calorific Value** † | | | |
| Higher Heating Value (MJ/kg) | 18.47 ± 0.06 $^{A}$ | 17.67 ± 0.05 $^{C}$ | 17.92 ± 0.13 $^{B}$ |

† - dry basis
Different superscripts within row indicate significant differences as determined using one-way ANOVA with Tukey's *post*-hoc test for multiple comparisons ($p$<0.05**)**

Sugarcane bagasse demonstrated higher glucan, acetyl group and lignin contents and lower extractives and ash contents relative to the CLM ($p < 0.05$). Based on the glucan and xylan contents,

the potential monomeric sugar (glucose + xylose) recovery from of bagasse and CLM is 72.48 ± 0.6 and 69.75 ± 0.9 kg per 100 kg RDM, respectively, making both materials promising feedstocks for ethanol production. The lower ash content and higher HHV of the bagasse ($p < 0.05$) suggests that it may be a more suitable source candidate for cogeneration operations in common mill boilers [27]. High ash content boiler feeds are understood to contribute to slagging, corrosion and fouling formation within the boiler [26,251]. Other than washing the CLM to remove mineral impurities collected from harvesting the CLM, mixing with bagasse (at appropriate ratios) may provide a simpler way of reducing the ash content of sugar mill boiler feeds. Moreover, given the availability of sugarcane bagasse at elevated moisture levels (> 50%, w/w) as an end-of-process product compared to the modest moisture content of on-field dried CLM (~15%, w/w), mixing the two feedstocks may also be an effective strategy of reducing the moisture content and increasing the efficiency of sugarcane mill boiler feeds.

### 4.3.2 *Pretreatment*

#### 4.4.2.1 *Steam Explosion*

The overall glucose and xylose yields from StEx pretreatment at temperatures ranging from 185 to 215 °C and residence times from 10 to 15 min are presented in Figure 4.2. The combined sugar yield was determined from the soluble monomeric and oligomeric sugars (glucose + xylose) in the pretreatment liquor and the soluble monomeric sugars (glucose + xylose) released after enzymatic hydrolysis of washed solids, performed at low solid loading (section 4.2.6). A summary of the compositions of the pretreated water insoluble solids, pretreatment liquor and major phenolic compounds in the liquor is presented in Table S4.1 (Supplementary Information).

As is common with acid-based pretreatments, increased pretreatment severity successively increased the solubilization of hemicellulose from the plant cell wall matrix, thus enriching the pretreated solids in cellulose and lignin for both bagasse and CLM [104,110]. Within the evaluated conditions, the highest combined sugar yield for bagasse was obtained at intermediate severity ($LogR_o$ = 4.22), amounting to 55.3 kg sugar/100 kg RDM (77% of the theoretical maximum). StEx pretreatment of bagasse at this severity facilitated significant hydrolysis of ester linkages in the acetyl group side

branches of the xylan backbone as evidenced by an acetic acid yield of 3.36 kg/100 kg RDM in the pretreatment liquor (Table S4.1).

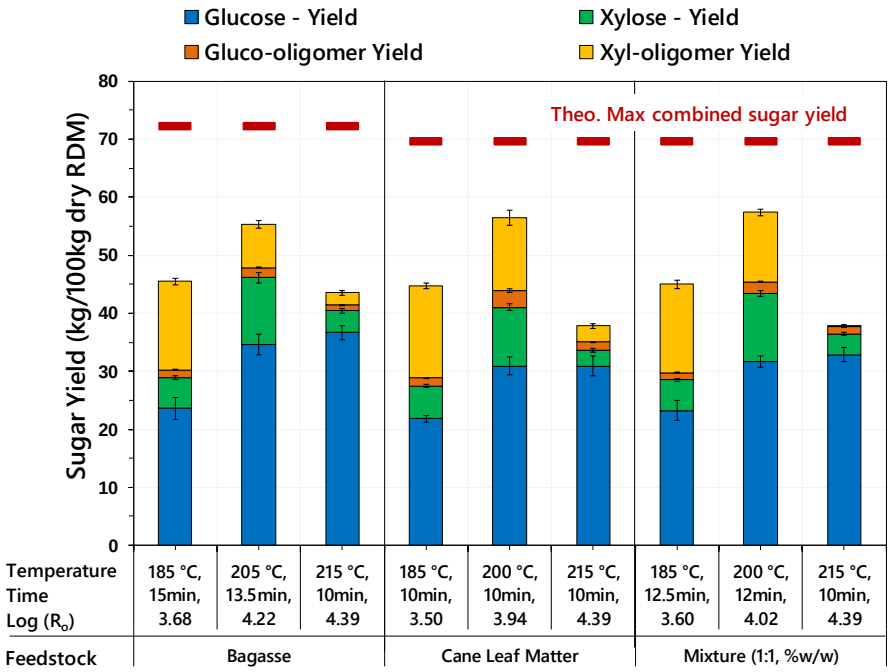



**Figure 4.2:** The evaluation of the impact of pretreatment conditions on glucose and xylose yield from sugarcane bagasse, cane leaf matter (CLM), and a bagasse: CLM mixture (1:1 w/w). **A** – Steam explosion sugar yield as a function of pretreatment severity. Enzymatic hydrolysis performed at 2% (w/v) WIS loading and incubated 50 °C, for 72 h using 22 mg CTec2 and 11 mg HTec2. **Theo.:** Theoretical; **Max.:** Maximum **RDM:** raw dry material; **Log ($R_o$):** severity factor

The accumulation of acetic acid (and other aliphatic and aromatic acids) in the aqueous solution and the presence of hydronium ions from the self-ionization of water at the pretreatment temperature (205 °C, intermediate severity) were reported to catalyse the partial hydrolysis of hemicellulose to soluble hemicellulose monomeric and oligomeric sugars [101,252]. Accordingly, the total monomeric and oligomeric xylose yield at this condition was 18.9 kg/100 kg RDM (66.1% of the theoretical maximum), with approximately 40% of the xylose recovered in oligomeric form. In comparison, pretreatment at lower severity resulted in a xylose yield of 20.51 kg/100 kg RDM, with more than 74% of the recovered xylose in oligomeric form. However, pretreatment at low severity conditions did not enhance cellulose digestibility as much as the intermediate condition, as demonstrated by a lower glucose yield (57% of the theoretical maximum). Pretreatment at higher severity resulted in the highest glucose yield (86% of the theoretical maximum), but also significant xylan degradation products were produced, likely from the dehydration of xylose and thereby lowered



the combined sugar yield. Although the intermediate pretreatment severity resulted in the highest combined sugar yield, unavoidable degradation products were nonetheless present in the pentose-rich liquor (Table S4.1).

The highest combined sugar yield for the StEx-treated sugarcane CLM was also obtained at the intermediate severity condition ($LogR_o$ = 3.94), corresponding to 56.5 kg sugar/100 kg RDM (81% of the theoretical maximum). However, unlike StEx-treated bagasse, the highest xylose yield (80.7% of the theoretical maximum) was also obtained at the intermediate severity, with more than 60% of the soluble xylose in oligomeric form. Biomass with high ash content has been previously reported to have some neutralizing/buffering capacity in acidic pretreatments [101,252]. Untreated CLM is composed of more than 7% ash and about 2% acetyl group content, and therefore, the proton concentration (or $[H_3O^+]$) in the aqueous pretreatment slurry is dependent on competing neutralization, de-acetylation and water ionization reactions. The hydrolysis of insoluble xylan to soluble oligomers is generally observed when the pretreatment temperatures are low or the pH is closer to neutral and the hydrolysis of soluble oligomers to monomeric sugars occurs rapidly under more acidic conditions [110]. As a result, the high ash content and low acetyl group content of CLM may indirectly contribute to the formation of soluble xylan oligomers instead of monomeric xylose, which is prone to dehydration at high temperatures. In support of this hypothesis, we found that the final pH after pretreatment of the CLM at the intermediate severity was 3.7 compared to 3.08 for the bagasse. Consequently, the CLM resulted in higher xylose yield and lower furfural yield relative to the bagasse (Table S4.1). Ferrierra-Leitão *et al.,* [45] reported a similar finding, with higher buffering capacity and sugar recoveries from CLM relative to sugarcane bagasse for autocatalyzed and $CO_2$-impregnated StEx, at pretreatment temperatures similar to those used in this work. Further, pretreating a mixture of bagasse and CLM (at 1:1 ratio on a dry weight basis) at 200 °C and 12 min resulted in a combined sugar yield of 57.4 kg sugar/100 kg RDM (82.2% of the theoretical maximum). This outcome suggests that StEx could still be effective for pretreating mixtures of bagasse and CLM when the mean moisture content of the mixture is in the range of 65-75% (w/w).



Based on the combined sugar yield results, the intermediate StEx pretreatment severity for both sugarcane bagasse and CLM were selected as the preferred pretreatment conditions and henceforth used in enzyme cocktail optimization, high solids loading enzymatic hydrolysis and fermentation studies.

#### 4.4.2.2 *AFEX™*

To understand the interaction of pretreatment parameters and potentially minimize ammonia loading during AFEX™ pretreatment, a wide range of pretreatment conditions were evaluated and statistically modelled. Contour plots and regression models depicting the impact of the pretreatment temperature, ammonia loading and water loading on the combined monomeric sugar (glucose + xylose) yield, following low solids loading enzymatic hydrolysis, are presented in Figure S4.1A and Figure S4.1B (Supplementary Information). The main effects of ammonia loading, pretreatment temperature and water loading were statistically significant, with a quadratic, second order model deemed sufficient to describe the release of fermentable sugars during enzymatic hydrolysis for AFEX™-treated sugarcane bagasse and CLM, as evident from insignificant lack of fit. The statistically-derived regression models were validated by performing additional experiments not included in the original CCD statistical design and subsequently used to predict the combined sugar yield at various ammonia loading conditions, *i.e.* low, intermediate and high ammonia loadings, as presented in Figure 4.3 (next page).

High temperature and high ammonia loading AFEX™ pretreatment resulted in the highest monomeric glucose and xylose yields for both sugarcane bagasse and CLM. The combined monomeric sugar yields achieved at an ammonia loading of 1.5 g $NH_3$/g DM were 61.4 kg sugar/100 kg RDM (84.8% of the theoretical maximum) and 57.1 kg sugar/100 kg RDM (81.7% of the theoretical maximum) for sugarcane bagasse and CLM, respectively. High ammonia loading AFEX™ treatment has been shown to enhance the cleavage of ester-linked phenolic compounds in the plant cell wall of monocots, particularly ferulates and coumarates, through ammonolysis reactions [78,123]. These reactions correlate with higher enzymatic digestion of agricultural grasses [253]. However, high ammonia

loadings also translate into higher energy and capital costs for ammonia recovery operations [130,253].

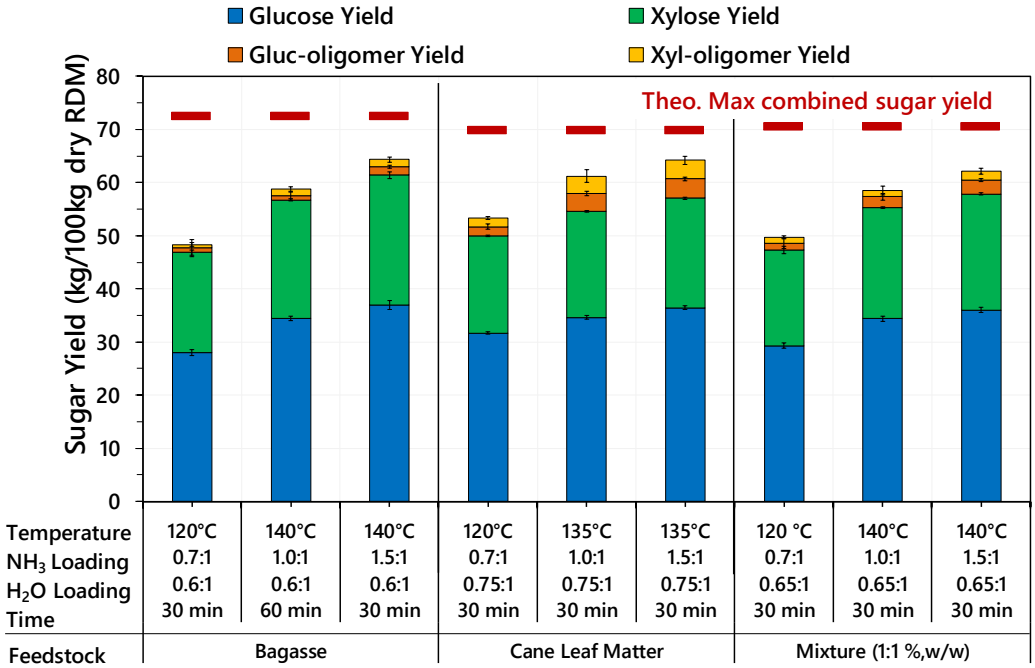



**Figure 4.3:** The evaluation of the impact of pretreatment conditions on glucose and xylose yield from sugarcane bagasse, cane leaf matter (CLM), and a bagasse: CLM mixture (1:1 w/w). AFEX™ sugar yield as a function of temperature, ammonia loading, water loading and residence time. Enzymatic hydrolysis performed at 1% (w/v) glucan loading and incubated 50 °C, for 72hrs using 10 mg CTec3 and 5 mg HTec3. **Theo.:** Theoretical; **Max.:** Maximum **RDM:** raw dry material; **Log ($R_o$):** severity factor

In comparison, pilot-scale AFEX™ pretreatment of corn stover is typically performed at ammonia loadings lower than 1 g $NH_3$/g DM [30]. Limiting the ammonia loading to 1 g $NH_3$/g DM resulted in combined monomeric sugar yields of 56.8 kg sugar/100 kg RDM (78.3% theoretical maximum) and 54.6 kg sugar/100 kg RDM (78.2% of the theoretical maximum) for sugarcane bagasse and CLM, respectively. While the ammonia loading was reduced by 33%, the combined monomeric sugar yield only reduced by 6.5% and 3.5% for bagasse and CLM, respectively. Although the combined sugar yields for bagasse and CLM were quite similar, the CLM glucan conversion (83% of the theoretical maximum) was less sensitive to the reduced ammonia loading relative to the bagasse (77% of the theoretical maximum). Oligomeric analysis of the CLM enzymatic hydrolysate revealed significant quantities of xylooligomers, hinting at the possible absence of some auxiliary activities in the enzyme cocktail employed, which may be required to further increase the combined sugar yields for CLM from AFEX™ pretreatment [254].



The statistically-derived regression models were used to identify pretreatment conditions that would be suitable for the AFEX™ pretreatment of a mixture of bagasse and CLM (Figure S4.2, Supplementary Information). AFEX™ pretreatment of a bagasse-CLM mixture (composed of 1:1 w/w ratio) at 140 °C, 1 g $NH_3$/g DM, 0.65 g $H_2O$/g DM and 30 min residence time produced a combined monomeric sugar yield of 55.3 kg sugar/100 kg RDM (78.3% of the theoretical maximum). Like StEx pretreatment, this result demonstrates the suitability of AFEX™ to sugarcane residue mixtures, provided the initial moisture of the mixture prior to ammonia addition is approximately 0.65 g $H_2O$/g DM.

In its most mature design, packed-bed AFEX™ pretreatment on pilot-scale is designed to receive biomass with an initial moisture content of approximately 30% before being pre-steamed to simultaneously preheat the biomass and adjust the moisture content to an optimized water loading (typically 60-70%) [30]. In industry, sugarcane bagasse fed into mill boilers is obtained after juice extraction, warm washing and dewatering operations and usually has a moisture content of approximately 50-60%. Surplus bagasse is typically stockpiled for storage and occasionally mildly irrigated to minimise the risk of spontaneous combustion [44]. Therefore, for current AFEX™ pretreatment designs, energy would need to be expended to dry the bagasse towards a lower moisture content prior to pretreatment. Previously, it was shown that AFEX™ pretreatment of high moisture content bagasse required an ammonia to biomass loading of 2.0 g $NH_3$/g DM to achieve glucan conversions greater than 75%, demonstrating the necessity of lowering the moisture content of bagasse prior to pretreatment [46]. In contrast, CLM is likely to be left on the field and allowed to dry down to moisture levels where it can be easily managed. In general, dried CLM typically has a much lower moisture content (about 15%) and therefore would be at a much more suitable moisture content for direct use in AFEX™ pretreatment. Alternatively, mixing these two substrates may negate the need for expending significant energy for drying the bagasse and/or minimize water consumption for adjusting the initial moisture of the CLM prior to AFEX™ pretreatment. As suggested by the results in this section, mixing these two substrates at appropriate ratios would not significantly affect the

pretreatment effectiveness as measured by the combined monomeric sugar yields from downstream enzymatic hydrolysis, thus making AFEX™ also agnostic to sugarcane residues. Ultimately, local biomass harvesting techniques (manual vs mechanical), logistics, handling and storage infrastructure available at the biorefinery will likely define processing decisions (*e.g.* on-field CLM drying, milling operations) necessary to minimize energy expenditure for maximising AFEX™ or StEx pretreatment efficiency.

### 4.3.3 *High solids loading enzymatic hydrolysis*

Due to uncertainties regarding the cost of enzymes, minimizing the enzyme dosage would ensure that AFEX™ or StEx-based biorefineries would be less sensitive to fluctuations in enzyme purchase or production costs [54]. High solids loading enzymatic hydrolysis (HSL-EH) was evaluated to compare the effect of enzyme dosage and solids processing option on monomeric sugar yields based on configurations defined in Figure 4.1. Optimal commercial enzyme combinations (CTec3: HTec3: Pectinex Ultra-SP) for AFEX™ and StEx treated bagasse and CLM were used to maximize the saccharification yields for each pretreated substrate (Fig. S4.3, Supplementary Information). The corresponding glucose and xylose yields were based on the weight of monomeric sugars recovered relative to the total weight of the corresponding carbohydrate loaded.

The monomeric glucose and xylose yields as a function of the enzyme dosage are presented in Figure 4.4. AFEX™-treated bagasse and CLM (Process I) achieved glucose yields of 77% and 81.5% at the inflection enzyme dosage of 25 mg /g glucan, respectively. However, an additional 16% and 14% of the total sugars released from AFEX™ treated bagasse and CLM were in oligosaccharide form, respectively (data not shown). At lower enzyme loadings, the accumulation of these soluble oligosaccharides was even more pronounced. For example, the monomeric glucose and xylose yields for AFEX™ treated CLM at 15 mg enzyme/g glucan were 65% and 63%, respectively. However, an additional 14% G-OS and 21% X-OS were recovered in the enzymatic hydrolysate. The accumulation of oligomeric sugars is not unique to AFEX™ pretreatment and has also been demonstrated for dilute acid and ionic liquid pretreated corn stover [254]. These soluble oligomeric sugars not only inhibit the

activity of commercial enzyme mixtures, but they also represent lost yield since most ethanologens only consume monomeric sugars [97]. The discovery of enzyme activities that are absent from current commercial cocktail mixtures for converting recalcitrant oligosaccharides to fermentable monomeric sugars, can potentially generate higher fermentable sugar yields or even reduced enzyme requirements for these AFEX™-treated sugarcane residues [255].

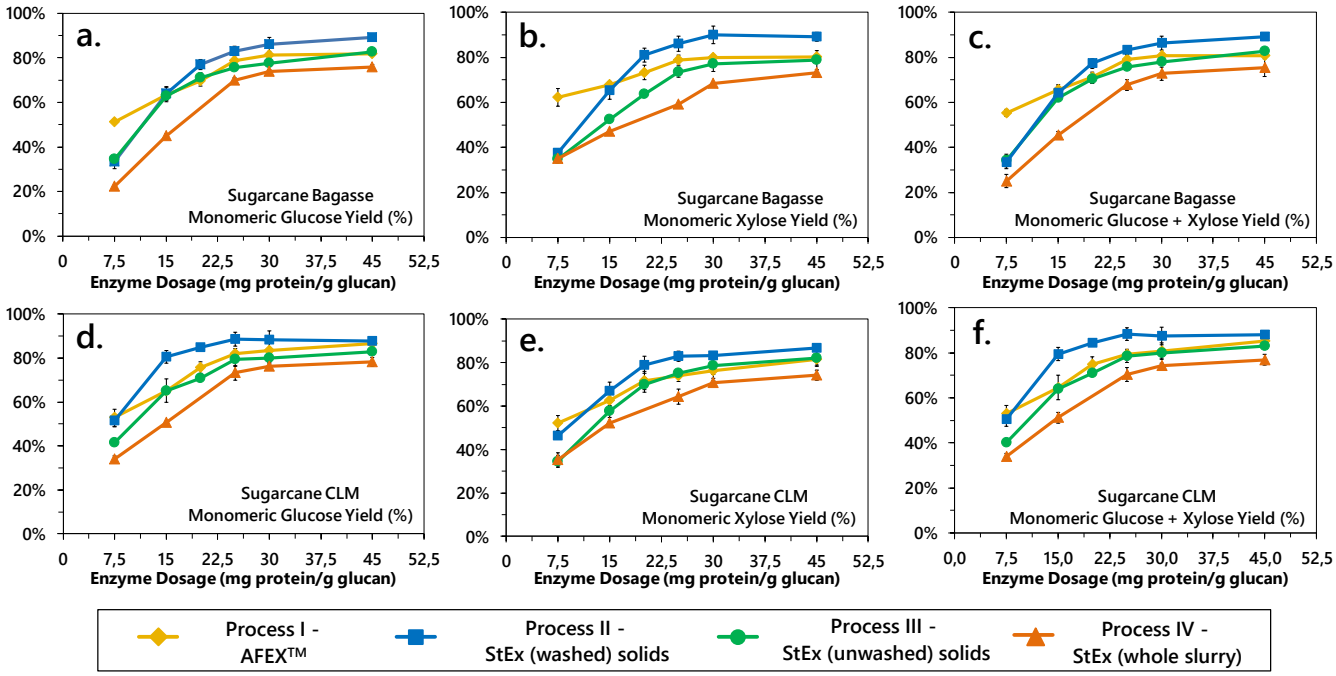



**Figure 4.4:** Glucose and Xylose yields for high solids loading enzymatic hydrolysis of AFEX™-treated, steam exploded (washed solids), and steam exploded solids (unwashed) of bagasse and CLM. Enzymatic hydrolysis was performed using optimized mixtures of CTec3, HTec3, and Pectinex Ultra-SP. The solids loading was maintained at 10% carbohydrate loading, pH 4.8 and incubated at 50 °C for 96 h.

For StEx pretreatment, the presence of organic acids, furan aldehydes, phenolic compounds, and soluble sugars (monomeric and oligomeric) limited the enzyme activity and subsequently required high enzyme dosages to achieve high sugar yields (Table S4.1). This was evident as separating and washing StEx pretreated bagasse or CLM solids (Process II) resulted in higher combined glucose plus xylose yields relative to unwashed solids or the whole slurry processing options (Process III and IV). Washing StEx solids has been reported to remove some of the inhibitory components including soluble carbohydrates (especially X-OS and monomeric sugars), soluble organic acids, water-soluble aromatics and furan derivatives that might have adsorbed onto the solid biomass during pretreatment [112,147,256]. Interestingly, for CLM, washing the solids had a larger impact on the glucose and xylose

yields at lower enzyme loadings. At 15 mg/g glucan, the combined glucose plus xylose yield for StEx-CLM (washed) solids was 80% relative to 64% and 51% for unwashed solids and whole slurry, respectively (Figure 4.4-f). StEx-treated CLM produced a pretreatment liquor that was rich in oligosaccharides (particularly X-OS) that strongly inhibit cellulases (particularly CBH I and CBH II) [154,254]. Hence, by introducing a solid-liquid separation step and/or washing the StEx pretreated CLM solids, the effect of enzyme inhibition by soluble X-OS or degradation products can be minimized, and enzyme loadings can be significantly reduced. At lower enzyme loadings (< 15 mg/g glucan), the glucose and xylose yields from StEx-treated bagasse and CLM both with-and without-washing decreased sharply. This effect could be due to end-product inhibition, enzyme access blockage by lignin and/or non-productive binding of the hydrolytic enzymes to lignin [153].

Given that the enzyme costs were previously estimated to account for 15.7% of the total costs even at enzyme loadings of 20 mg/g glucan, it may be necessary to explore processing options that further reduce the required enzyme dosage [92]. As demonstrated in this work, depending on the pretreatment conditions and the pretreated biomass, investing in solid-liquid separation and/or washing steps may reduce the enzyme loadings. However, an economic and environmental impact assessment may be necessary to decide whether the enzyme savings for using washed solids outweigh the requirements for additional capital and operating costs for solid-liquid separation and/or washing operations. Similarly, lowering the enzyme loading for AFEX™-treated bagasse or CLM may also require an economic and environmental impact assessment given that by altering the pretreatment conditions (*e.g.* using a higher ammonia loading during pretreatment), the enzyme loading requirements to reach target sugar yields can be lowered at the expense of higher capital and operational costs for ammonia recovery.

### 4.3.4 *Fermentation*

The fermentation profiles for converting enzymatic hydrolysates and the StEx $C_5$-liquor (configurations shown in Figure 4.1) to ethanol are presented in Figure 4.5. A summary of the fermentation performance of xylose-fermenting *S. cerevisiae* 424A (LNH-ST) on the various process

streams is presented in Table 4.2. The extent of glucose or xylose consumption, ethanol metabolic yield, and ethanol titre were used as metrics for comparing the fermentability of the various streams.

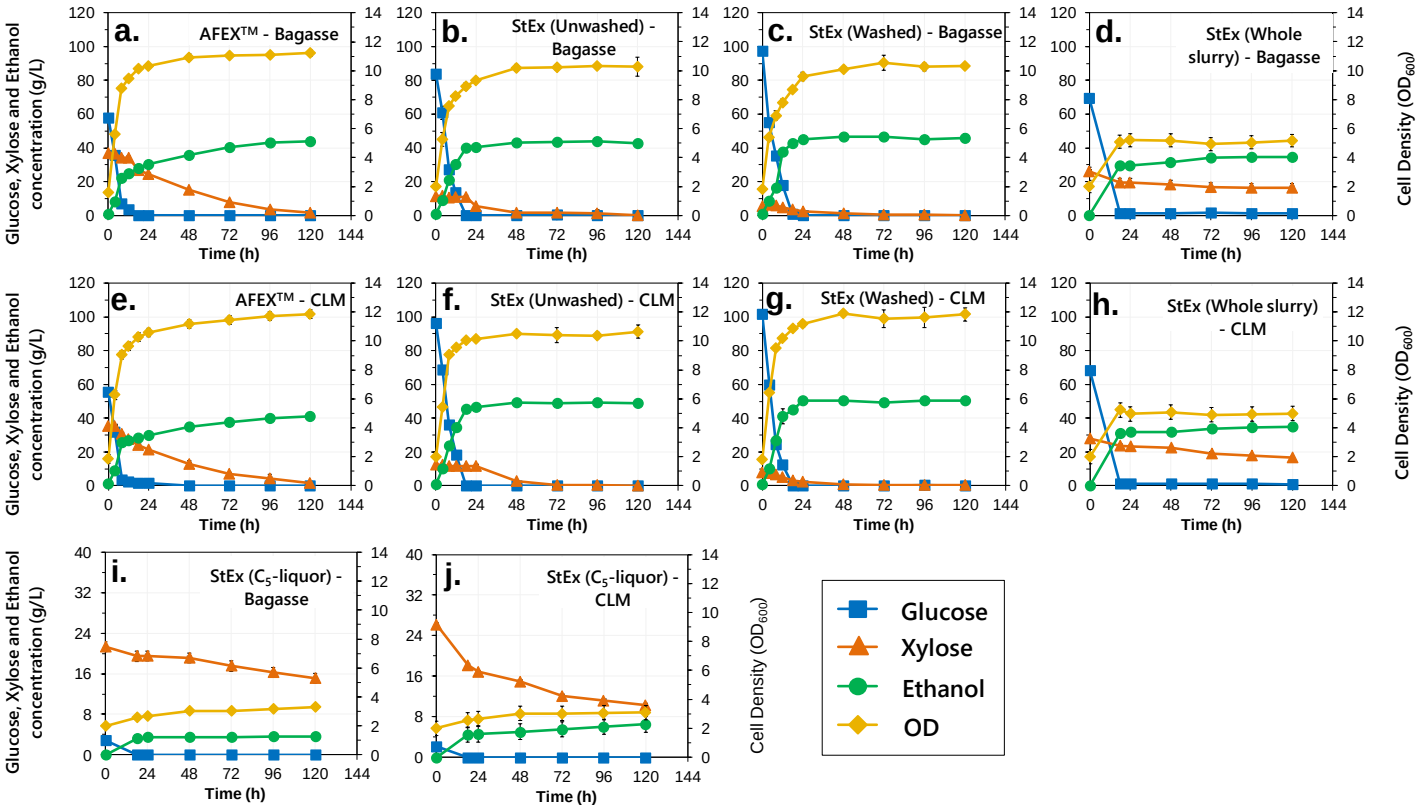



**Figure 4.5:** Fermentation time profiles for enzymatic hydrolysates obtained from AFEX™, StEx (Washed solids), StEx (Unwashed solids), StEx (whole slurry), and StEx ($C_5$-liquor) sugarcane bagasse and cane leaf matter. Fermentations were performed using S. cerevisiae 424A (LHN-ST) with an initial inoculum of 0.96g CDW/L at 30 °C, pH 5.5, and a shaking speed of 150 rpm for 120 h. All enzymatic hydrolysates were supplemented with 0.25% (w/w) corn steep liquor prior to fermentation. **Square** – Glucose (g/L), **Triangle** – Xylose (g/L), **Circle** – Ethanol (g/L), **Diamond** – $OD_{600nm}$

Like most native *S. cerevisiae* strains, the microbial strain used in this work typically demonstrates slow diauxic xylose fermentation due to the lack of high-affinity xylose transporters in the presence of glucose [257]. As a result, glucose was rapidly consumed from all process streams (Process I – IV) within 18 h (Figure 4.5). In agreement with previous reports, AFEX™-derived bagasse and CLM enzymatic hydrolysates (Process I) achieved near complete xylose consumption, with ethanol metabolic yields and ethanol titres greater than 89% and 40 $g.L^{-1}$, respectively [135,171]. Similarly, near complete xylose consumption was observed for washed and unwashed StEx-derived bagasse and CLM enzymatic hydrolysates (Process II and III), with approximately 90% metabolic yield and ethanol titres greater than 40 $g.L^{-1}$.



**Table 4.2:** Summary of fermentation parameters of steam exploded and AFEX™-treated bagasse and cane leaf matter. All hydrolysates were supplemented with 0.25% (w/w) corn steep liquor prior to fermentation. The $C_5$-liquor/s were acid hydrolysed to convert oligosaccharides to their monomeric counterparts prior to fermentation.

| Parameter | AFEX™ – Bagasse | StEx - Bagasse – Unwashed solids | StEx - Bagasse – Washed solids | StEx – Bagasse – Whole Slurry | StEx – Bagasse – $C_5$-liquor | AFEX™ – CLM | StEx - CLM – Unwashed solids | StEx - CLM – Washed solids | StEx- CLM – Whole Slurry | StEx – CLM – $C_5$-liquor |
|---|---|---|---|---|---|---|---|---|---|---|
| Initial glucose conc. (g/L) | 59.03 | 83.89 | 97.56 | 69.67 | 2.89 | 58.45 | 96.27 | 101.49 | 68.30 | 2.10 |
| Initial xylose conc. (g/L) | 37.01 | 11.22 | 5.60 | 26.09 | 21.19 | 35.15 | 12.38 | 7.70 | 27.95 | 26.18 |
| Glucose consumption (%) | 100% | 100% | 100% | 98% | 100% | 100% | 100% | 100% | 99% | 100% |
| Xylose consumption (%) | 96% | 90% | 95% | 37% | 29% | 95% | 97% | 96% | 41% | 61% |
| $\mu_{max}$ ($h^{-1}$) † | 0.31 | 0.24 | 0.27 | 0.05 | 0.013 | 0.3 | 0.25 | 0.32 | 0.055 | 0.015 |
| $Y_{x/s}$ (g CDW/ g sugar) ‡ | 0.05 | 0.04 | 0.04 | 0.03 | 0.02 | 0.05 | 0.04 | 0.04 | 0.03 | 0.01 |
| Metabolic yield (%) ϕ | 92% | 91% | 89% | 87% | 69% | 89% | 89% | 90% | 87% | 71% |
| $Y_{p/s}$ (g EtOH /g sugar) ψ | 0.46 | 0.45 | 0.45 | 0.36 | 0.13 | 0.44 | 0.45 | 0.46 | 0.36 | 0.23 |
| EtOH conc. (g/L) | 44.17 | 43.48 | 46.75 | 34.62 | 3.18 | 41.70 | 49.24 | 50.21 | 35.07 | 6.48 |
| EtOH yield (kg/100kg DM) λ | 25.60 | 16.54 | 16.84 | 16.23 | 1.69 | 24.90 | 15.98 | 16.58 | 16.67 | 3.75 |

Data represents the averages of independent duplicate fermentation cultivations. All standard errors were less than 5%.

† – Maximum specific growth rate: maximum growth rate calculated in the exponential growth phase

‡ – Cell biomass yield: gram of cell dry weight per gram of sugar (glucose + xylose) consumed during fermentation

ϕ – Metabolic yield: gram of ethanol produced per gram of sugar (glucose + xylose) consumed during fermentation

ψ – Ethanol yield: gram ethanol produced per gram of sugar (glucose + xylose) at the beginning of fermentation

λ – Ethanol yield per 100 kg of untreated dry material

Moreover, the fermentation of these enzymatic hydrolysates was complete after 48 h owing to their low initial xylose concentrations (< 15 $g.L^{-1}$) and the supplementation with corn steep liquor. This observation is supported by previous work that demonstrated that xylose fermentation performance of *S. cerevisiae* 424A (LNH-ST) was influenced by nutrient availability in the fermentation media [40]. The fermentation of whole slurry enzymatic hydrolysates (Process IV) resulted in significantly lower xylose consumption and slightly lower metabolic yield for both StEx-treated bagasse and CLM. Whole slurries derived from acidic pretreatments are typically rich in various pretreatment inhibition products, including aliphatic and aromatic carboxylic acids, furan aldehydes, and phenolic compounds [112]. Moreover, because glucose fermentation occurs before xylose fermentation, ethanol and other accumulated fermentation metabolites generated during the glucose consumption phase further inhibit xylose fermentation. The presence of fermentation metabolites has been previously shown to play a critical role in inhibiting xylose uptake by *S. cerevisiae* 424A (LNH-ST) [122,160]. Therefore, the limited xylose fermentation performance for StEx-whole slurries may be attributed to the inability of this strain to buffer redox changes caused by the synergistic/combined effect of pretreatment inhibitors, ethanol and fermentation metabolites [258,259]. Nonetheless, even in the presence of microbial inhibition, ethanol titres of approximately 35 $g.L^{-1}$ at metabolic yields greater than 85% were achieved for both StEx bagasse and CLM whole slurries. In comparison, Mosier *et al.,* [85] reported metabolic yields and a final ethanol concentration of 88% and 22.5 $g.L^{-1}$ , respectively, for the fermentation of non-detoxified liquid hot water treated corn stover whole slurry hydrolysate by *S. cerevisiae* 424A (LNH-ST).

The StEx bagasse and CLM $C_5$-liquor streams were poorly fermented by *S. cerevisiae* 424A (LNH-ST), as demonstrated by low specific growth rate, xylose consumption and ethanol yield compared to the enzymatic hydrolysates. Like the StEx whole slurries, it appears that this yeast strain's fermentation performance was limited by degradation product inhibition. Although $C_5$-liquor fermentation was limited due to microbial inhibition, xylose consumption was not completely arrested as xylose was still being consumed albeit at a significantly slower rate (approx. 0.05 $g.L^{-1}.h^{-1}$) after 120



h (see Fig. 4 i-j). Recently, recombinant *S. cerevisiae* strains MEC1122 and LF1 demonstrated ethanol yields up to 0.42 g/g in non-detoxified liquid hot water-treated corn cob $C_5$-liquor and StEx-treated corn stover hydrolysate, respectively [260,261]. Therefore, developing hardened xylose-fermenting mutant strains with higher tolerance of pretreatment-derived inhibitors and fermentation metabolites could hypothetically improve fermentation yields and ethanol titres from the $C_5$-liquor streams [262].

The recovery of XOS via a dilute acid post-hydrolysis of the $C_5$-rich liquid fraction is an example of a process that can be performed in a simple stirred-tank or plug flow reactor in a commercial setting, without the need for a complex high solids reactor configuration [79]. This option is particularly important because smaller reaction volumes will be necessary since only the pseudo-homogenous liquid fraction will be hydrolyzed. Moreover, the post hydrolysis is performed at much lower reaction temperatures (~120 °C) without the threat of significant ash neutralization by high ash content biomass slurries. Hence, capital expenses can be reduced due to requirement of a significantly smaller reactor that is lined with resistant but high cost anti-corrosion alloys. Another pertinent issue with the StEx $C_5$-liquor stream is the dilute concentration of total sugars available for fermentation. The sugar concentration of this stream can potentially be increased by increasing the solids loading during StEx pretreatment. However, increasing the solids loading is usually coupled with lower pretreatment efficiency and higher concentrations of organic acids, particularly acetic acid, which is a major microbial inhibitory compound. On the other hand, the $C_5$-liquor stream could be concentrated using thermal evaporation technology, similar to that applied for concentrating cane juice, to remove water and volatile products such as acetic acid and furan derivatives. However, such an approach would likely increase operating costs and enrich the $C_5$-liquor stream with other non-volatile inhibitory compounds such as vanillin and coniferyl aldehyde [112].

For 2G biorefineries annexed to 1G autonomous distilleries or existing sugar mills, the $C_5$-liquor stream could be mixed with molasses or sugarcane juice to simultaneously increase the total stream sugar concentration and dilute the concentration of the pretreatment derived inhibitors.



Losordo *et al.,* [263] reported that up to 37% more ethanol could be produced without affecting sugar coproduction when the $C_5$-sugars from StEx are combined with molasses. Therefore, with adequate process integration and yeast development, there are potential avenues to convert the $C_5$-sugars produced during StEx pretreatment into ethanol or other commodity chemicals.

### 4.3.5 *Process Mass Balances*

The results from pretreatment, high solids loading enzymatic hydrolysis at 25 mg/g glucan and fermentation were used to develop mass balances for each biomass material in each process configuration (Process I – IV). The carbohydrate recovery of monomeric and oligomeric sugars from pretreatment and HSL-EH relative to the initial untreated dry material are presented in Figure 4.6A, whereas the ethanol yield from the recovered carbohydrates is presented in Figure 4.6B (next page). Detailed process flow diagrams are presented in Fig 4.4S (Supplementary Information).

For bagasse, AFEX™ pretreatment ultimately generated the highest carbohydrate recovery (609 kg sugar/tonne RDM or 84% theoretical maximum), owing to absence of significant polysaccharide degradation during pretreatment and high enzymatic hydrolysis conversion of both glucan and xylan. AFEX™ consumed about 15 kg of ammonia per Mg RDM, primarily due to ammonolysis reactions with the biomass and residual ammonia chemically bound to the biomass, which would have to be replenished after every cycle on an industrial scale. The remaining ammonia can be recycled and reused as demonstrated at MBI International's pilot scale operation [120]. About 7% of the recovered carbohydrates were in oligomeric form, which highlights the importance of identifying enzyme activities absent from current commercial enzyme mixtures required to maximize ethanol production from these residues. For StEx-pretreated bagasse, unwashed solids generated the highest carbohydrate recovery (546 kg sugar/Mg RDM or 75% theoretical maximum). Although washed solids achieved the highest enzymatic hydrolysis conversions, washing the solids removed about 21 kg water soluble monomeric sugars and oligosaccharides per Mg RDM. Moreover, washing the StEx solids with water heated to 50 °C consumed approximately 10 kg of water per kg of unwashed solids, thereby increasing the overall process water consumption. Although we considered the water-

soluble sugars as "lost" sugars in carbohydrate recovery calculations, in a biorefinery setting, it is likely that these sugars would be sent directly to an anaerobic digestion-based waste water treatment to produce process energy in the form of methane.

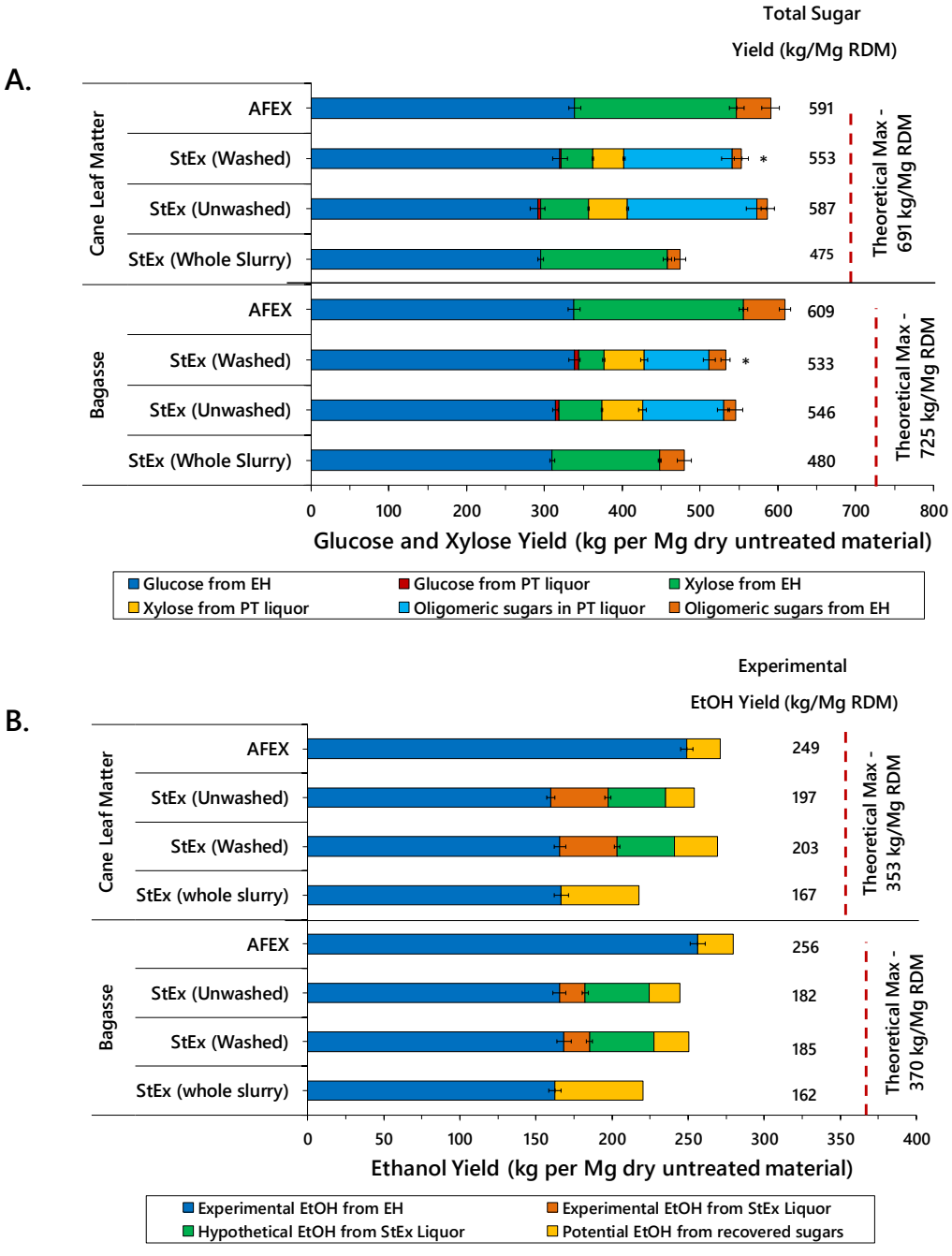



**Figure 4.6:** Comparison of bagasse and CLM carbohydrate recovery **(A)** and ethanol yields **(B)** per ton dry biomass from AFEX™ and StEx pretreatment coupled with various solids processing options. The theoretical maximum carbohydrate recovery and ethanol yields (red dotted line) were calculated based on the initial glucan and xylan content in untreated bagasse and CLM.).

The StEx-whole slurry produced the lowest carbohydrate recovery (480 kg sugar/Mg RDM or 66% theoretical maximum) due to significant enzyme inhibition during HSL-EH. Moreover,



approximately 4% of the solubilized sugars were retained in oligomeric form (the highest among the StEx solids processing options).

For CLM, AFEX™ and StEx-unwashed solids resulted in the highest carbohydrate recoveries of 591 kg sugar/Mg RDM and 587 kg sugar/Mg RDM, respectively. The difference between the two process configurations, Process I and III, was statistically insignificant ($P > 0.05$). Similar to the case of StEx-treated bagasse, washing StEx-treated CLM removed approximately 37 kg of soluble sugars per Mg RDM and therefore resulted in the recovery of 553 kg sugar/Mg RDM. However, these mass balances were performed at relatively higher enzyme loadings (25 mg/g glucan), which implies that the benefit of washing the StEx solids on the carbohydrate recovery may become more apparent at lower enzyme loadings (*e.g.* at 15 mg/g glucan). Finally, StEx-CLM whole slurries recovered the least glucan and xylan due to a combination of sugar degradation during pretreatment and enzyme inhibition during HSL-EH.

The ethanol yield per ton of RDM provides a means of quantifying the combined effect of biomass recalcitrance, enzyme inhibition and microbial inhibition for the various AFEX™ and StEx process configurations. AFEX™-treated bagasse and CLM (Process I) generated the highest ethanol yields due to higher sugar recovery and superior fermentability of the AFEX™-hydrolysates by *S. cerevisiae* 424A (LNH-ST) without detoxification. The estimated ethanol yield for AFEX™ bagasse and CLM were 256 and 249 kg ethanol/Mg RDM, respectively. The poor fermentability of the StEx $C_5$-liquor and whole slurry significantly impacted the ethanol yield for StEx bagasse, resulting in 185, 182 and 162 kg ethanol/Mg RDM recovered from Processes II, III and IV, respectively. The experimental ethanol yields for StEx-CLM were 203, 197, and 167 kg ethanol/Mg RDM for Process II, III and IV, respectively. These ethanol yields were slightly higher than those of bagasse due to higher ethanol yield from the $C_5$-liquor derived from CLM relative to that derived from bagasse. In general, the StEx bagasse $C_5$-liquor contained higher concentrations of well-known microbial inhibitors, including organic acids and phenolic compounds, thus producing lower ethanol yields relative the StEx CLM generated liquor (Table S4.1). Nonetheless, the lower StEx ethanol yields relative to AFEX™ demonstrates the



compounded consequences of sugar loss due to degradation during StEx pretreatment, the degree of enzyme inhibition due to the solids processing option, and microbial inhibition of *S. cerevisiae* 424A (LNH-ST) due to the presence of pretreatment-derived inhibitors.

### 4.3.6 *Estimation of 2G ethanol yields per sugarcane cultivation area*

In this work, we developed comprehensive process mass balances, based on experimental data, to estimate the potential ethanol yields that can be achieved at industrially-relevant conditions from 2G sugarcane based biorefineries using mature technologies available today (Fig 4S). Assuming a commercial average sugarcane yield of 80 metric tonnes of wet cane per hectare, it was estimated that AFEX™-based biorefineries (Process I) would generate higher ethanol yields per sugarcane cultivation area (4496 L/ha) relative to StEx-based biorefineries (3416-3341 L/ha), irrespective of the StEx processing configuration (Table 4.3) [264]. As previously discussed, StEx process bottlenecks that lowered the ethanol yields were mainly associated with sugar degradation during pretreatment, enzyme inhibition and the inability of recombinant *S. cerevisiae* 424A (LNH-ST) to efficiently convert the sugars in the $C_5$-liquor to ethanol.

**Table 4.3:** Estimated ethanol yield per hectare of sugarcane cultivation area

| Sugarcane Crop Segment | Yield |
|---|---|
| Average Cane Yield (Mg wet cane/ha) | 80.0 |
| Bagasse (kg dry fiber/Mg wet cane) † | 140.0 |
| Available Bagasse (Mg dry fiber/ha) ‡ | 8.4 |
| Cane Leaf Matter (kg dry fiber/Mg wet cane) † | 140.0 |
| Available Cane Leaf Matter (Mg dry fiber/Mg wet cane) ϕ | 5.6 |
| **2G Bagasse + CLM - Ethanol Yield (L/ha) ψ** | |
| AFEX™ - Process I | 4496 |
| StEx (washed solids + $C_5$-liquor) - Process II | 3416 |
| StEx (unwashed solids + $C_5$-liquor) - Process III | 3341 |
| StEx (whole slurry) - Process IV | 2911 |

† - Estimated sugarcane bagasse and cane leaf matter yield per ton wet cane [16].

‡ - Assuming 75% of bagasse collected from the sugar mill is allocated to biofuel production and the remainder will supplement lignin-rich enzymatic residues for energy cogeneration.
ϕ - Assuming 50% of the CLM harvested on the field can be removed without significantly affecting soil fertility [18-21].
ψ – Ethanol yield calculated by multiplying available bagasse or CLM (Mg dry fiber/ha) with the experimental ethanol yield (L/ton).

A single variable sensitivity analysis was carried out to project the effect of sugarcane bagasse allocation, variation in enzyme dosage, the extent of xylose conversion to ethanol in the StEx $C_5$-liquor, and the conversion of oligosaccharides present in the enzymatic hydrolysate on the estimated ethanol yields from AFEX™ or StEx per sugarcane cultivation area (Figure 4.6).

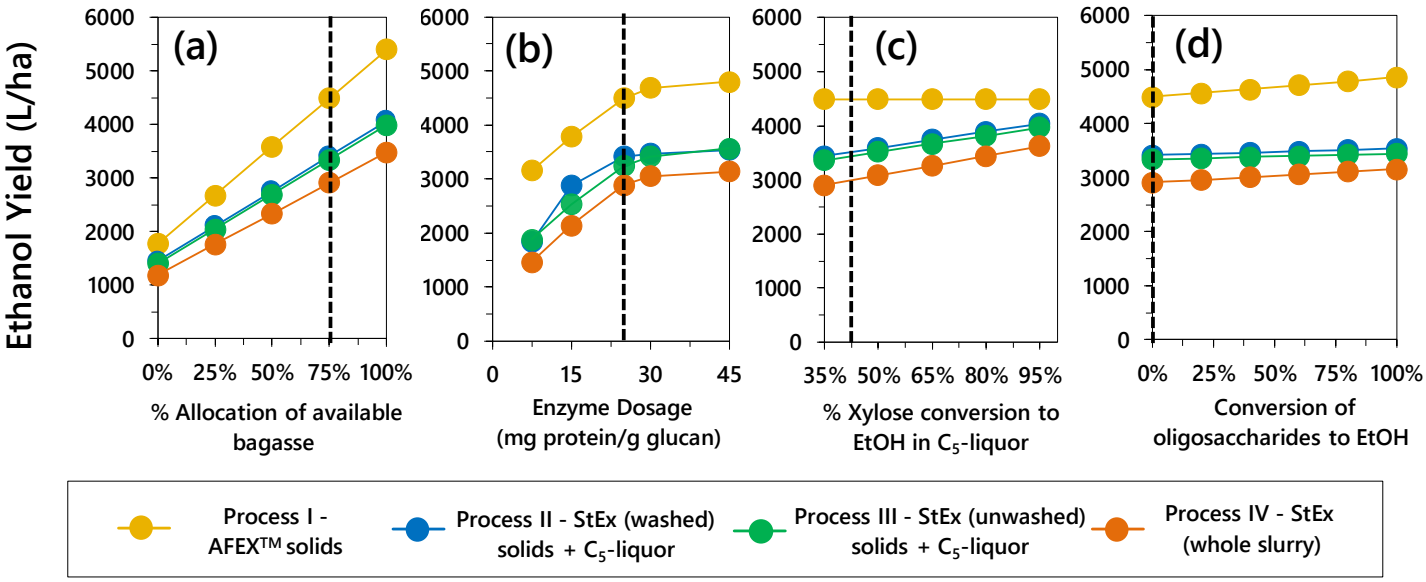



**Figure 4.7:** Sensitivity analysis on the ethanol yield per hectare of sugarcane cultivation area considering: **a)** percent allocation of available bagasse, **b)** the enzyme dosage, **c)** percent of xylose conversion to ethanol from the $C_5$-liquor, **d)** the conversion of the oligosaccharides in the enzymatic hydrolysate to ethanol. Black dotted line indicates baseline conditions which were selected based on experimental data presented in **Table 4.2** and the assumptions presented in **Table 4.3**.

The quantity of bagasse allocated to biofuel production had the highest impact on the ethanol yield, with AFEX™ yields decreasing from 4496 to 3586 L/ha when the quantity of available bagasse allocated to ethanol production is reduced from 75% to 50% (Figure 4.7a). End-of-process sugarcane bagasse from the sugar mill will be required to supplement lignin-rich enzymatic hydrolysis residues to provide process energy for the 2G biorefinery, hence the amount of bagasse available for biofuel production will be limited by factors such as the state of mill boiler technology and the biorefinery plant size [32]. A recent study estimated that bagasse allocation for ethanol production in Brazil ranges from 64 – 84% depending on the electricity production cost, ethanol production cost, plant size, and regulation of the electricity/biofuel markets [265]. A similar study revealed that 65% of the available sugarcane residues (bagasse and CLM) could be allocated to biofuel production, with the remainder



being diverted towards energy cogeneration operations to ensure that South African sugar mills meet their steam and energy demands [32].

Reducing the enzyme dosage below 25 mg/g glucan lowered the ethanol yields for all processes (Process I to IV), with Process IV (whole slurry) obtaining the lowest yield of 1458 L/ha at an enzyme dosage of 7.5 mg protein/g glucan (Figure 4.7b). In comparison, AFEX™ (Process I) was estimated to achieve an ethanol yield of 3154 L/ha at the same enzyme dosage (more than double that of Process IV). This result demonstrates again the compounded effect of enzyme and microbial inhibition during whole slurry processing. Increasing xylose to ethanol conversion from the $C_5$-liquor improved ethanol yields for Processes II to IV from 2849 to 4045 L/ha when hypothetical xylose consumption and metabolic yield scenarios of 95% and 90% were considered, respectively. Therefore, by using a suitable hardened xylose-fermenting yeast or even exploring process integration strategies such as mixing the $C_5$-liquor stream with sugarcane molasses, ethanol yields can be significantly improved for StEx-treated sugarcane residues to approach those achieved by AFEX™-treated residues. Lastly, the conversion of all recalcitrant oligosaccharides from enzymatic hydrolysates to ethanol would significantly improve ethanol yields from AFEX™-treated residues from 4496 to 4860 L/ha. In contrast, minor increments in the ethanol yield would be achieved with increasing oligosaccharide conversion to ethanol in StEx hydrolysates. Hence, identifying auxiliary enzymatic activities missing from the commercial cocktails used in this work could benefit AFEX™-treated residues more than StEx-treated residues.

## 4.4 Conclusions

In the context of expanding the sugar industry towards a diversified bioeconomy, the use of sugarcane harvest residues (including bagasse and cane leaf matter) in a 2G biorefinery presents an attractive opportunity for increasing ethanol yields per unit of land cultivated, whilst facilitating the sharing of existing logistics and supply chain infrastructure with the sugar industry. In this work, we evaluated the ethanol production potential for future sugarcane residue-based biorefineries with



AFEX™ or StEx as the central pretreatment technologies. AFEX™ proved to be the more effective pretreatment technology for maximising ethanol yields from sugarcane residues, resulting in ethanol yields of 249 and 256 kg per Mg RDM (equivalent of 316-325 L/Mg RDM) for sugarcane bagasse and CLM, respectively. In comparison, steam explosion-pretreated sugarcane bagasse and CLM generated 162 – 203 kg of ethanol per Mg RDM (205-257 L/Mg RDM) depending on the solids processing option chosen to follow pretreatment.

Although both pretreatments were agnostic for sugarcane residues, we identified some process limitations for both technologies. Currently both pretreatments required relatively high enzyme loadings (~ 25 mg/g glucan) to reach carbohydrate conversions greater than 75%, even with some of the most efficient commercial enzyme combinations. Due to uncertainties in the enzyme cost, the enzyme usage for both pretreatments would need to be reduced to decrease the sensitivity of these biorefineries to enzyme cost fluctuations. Moreover, ethanol yields from StEx-treated bagasse and CLM were limited by a combination of sugar degradation during pretreatment, enzyme inhibition and the inhibition of recombinant *S. cerevisiae* 424A (LNH-ST) due to pretreatment derived inhibitors. On the other hand, hydrolysis of AFEX™-treated bagasse and CLM left more than 7% of the total sugars in oligomeric form, thereby reducing the overall sugar and ethanol yields.

Overall, selecting the preferred pretreatment technology is primarily an economic and environmental impact issue. Hence, estimating the cost of ethanol production ($USD/ L ethanol) through techno-economic analysis and environmental impacts through a life-cycle analysis would provide the necessary basis for comparing 2G sugarcane biorefineries centred on AFEX™ or StEx pretreatment. This work provides insights that will enable later economic and environmental evaluations of the impacts of the various AFEX™/StEx processing options on the cost of ethanol production.

## 4.5 Supplementary Information – CHAPTER 4

**Table S4.1:** Composition of the liquid and solid fractions after water-impregnated steam explosion of sugarcane bagasse, cane leaf matter and a bagasse-CLM mixture (at 1:1 w/w ratio). Each pretreatment condition was performed in duplicate or triplicate. Mean and standard deviation of triplicate experiments are reported.

| Pretreatment Conditions | Bagasse | | | | CLM | | | | Bagasse + CLM Mixture | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Raw | LS - StEx | MS-StEx | HS - StEx | Raw | LS - StEx | MS-StEx | HS - StEx | Raw | LS - StEx | MS-StEx | HS - StEx |
| Temperature (°C) | - | 185.0 | 205.0 | 215.0 | - | 185.0 | 200.0 | 215 | - | 185.0 | 200.0 | 215.0 |
| Residence Time (min) | - | 15.0 | 13.5 | 10.0 | - | 10.0 | 10.0 | 10.0 | - | 12.5 | 12.0 | 10.0.0 |
| Severity Factor | - | 3.68 | 4.22 | 4.39 | - | 3.50 | 3.94 | 4.39 | - | 3.60 | 4.02 | 4.39 |
| **Solids Calorific Value (MJ/kg)** | | | | | | | | | | | | |
| HHV | 18.5 ± 0.06 | 19.2 ± 0.08 | 19.9 ± 0.10 | 20.2 ± 0.09 | 17.7 ± 0.10 | 18.5 ± 0.03 | 18.9 ± 0.06 | 19.4 ± 0.00 | 17.9 ± 0.10 | 18.9 ± 0.0 | 19.4 ± 0.15 | 19.9 ± 0.00 |
| **Component in Solids (g/100g RDM)** | | | | | | | | | | | | |
| WIS Recovery | 100.0 | 68.9 ± 1.92 | 63.1 ± 1.6 | 59.3 ± 1.22 | 100.0 | 71.6 ± 1.02 | 59.9 ± 1.58 | 58.1 ± 1.00 | 100.0 | 67.7 ± 0.62 | 63.2 ± 2.78 | 60.2 ± 1.67 |
| Glucan | 39.50 ± 0.41 | 52.49 ± 0.53 | 58.35 ± 0.3 | 60.55 ± 0.54 | 37.45 ± 0.6 | 49.52 ± 0.59 | 54.25 ± 0.12 | 56.62 ± 1.19 | 38.11 ± 0.1 | 53.60 ± 1.0 | 56.74 ± 1.31 | 60.07 ± 1.31 |
| Xylan | 25.21 ± 0.13 | 7.92 ± 1.35 | 4.88 ± 0.41 | 1.72 ± 0.09 | 24.81 ± 0.4 | 10.13 ± 0.66 | 6.62 ± 0.22 | 1.61 ± 0.08 | 24.21 ± 0.2 | 8.88 ± 0.19 | 4.53 ± 0.44 | 1.73 ± 0.24 |
| Arabinan | 1.23 ± 0.38 | 0.21 ± 0.04 | 0.21 ± 0.02 | 0.00 ± 0.00 | 2.73 ± 0.1 | 0.69 ± 0.06 | 0.36 ± 0.04 | 0.00 ± 0.00 | 1.48 ± 0.24 | 0.39 ± 0.05 | 0.28 ± 0.06 | 0.00 ± 0.00 |
| Acetyl | 3.43 ± 0.04 | 2.06 ± 0.37 | 1.24 ± 0.24 | 0.39 ± 0.01 | 2.21 ± 0.06 | 2.07 ± 0.09 | 1.23 ± 0.11 | 0.29 ± 0.03 | 4.32 ± 0.18 | 2.27 ± 0.05 | 1.43 ± 0.19 | 0.73 ± 0.08 |
| Lignin | 19.35 ± 0.06 | 29.41 ± 0.72 | 29.51 ± 0.4 | 33.29 ± 1.58 | 16.17 ± 0.8 | 25.78 ± 1.82 | 27.30 ± 0.34 | 30.64 ± 1.20 | 19.5 ± 0.59 | 28.5± 2.00 | 28.3 ± 0.29 | 32.6 ± 0.80 |
| Ash | 2.89 ± 0.65 | N. D | N.D | N.D | 7.34 ± 0.21 | N.D | N.D | N.D | 5.21 ± 0.71 | N.D | N.D | N.D |
| Extractives | 6.02 ± 0.42 | N. D | N.D | N.D | 12.07 ± 1.5 | N.D | N.D | N.D | 10.32 ± 0.4 | N.D | N.D | N.D |
| Water soluble sugars | | | | | | | | | | | | |
| Sucrose + fructose | 0.41 ± 0.01 | - | - | - | 1.02 ± 0.02 | - | - | - | 0.70 ± 0.02 | - | - | - |
| glucose + G-OS | 0.33 ± 0.01 | - | - | - | 0.44 ± 0.01 | - | - | - | 0.36 ± 0.01 | - | - | - |
| xylose + X-OS | 0.37 ± 0.01 | - | - | - | 0.40 ± 0.01 | - | - | - | 0.36 ± 0.02 | - | - | - |
| **Component in Liquor + WSS (g/100g RDM)** | | | | | | | | | | | | |
| Monomeric Glucose | - | 0.02 ± 0.00 | 0.64 ± 0.08 | 0.77 ± 0.06 | - | 0.04 ± 0.01 | 0.39 ± 0.03 | 0.45 ± 0.04 | - | 0.03 ± 0.00 | 0.34 ± 0.05 | 0.47 ± 0.05 |
| G-OS | - | 1.33 ± 0.04 | 1.74 ± 0.15 | 1.10 ± 0.15 | - | 1.42 ± 0.08 | 2.10 ± 0.36 | 1.43 ± 0.08 | - | 1.24 ± 0.05 | 2.05 ± 0.05 | 1.23 ± 0.05 |
| Monomeric Xylose | - | 1.69 ± 0.03 | 6.74 ± 0.47 | 2.74 ± 0.17 | - | 0.99 ± 0.04 | 5.01 ± 0.11 | 1.73 ± 0.05 | - | 1.52 ± 0.09 | 6.09 ± 0.60 | 2.49 ± 0.06 |
| X-OS | - | 15.22 ± 0.54 | 7.49 ± 0.62 | 2.04 ± 0.13 | - | 15.78 ± 0.20 | 12.60 ± 1.29 | 2.78 ± 0.21 | - | 15.27 ± 0.8 | 11.94 ± 0.94 | 2.10 ± 0.14 |
| Monomeric Arabinose | - | 0.60 ± 0.07 | 0.71 ± 0.05 | 0.19 ± 0.11 | - | 1.00 ± 0.05 | 0.84 ± 0.09 | 0.15 ± 0.03 | - | 0.92 ± 0.05 | 0.75 ± 0.08 | 0.2 ± 0.01 |
| A-OS | - | 0.19 ± 0.04 | 0.21 ± 0.05 | 0.10 ± 0.08 | - | 0.75 ± 0.14 | 0.54 ± 0.28 | 0.10 ± 0.02 | - | 0.64 ± 0.15 | 0.42 ± 0.16 | 0.15 ± 0.07 |
| Furfural | - | 0.17 ± 0.00 | 0.51 ± 0.10 | 0.96 ± 0.03 | - | 0.20 ± 0.00 | 0.26 ± 0.04 | 0.80 ± 0.00 | - | 0.18 ± 0.00 | 0.32 ± 0.03 | 0.93 ± 0.01 |
| 5-HMF | - | 0.03 ± 0.00 | 0.15 ± 0.02 | 0.26 ± 0.01 | - | 0.03 ± 0.01 | 0.08 ± 0.009 | 0.30 ± 0.03 | - | 0.03 ± 0.01 | 0.07 ± 0.01 | 0.22 ± 0.00 |
| Acetic Acid | - | 2.78 ± 0.10 | 3.36 ± 0.17 | 3.68 ± 0.14 | - | 1.79 ± 0.06 | 2.13 ± 0.09 | 2.60 ± 0.04 | - | 2.38 ± 0.01 | 2.82 ± 0.06 | 3.37 ± 0.04 |
| pH | - | 3.60 ± 0.03 | 3.08 ± 0.03 | 3.05 ± 0.03 | - | 3.90 ± 0.01 | 3.63 ± 0.03 | 3.26 ± 0.01 | - | 3.76 ± 0.01 | 3.43 ± 0.00 | 3.17 ± 0.01 |

| Pretreatment Conditions | Bagasse | | | | Cane Leaf Matter | | | | Bagasse + CLM Mixture | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Raw | LS - StEx | MS-StEx | HS - StEx | Raw | LS - StEx | MS-StEx | HS - StEx | Raw | LS - StEx | MS-StEx | HS - StEx |
| **Major phenolic compounds in liquor (mg/L)** | | | | | | | | | | | | |
| Vanillic Acid | - | 1.34 | 8.08 | 16.3 | - | 0.92 | 11.19 | 17.98 | - | 0.88 | 11.20 | 16.31 |
| Vanillin | - | 0.38 | 57.2 | 146.6 | - | 0.16 | 63.48 | 84.8 | - | 0.25 | 50.34 | 100.6 |
| Syringic Acid | - | 0.61 | 174.4 | 200.2 | - | 0.37 | 121.8 | 128.75 | - | 0.72 | 151.0 | 161.0 |
| Syringaldehyde | - | 0.00 | 21.2 | 25.2 | - | 0.00 | 13.60 | 14.9 | - | 0.00 | 14.31 | 14.9 |
| *p*-Coumaric Acid | - | 0.89 | 26.3 | 99.7 | - | 0.14 | 16.10 | 47.14 | - | 0.31 | 20.78 | 63.11 |
| Ferulic Acid | - | 0.54 | 6.99 | 53.5 | - | 0.00 | 6.47 | 23.52 | - | 0.22 | 6.77 | 34.4 |
| Coniferyl Aldehyde | - | 0.00 | 4.07 | 8.66 | - | 0.00 | 4.46 | 5.14 | - | 0.00 | 4.35 | 8.11 |
| 3,4-Dihydrobenzoic acid | - | 0.00 | 7.53 | 18.22 | - | 0.00 | 10.33 | 17.09 | - | 0.00 | 7.01 | 17.53 |

StEx – Steam explosion, LS – low severity, MS – Mild or Intermediate severity, HS – High severity; G-OS – glucoligosaccharide; XOS – xyloligosaccharide; WIS – water insoluble solids; WSS – water soluble solids;



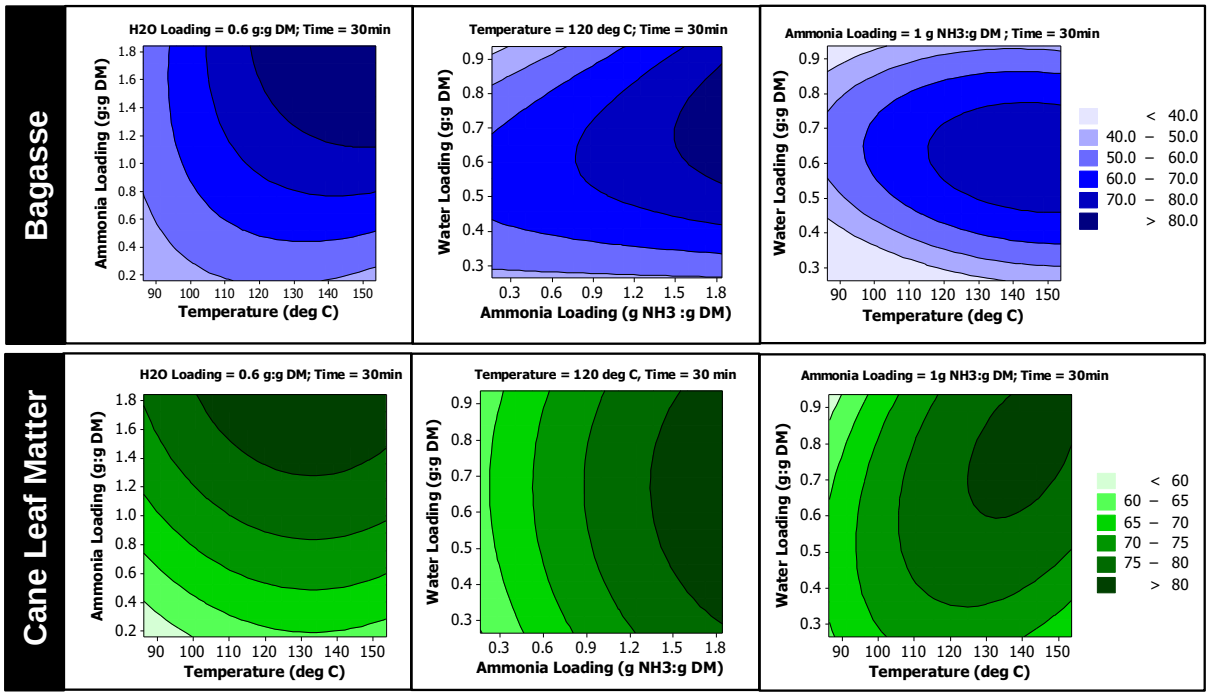
**Table S4.2-A:** AFEX™ -bagasse pretreatment conditions used for evaluating the effect of pretreatment conditions on the monomeric glucose, xylose and combined sugar yield using a central composite design of experiments (DOE). For all experiments, the pretreatment residence time was fixed at 30 min.

| High throughput AFEX™ pretreatment - Bagasse (DOE) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Run Order** | **Point Type** | **Blocks** | **Temperature (° C)** | **$NH_3$ Loading (g $NH_3$/g DM)** | **$H_2O$ Loading (g $H_2O$:g DM)** | **Glucose Yield (%)** | **Xylose Yield (%)** | **Combined Sugar Yield (%)** |
| 1 | -1 | 1 | 120.00 | 1.00 | 0.26 | 29.61 | 30.5 | 29.96 |
| 2 | 1 | 1 | 140.00 | 1.50 | 0.80 | 82.25 | 79.000 | 80.98 |
| 3 | 1 | 1 | 100.00 | 1.50 | 0.80 | 57.21 | 75.17 | 64.21 |
| 4 | 1 | 1 | 100.00 | 1.50 | 0.40 | 39.56 | 47.15 | 42.52 |
| 5 | -1 | 1 | 120.00 | 0.16 | 0.60 | 50.4 | 54.69 | 52.07 |
| 6 | -1 | 1 | 86.36 | 1.00 | 0.60 | 48.57 | 62.68 | 54.07 |
| 7 | -1 | 1 | 120.00 | 1.84 | 0.60 | 79.62 | 86.81 | 82.42 |
| 8 | -1 | 1 | 153.64 | 1.00 | 0.60 | 75.37 | 73.55 | 74.66 |
| 9 | 1 | 1 | 100.00 | 0.50 | 0.40 | 41.02 | 52.15 | 45.36 |
| 10 | 1 | 1 | 140.00 | 1.50 | 0.40 | 72.86 | 76.01 | 74.09 |
| 11 | 1 | 1 | 100.00 | 0.50 | 0.80 | 34.43 | 41.62 | 37.23 |
| 12 | 0 | 1 | 120.00 | 1.00 | 0.60 | 69.33 | 72.91 | 70.72 |
| 13 | 1 | 1 | 140.00 | 0.50 | 0.40 | 51.42 | 53.94 | 52.40 |
| 14 | -1 | 1 | 120.00 | 1.00 | 0.94 | 48.6 | 52.52 | 50.13 |
| 15 | 0 | 1 | 120.00 | 1.00 | 0.60 | 71.42 | 73.54 | 72.25 |
| 16 | 0 | 1 | 120.00 | 1.00 | 0.60 | 70.5 | 73.16 | 71.54 |
| 17 | 1 | 1 | 140.00 | 0.50 | 0.80 | 47.68 | 51.51 | 49.17 |
| 18 | 1 | 2 | 110.00 | 1.00 | 0.80 | 56.06 | 69.08 | 61.13 |
| 19 | 1 | 2 | 130.00 | 1.00 | 0.60 | 72.8 | 74.6 | 73.50 |
| 20 | -1 | 2 | 100.00 | 1.00 | 0.40 | 44.55 | 51.97 | 47.44 |
| 21 | 1 | 2 | 140.00 | 1.20 | 0.60 | 82.5 | 79.5 | 81.33 |
| 22 | 1 | 2 | 140.00 | 1.00 | 0.60 | 78.5 | 79.5 | 78.89 |

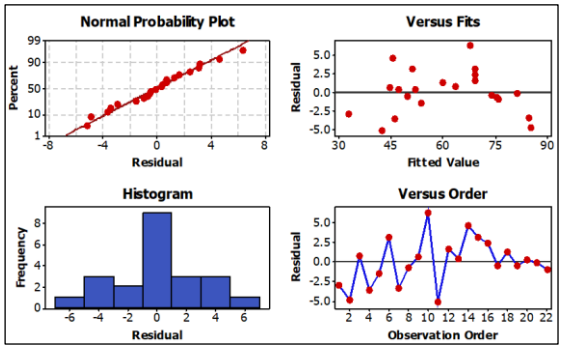


**Table S4.2-B:** AFEX™ -CLM pretreatment conditions used for evaluating the effect of pretreatment conditions on the monomeric glucose, xylose and combined sugar yield using a central composite design of experiments (DOE). For all experiments, the pretreatment residence time was fixed at 30 min.

| High throughput AFEX™ pretreatment - Cane Leaf Matter (DOE) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Run Order** | **Point Type** | **Blocks** | **Temperature (° C)** | **$NH_3$ Loading (g $NH_3$/g DM)** | **$H_2O$ Loading (g $H_2O$/g DM)** | **Glucose Yield (%)** | **Xylose Yield (%)** | **Combined Sugar Yield (%)** |
| 1 | 1 | 1 | 140.00 | 1.50 | 0.40 | 83.24 | 69.55 | 78.05 |
| 2 | 0 | 1 | 120.00 | 1.00 | 0.60 | 81.77 | 70.28 | 77.47 |
| 3 | 0 | 1 | 120.00 | 1.00 | 0.60 | 80.84 | 69.61 | 76.65 |
| 4 | 1 | 1 | 140.00 | 0.50 | 0.40 | 70.47 | 59.14 | 66.24 |
| 5 | -1 | 1 | 86.36 | 1.00 | 0.60 | 71.33 | 63.00 | 68.31 |
| 6 | 1 | 1 | 100.00 | 1.50 | 0.80 | 80.29 | 71.54 | 77.10 |
| 7 | 1 | 1 | 140.00 | 0.50 | 0.80 | 77.07 | 65.52 | 72.74 |
| 8 | 1 | 1 | 140.00 | 1.50 | 0.80 | 88.79 | 73.43 | 82.93 |
| 9 | 0 | 1 | 120.00 | 1.00 | 0.60 | 80.81 | 69.18 | 76.46 |
| 10 | -1 | 1 | 120.00 | 0.16 | 0.60 | 66.64 | 57.85 | 63.43 |
| 11 | -1 | 1 | 120.00 | 1.00 | 0.26 | 78.62 | 68.31 | 74.80 |
| 12 | -1 | 1 | 120.00 | 1.84 | 0.60 | 89.66 | 73.77 | 83.58 |
| 13 | 1 | 1 | 100.00 | 1.50 | 0.40 | 80.09 | 70.99 | 76.42 |
| 14 | 1 | 1 | 100.00 | 0.50 | 0.40 | 67.02 | 59.29 | 63.90 |
| 15 | 0 | 1 | 120.00 | 1.00 | 0.60 | 80.15 | 68.77 | 75.55 |
| 16 | 0 | 1 | 120.00 | 1.00 | 0.60 | 81.48 | 69.58 | 77.36 |
| 17 | -1 | 1 | 153.64 | 1.00 | 0.60 | 80.56 | 66.56 | 75.25 |
| 18 | 1 | 1 | 100.00 | 0.50 | 0.80 | 68.76 | 60.74 | 65.52 |
| 19 | -1 | 1 | 120.00 | 1.00 | 0.94 | 74.76 | 67.61 | 72.23 |
| 20 | 0 | 1 | 120.00 | 1.00 | 0.60 | 78.85 | 68.46 | 74.66 |
| 21 | 1 | 2 | 110.00 | 1.00 | 0.80 | 77.11 | 66.51 | 73.52 |
| 22 | 1 | 2 | 130.00 | 1.00 | 0.60 | 80.59 | 64.94 | 76.16 |
| 23 | -1 | 2 | 100.00 | 1.00 | 0.40 | 73.67 | 63.78 | 70.36 |
| 24 | 1 | 2 | 137.00 | 1.20 | 0.77 | 84.00 | 71.50 | 80.00 |
| 25 | 1 | 2 | 140.00 | 1.00 | 0.70 | 83.00 | 71.00 | 77.00 |




a. AFEX™-Bagasse: Residual plots for combined sugar yield



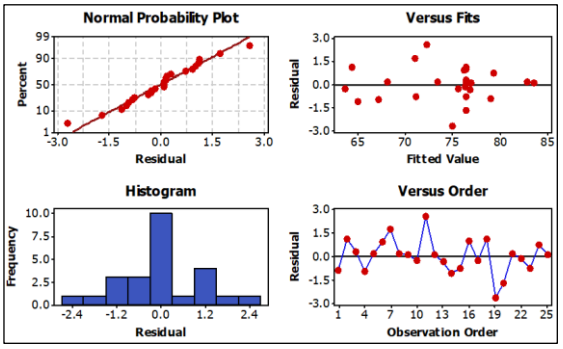

b. AFEX™-Bagasse: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| Constant | 70,01 | 1,45 | 48,30 | 0,000 |
| Block | -0,86 | 0,97 | -0,89 | 0,392 |
| Temperature ( °C) | 7,23 | 0,91 | 7,96 | 0,000 |
| Ammonia Loading (g:g DM) | 9,23 | 1,00 | 0,92 | 0,000 |
| Water Loading (g:g DM) | 3,78 | 0,94 | 4,02 | 0,001 |
| Temperature * Temperature | -2,10 | 1,03 | -2,04 | 0,062 |
| Water Loading * Water Loading | -10,57 | 1,02 | -10,40 | 0,000 |
| Temperature * Ammonia Loading | 3,58 | 1,30 | 2,75 | 0,017 |
| Ammonia Loading * Water Loading | 4,92 | 1,32 | 3,75 | 0,002 |

| Source | DF | Adj SS | Adj MS | F |
|---|---|---|---|---|
| Regression | 8 | 4809,18 | 601,15 | 43,68 |
| Lack of Fit | 11 | 117,76 | 16,16 | 27,94 |

S = 3.70981 PRESS = 691.829
$R^2$ = 96.41 % $R^2_{(predicted)}$ = 86.13 % $R^2_{(adjusted)}$ = 94.21%

c. AFEX™-CLM: Residual plots for combined sugar yield




d. AFEX™-CLM: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| Constant | -2,0141 | 15,0176 | -0,134 | 0,895 |
| Block | 0,3724 | 0,3717 | 1,002 | 0,331 |
| Temperature ( °C) | 0,9065 | 0,217 | 4,177 | 0,001 |
| Ammonia Loading (g:g DM) | 19,548 | 2,9433 | 6,641 | 0,000 |
| Water Loading (g:g DM) | 0,5785 | 16,7426 | 0,035 | 0,973 |
| Temperature * Temperature | -0,004 | 0,0009 | -4,575 | 0,000 |
| Ammonia Loading * Ammonia Loading | -3,896 | 1,4243 | -2,735 | 0,015 |
| Water Loading * Water Loading | -24,6201 | 8,6877 | -2,8340 | 0,012 |
| Ammonia Loading * Water Loading | 0,2747 | 0,1109 | 2,476 | 0,025 |

| Source | DF | Adj SS | Adj MS | F |
|---|---|---|---|---|
| Regression | 8 | 660,33 | 82,54 | 45,05 |
| Lack of Fit | 11 | 23,44 | 2,13 | 1,81 |

S = 1.35357 PRESS = 106.715
$R^2$ = 95.75 % $R^2_{(predicted)}$ = 84.53 % $R^2_{(adjusted)}$ = 93.62%

**Figure S4.1:** Contour plots, residual plots and regression coefficients used to validate ANOVA assumptions in evaluating the effect of AFEX™ pretreatment conditions on the monomeric combined sugar yield from sugarcane bagasse and CLM. Abbreviations: **S** - Standard Error of the Regression, **PRESS** - Prediction Sum of Squares.

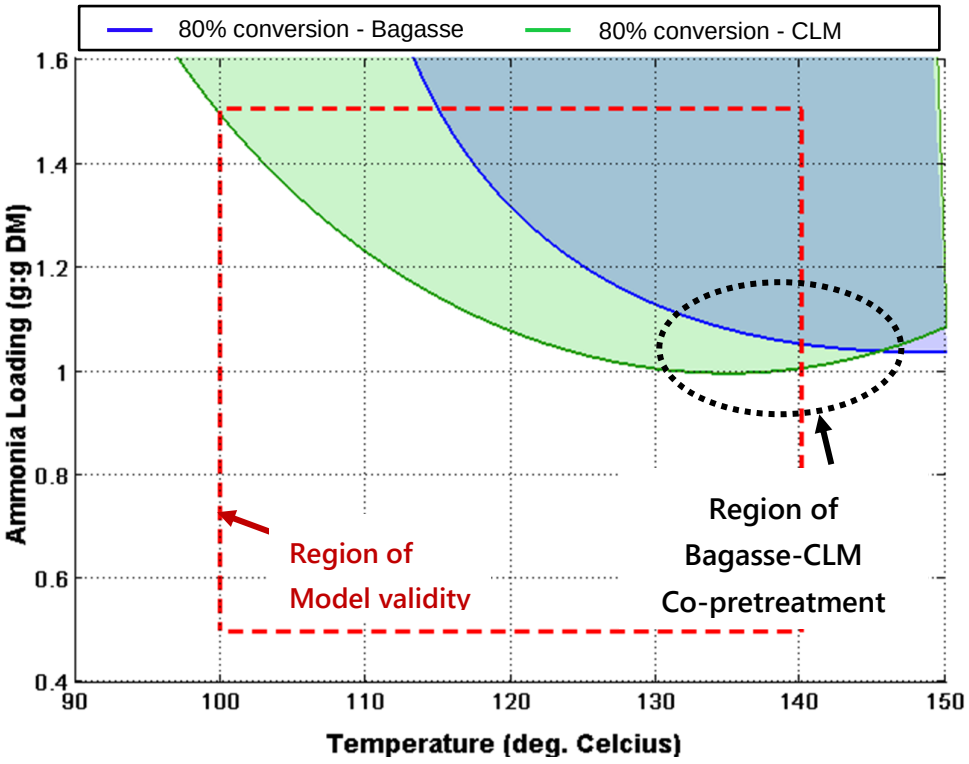
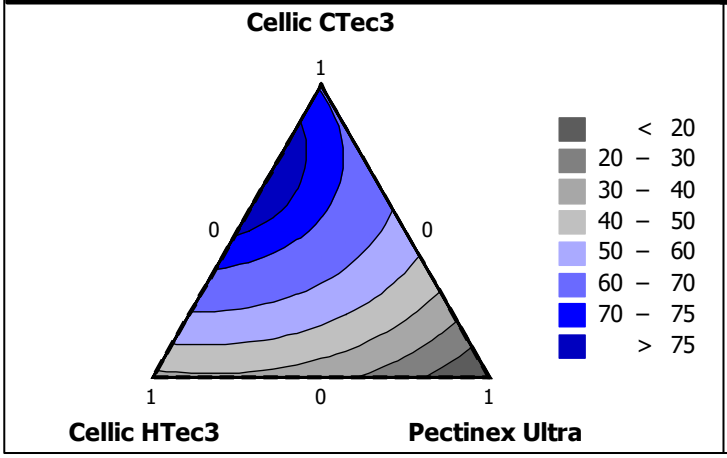
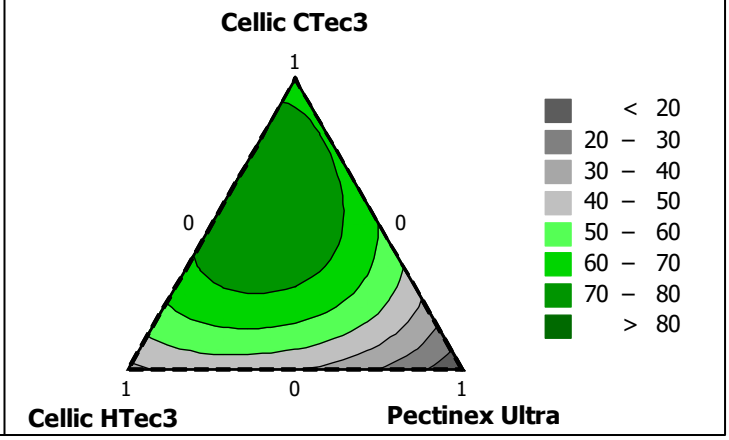



**Figure S4.2***:* Profiling the effect of ammonia loading and temperature on the combined glucose and xylose yields for AFEX™-treated bagasse and CLM after 1% glucan loading enzymatic hydrolysis with 15mg protein per gram glucan. Pretreatment water loading and residence time were fixed at 0.65 g $H_2O$ per gram DM and 30 minutes, respectively.

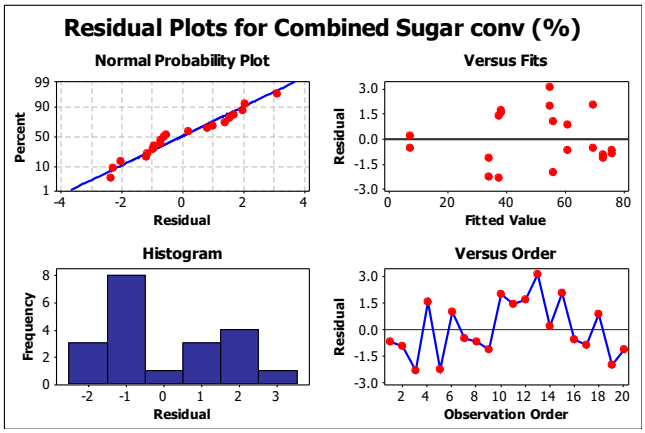
## AFEX™- Bagasse




## AFEX™- CLM




### a. AFEX™-Bagasse: Residual plots for Enzyme Optimization

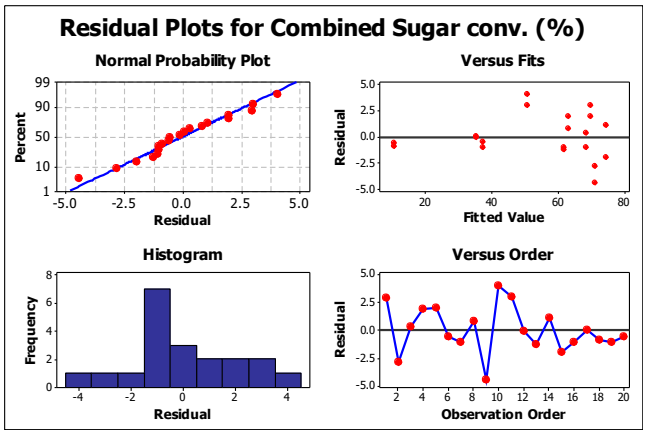



### c. AFEX™-Bagasse: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| CTec3 | 69 | 1.26 | * | 0.000 |
| HTec3 | 37 | 1.26 | * | 0.000 |
| Pectinex | 7 | 1.26 | * | 0.000 |
| CTec3*HTec3 | 90 | 5.80 | 15.50 | 0.000 |
| CTec3*Pectinex | 70 | 5.80 | 12.13 | 0.000 |
| HTec3*Pectinex | 47 | 5.80 | 8.06 | 0.000 |
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F** |
| Regression | 5.0 | 8286.04 | 1657.21 | 487.44 |
| Lack-of-Fit | 4.0 | 29.73 | 7.43 | 4.16 |

S = 1.84385 PRESS = 104.938
$R^2$ = 99.43 % $R^2_{(predicted)}$ = 98.74 % $R^2_{(adjusted)}$ = 99.22%

### b. AFEX™-CLM: Residual plots for Enzyme Optimization



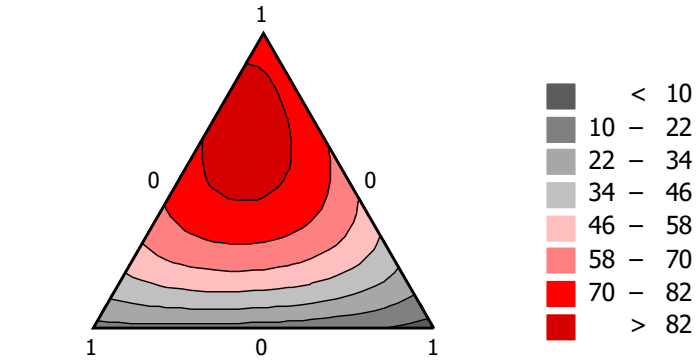

### c. AFEX™-CLM: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| Constant | 68 | 1839.00 | * | 0.000 |
| CTec3 | 35 | 1.80 | * | 0.000 |
| HTec3 | 10 | 1.80 | * | 0.000 |
| Pectinex | 90 | 8.98 | 10.06 | 0.000 |
| CTec3*HTec3 | 89 | 8.98 | 9.93 | 0.000 |
| CTec3*Pectinex | 58 | 9.02 | 6.47 | 0.000 |
| CTec3*HTec3*Pectinex | 629 | 107.57 | 5.85 | 0.000 |
| CTec3*CTec3*HTec3*Pectinex | -1347 | 269.56 | -5.00 | 0.000 |
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F** |
| Regression | 7.0 | 7711.00 | 1101.57 | 162.09 |
| Lack-of-Fit | 2.0 | 72.79 | 36.40 | 41.56 |

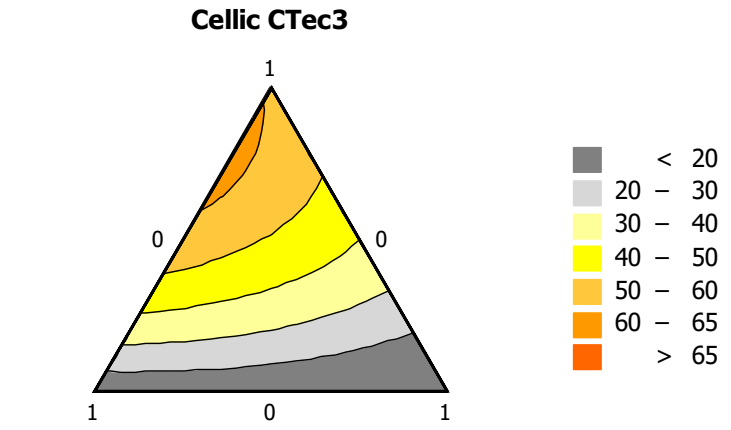
S = 2.60692 PRESS = 182.469
$R^2$ = 98.95 % $R^2_{(predicted)}$ = 97.66 % $R^2_{(adjusted)}$ = 98.34%

**Figure S4.3-A:** Contour plots, residual plots and regression coefficients (coded) used to validate ANOVA assumptions in evaluating the effect of commercial enzyme cocktail mixtures on the monomeric combined sugar yield from AFEX™-treated sugarcane bagasse and CLM. **Abbreviations: S** - Standard Error of the Regression, **PRESS** - Prediction Sum of Squares.

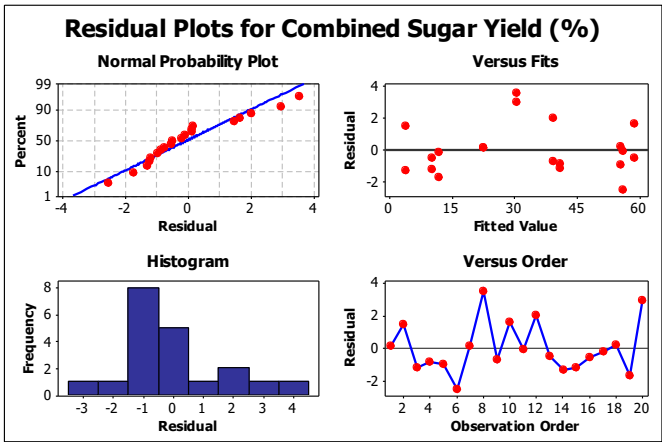
## StEx- Bagasse




## StEx- CLM




### e. StEx-Bagasse: Residual plots for Enzyme Optimization

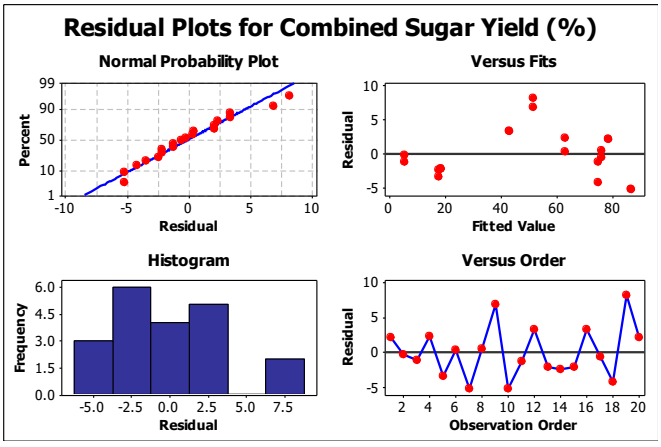



### f. StEx-Bagasse: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| CTec3 | 59 | 1.30 | * | 0.000 |
| HTec3 | 12 | 1.30 | * | 0.000 |
| Pectinex | 4 | 1.30 | * | 0.000 |
| CTec3*HTec3 | 81 | 6.54 | 12.39 | 0.000 |
| CTec3*Pectinex | 32 | 6.54 | 4.89 | 0.000 |
| HTec3*Pectinex | 9 | 6.54 | 1.40 | 0.019 |
| CTec3*HTec3*Pectinex | 71 | 43.11 | 1.65 | 0.012 |
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F** |
| Regression | 6.0 | 7465.71 | 1244.28 | 344.49 |
| Lack-of-Fit | 3.0 | 31.8 | 10.60 | 6.99 |

**S** = 1.90050 **PRESS** = 101.547
$R^2$ = 99.37 % $R^2_{(predicted)}$ = 98.65 % $R^2_{(adjusted)}$ = 99.09%

### g. StEx-CLM: Residual plots for Enzyme Optimization




### h. StEx-CLM: Regression coefficients and *p*-values

| Term | Coef. | SE Coef. | T | *p*-value |
|---|---|---|---|---|
| CTec3 | 78 | 3.02 | * | 0.000 |
| HTec3 | 18 | 3.02 | * | 0.000 |
| Pectinex | 5 | 3.02 | * | 0.000 |
| CTec3*HTec3 | 110 | 15.20 | 7.25 | 0.000 |
| CTec3*Pectinex | 84 | 15.20 | 5.56 | 0.000 |
| HTec3*Pectinex | 23 | 15.20 | 1.51 | 0.016 |
| CTec3*HTec3*Pectinex | 439 | 100.21 | 4.38 | 0.001 |
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F** |
| Regression | 6.0 | 7465.71 | 1244.28 | 344.49 |
| Lack-of-Fit | 3.0 | 31.8 | 10.60 | 6.99 |

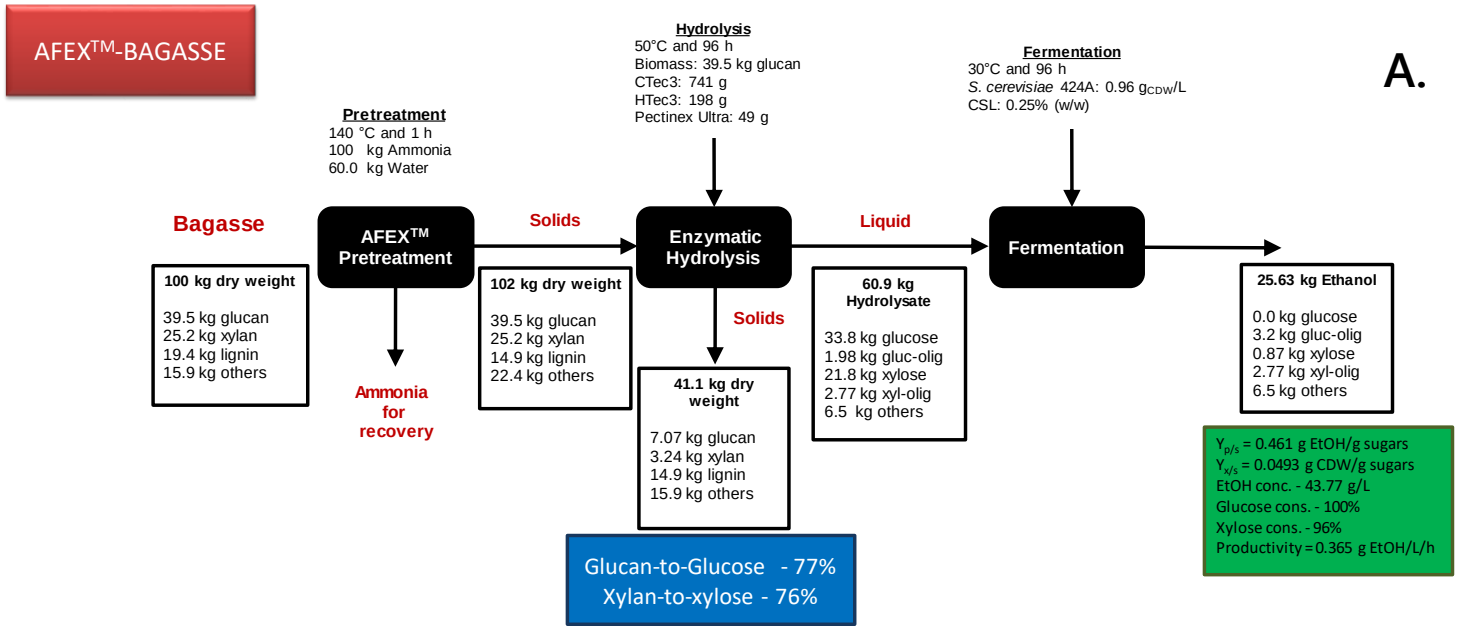
**S** = 4.41759 **PRESS** = 449.117
$R^2$ = 98.36 % $R^2_{(predicted)}$ = 97.10 % $R^2_{(adjusted)}$ = 97.60%

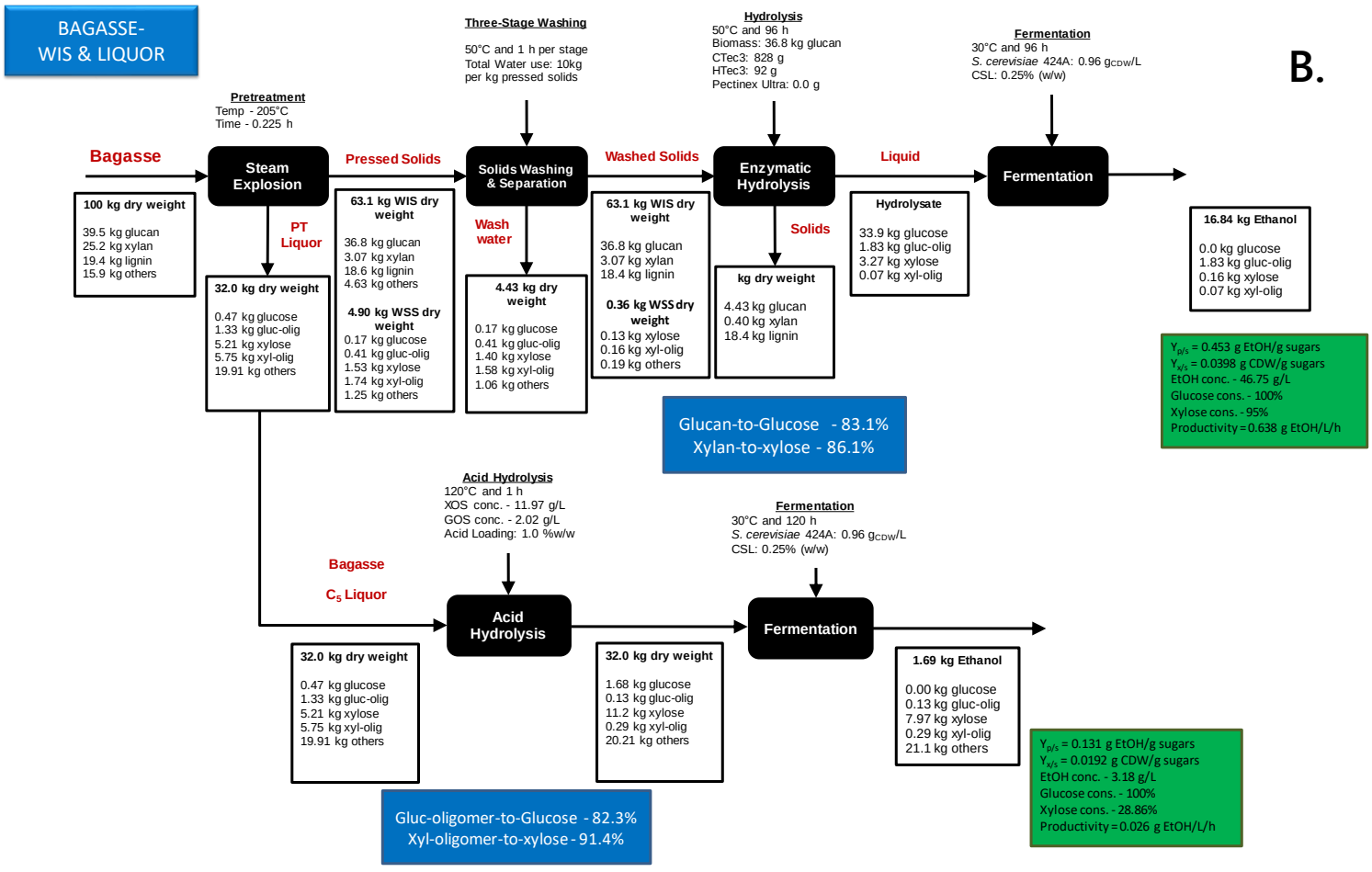
***Figure S4.3-B:*** Contour plots, residual plots and regression coefficients (coded) used to validate ANOVA assumptions in evaluating the effect of commercial enzyme cocktail mixtures on the monomeric combined sugar yield from StEx-treated sugarcane bagasse and CLM. **Abbreviations: S** - Standard Error of the Regression, **PRESS** - Prediction Sum of Squares.

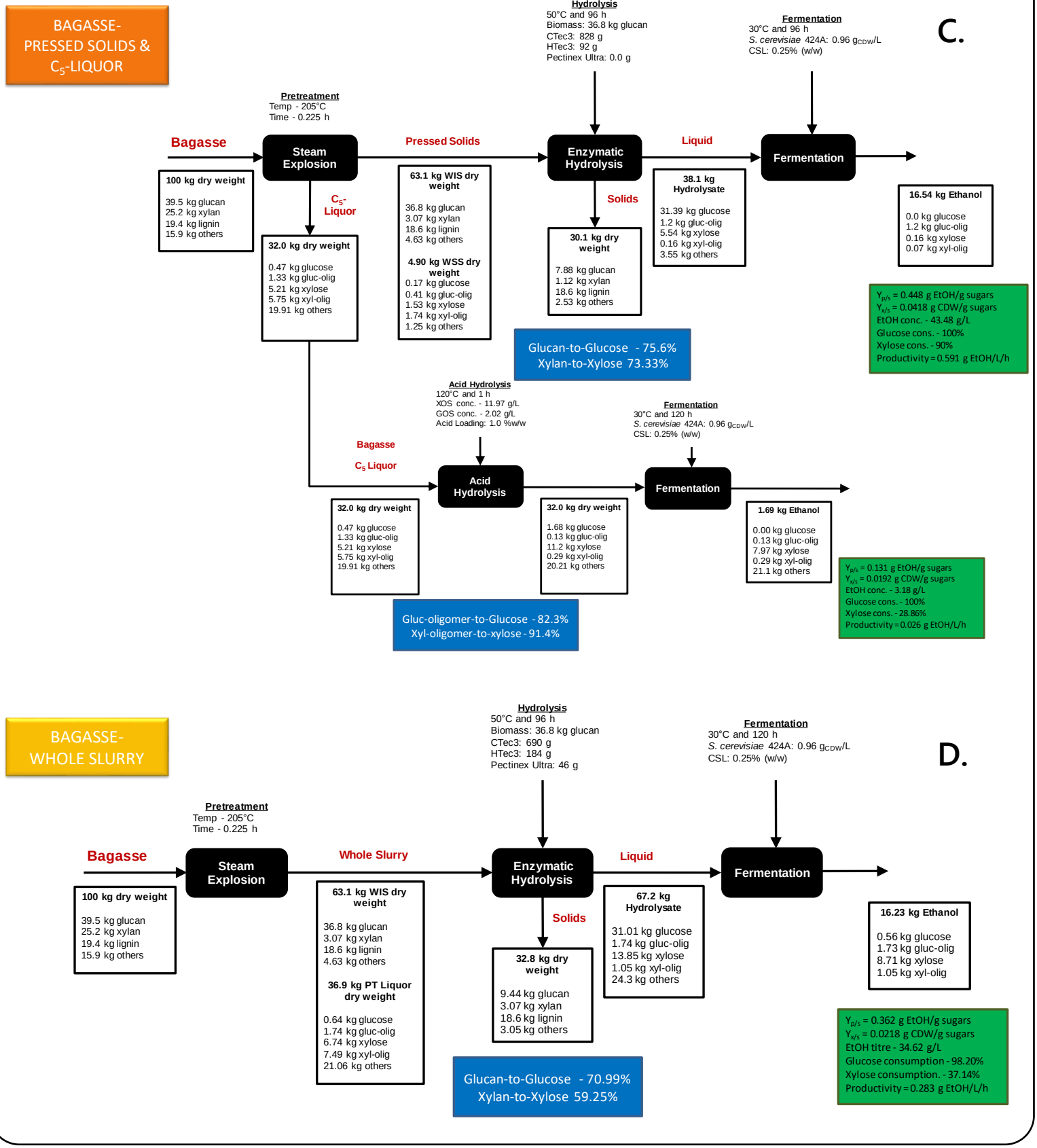
**Table S4.3:** Optimum combinations of Cellic® CTec3, Cellic ®HTec3, and Pectinex Ultra-SP for maximising the combined sugar yield after 1% glucan loading enzymatic hydrolysis performed at 50 °C, 250 rpm for 72 hrs. The total enzyme dosage was fixed at 15mg/g glucan in pretreated biomass

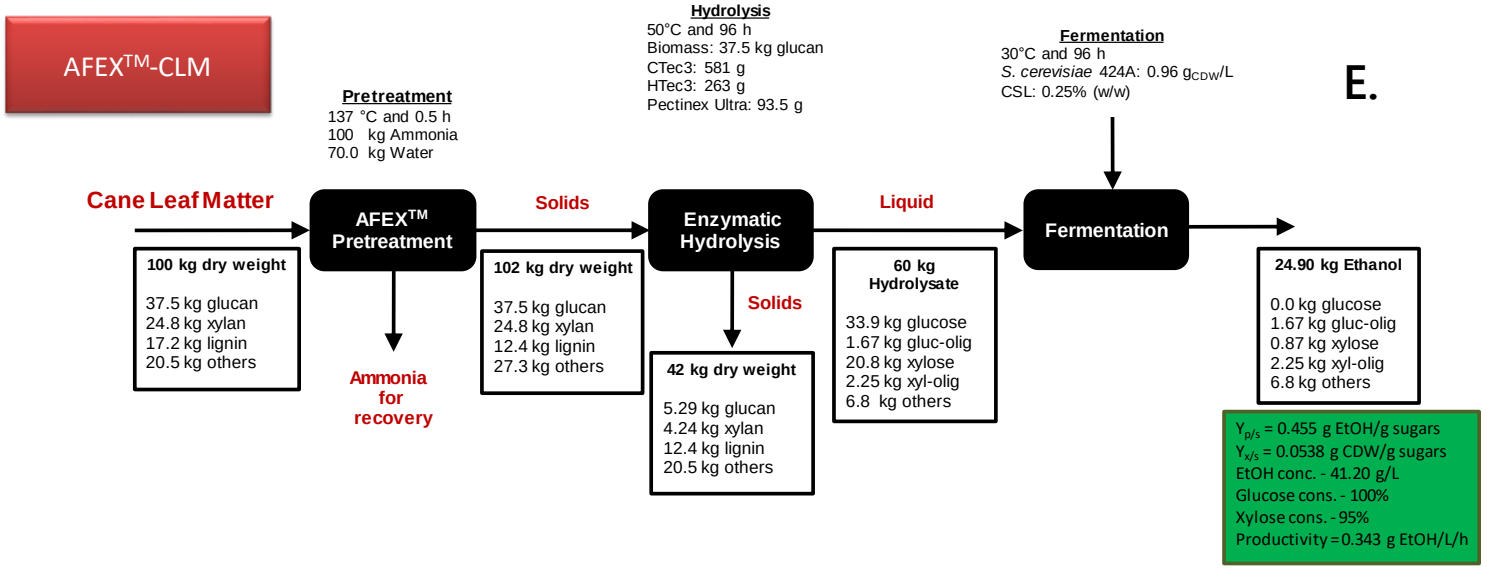
| Pretreatment | Biomass | Cocktail Mixture ($z_1$:$z_2$:$z_3$) | Glucose Yield (%) | Xylose Yield (%) | Combined Sugar Yield (%) |
|---|---|---|---|---|---|
| AFEX™ | Bagasse | 68% : 22% : 10% | 74.9 | 75.41 | 75.1 |
| AFEX™ | Tops & Leaves | 62% : 28% : 10% | 80.7 | 78.43 | 79.78 |
| Steam Explosion | Bagasse | 90% : 10% : 0% | 66.03 | 63.69 | 65.92 |
| Steam Explosion | Tops & Leaves | 72% : 20% : 8% | 83.62 | 81.3 | 83.47 |

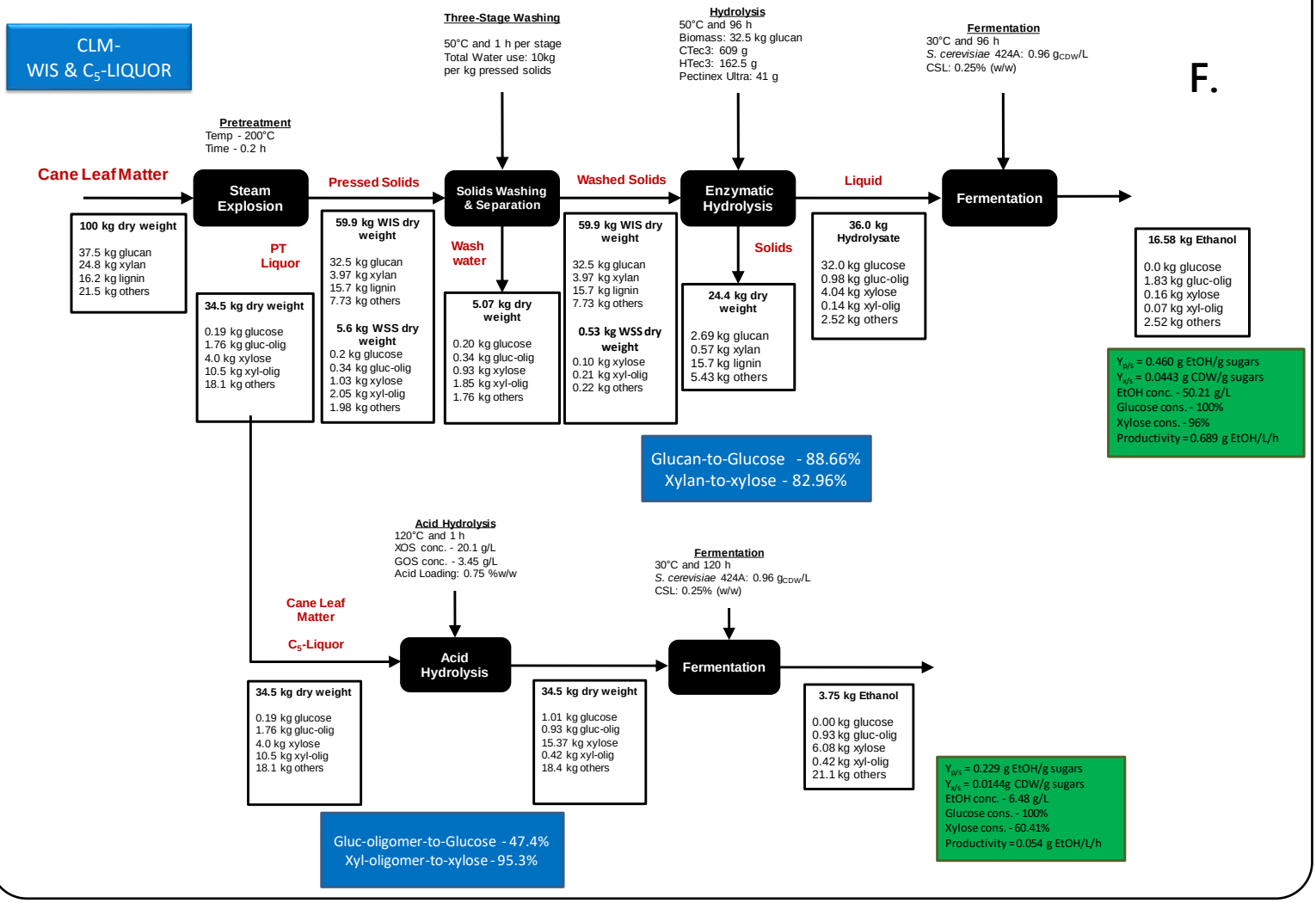
$z_1$ – Cellic® CTec3; $z_2$ – Cellic® HTec3; $z_3$ – Pectinex Ultra SP;


AFEX™-BAGASSE
A.
Pretreatment
140 °C and 1 h
100 kg Ammonia
60.0 kg Water
Hydrolysis
50°C and 96 h
Biomass: 39.5 kg glucan
CTec3: 741 g
HTec3: 198 g
Pectinex Ultra: 49 g
Fermentation
30°C and 96 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Bagasse
100 kg dry weight
39.5 kg glucan
25.2 kg xylan
19.4 kg lignin
15.9 kg others
AFEX™ Pretreatment
Ammonia for recovery
Solids
102 kg dry weight
39.5 kg glucan
25.2 kg xylan
14.9 kg lignin
22.4 kg others
Enzymatic Hydrolysis
Solids
41.1 kg dry weight
7.07 kg glucan
3.24 kg xylan
14.9 kg lignin
15.9 kg others
Liquid
60.9 kg Hydrolysate
33.8 kg glucose
1.98 kg gluc-olig
21.8 kg xylose
2.77 kg xyl-olig
6.5 kg others
Fermentation
25.63 kg Ethanol
0.0 kg glucose
3.2 kg gluc-olig
0.87 kg xylose
2.77 kg xyl-olig
6.5 kg others
Yp/s = 0.461 g EtOH/g sugars
Yx/s = 0.0493 g CDW/g sugars
EtOH conc. - 43.77 g/L
Glucose cons. - 100%
Xylose cons. - 96%
Productivity = 0.365 g EtOH/L/h
Glucan-to-Glucose - 77%
Xylan-to-xylose - 76%


BAGASSE-WIS & LIQUOR
B.
Pretreatment
Temp - 205°C
Time - 0.225 h
Three-Stage Washing
50°C and 1 h per stage
Total Water use: 10kg per kg pressed solids
Hydrolysis
50°C and 96 h
Biomass: 36.8 kg glucan
CTec3: 828 g
HTec3: 92 g
Pectinex Ultra: 0.0 g
Fermentation
30°C and 96 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Bagasse
100 kg dry weight
39.5 kg glucan
25.2 kg xylan
19.4 kg lignin
15.9 kg others
Steam Explosion
Pressed Solids
63.1 kg WIS dry weight
36.8 kg glucan
3.07 kg xylan
18.6 kg lignin
4.63 kg others
4.90 kg WSS dry weight
0.17 kg glucose
0.41 kg gluc-olig
1.53 kg xylose
1.74 kg xyl-olig
1.25 kg others
PT Liquor
32.0 kg dry weight
0.47 kg glucose
1.33 kg gluc-olig
5.21 kg xylose
5.75 kg xyl-olig
19.91 kg others
Solids Washing & Separation
Wash water
4.43 kg dry weight
0.17 kg glucose
0.41 kg gluc-olig
1.40 kg xylose
1.58 kg xyl-olig
1.06 kg others
Washed Solids
63.1 kg WIS dry weight
36.8 kg glucan
3.07 kg xylan
18.4 kg lignin
0.36 kg WSS dry weight
0.13 kg xylose
0.16 kg xyl-olig
0.19 kg others
Enzymatic Hydrolysis
Solids
kg dry weight
4.43 kg glucan
0.40 kg xylan
18.4 kg lignin
Liquid
Hydrolysate
33.9 kg glucose
1.83 kg gluc-olig
3.27 kg xylose
0.07 kg xyl-olig
Fermentation
16.84 kg Ethanol
0.0 kg glucose
1.83 kg gluc-olig
0.16 kg xylose
0.07 kg xyl-olig
Yp/s = 0.453 g EtOH/g sugars
Yx/s = 0.0398 g CDW/g sugars
EtOH conc. - 46.75 g/L
Glucose cons. - 100%
Xylose cons. - 95%
Productivity = 0.638 g EtOH/L/h
Glucan-to-Glucose - 83.1%
Xylan-to-xylose - 86.1%
Acid Hydrolysis
120°C and 1 h
XOS conc. - 11.97 g/L
GOS conc. - 2.02 g/L
Acid Loading: 1.0 %w/w
Fermentation
30°C and 120 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Bagasse
C5 Liquor
32.0 kg dry weight
0.47 kg glucose
1.33 kg gluc-olig
5.21 kg xylose
5.75 kg xyl-olig
19.91 kg others
Acid Hydrolysis
32.0 kg dry weight
1.68 kg glucose
0.13 kg gluc-olig
11.2 kg xylose
0.29 kg xyl-olig
20.21 kg others
Fermentation
1.69 kg Ethanol
0.00 kg glucose
0.13 kg gluc-olig
7.97 kg xylose
0.29 kg xyl-olig
21.1 kg others
Yp/s = 0.131 g EtOH/g sugars
Yx/s = 0.0192 g CDW/g sugars
EtOH conc. - 3.18 g/L
Glucose cons. - 100%
Xylose cons. - 28.86%
Productivity = 0.026 g EtOH/L/h
Gluc-oligomer-to-Glucose - 82.3%
Xyl-oligomer-to-xylose - 91.4%


BAGASSE-PRESSED SOLIDS & $C_5$-LIQUOR
C.
Hydrolysis
50°C and 96 h
Biomass: 36.8 kg glucan
CTec3: 828 g
HTec3: 92 g
Pectinex Ultra: 0.0 g
Fermentation
30°C and 96 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Pretreatment
Temp - 205°C
Time - 0.225 h
Bagasse
Steam Explosion
Pressed Solids
Enzymatic Hydrolysis
Liquid
Fermentation
100 kg dry weight
39.5 kg glucan
25.2 kg xylan
19.4 kg lignin
15.9 kg others
$C_5$-Liquor
32.0 kg dry weight
0.47 kg glucose
1.33 kg gluc-olig
5.21 kg xylose
5.75 kg xyl-olig
19.91 kg others
63.1 kg WIS dry weight
36.8 kg glucan
3.07 kg xylan
18.6 kg lignin
4.63 kg others
4.90 kg WSS dry weight
0.17 kg glucose
0.41 kg gluc-olig
1.53 kg xylose
1.74 kg xyl-olig
1.25 kg others
Solids
30.1 kg dry weight
7.88 kg glucan
1.12 kg xylan
18.6 kg lignin
2.53 kg others
38.1 kg Hydrolysate
31.39 kg glucose
1.2 kg gluc-olig
5.54 kg xylose
0.16 kg xyl-olig
3.55 kg others
16.54 kg Ethanol
0.0 kg glucose
1.2 kg gluc-olig
0.16 kg xylose
0.07 kg xyl-olig
Yp/s = 0.448 g EtOH/g sugars
Yx/s = 0.0418 g CDW/g sugars
EtOH conc. - 43.48 g/L
Glucose cons. - 100%
Xylose cons. - 90%
Productivity = 0.591 g EtOH/L/h
Glucan-to-Glucose - 75.6%
Xylan-to-Xylose 73.33%
Acid Hydrolysis
120°C and 1 h
XOS conc. - 11.97 g/L
GOS conc. - 2.02 g/L
Acid Loading: 1.0 %w/w
Fermentation
30°C and 120 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Bagasse
$C_5$ Liquor
Acid Hydrolysis
Fermentation
32.0 kg dry weight
0.47 kg glucose
1.33 kg gluc-olig
5.21 kg xylose
5.75 kg xyl-olig
19.91 kg others
32.0 kg dry weight
1.68 kg glucose
0.13 kg gluc-olig
11.2 kg xylose
0.29 kg xyl-olig
20.21 kg others
1.69 kg Ethanol
0.00 kg glucose
0.13 kg gluc-olig
7.97 kg xylose
0.29 kg xyl-olig
21.1 kg others
Yp/s = 0.131 g EtOH/g sugars
Yx/s = 0.0192 g CDW/g sugars
EtOH conc. - 3.18 g/L
Glucose cons. - 100%
Xylose cons. - 28.86%
Productivity = 0.026 g EtOH/L/h
Gluc-oligomer-to-Glucose - 82.3%
Xyl-oligomer-to-xylose - 91.4%
BAGASSE-WHOLE SLURRY
D.
Hydrolysis
50°C and 96 h
Biomass: 36.8 kg glucan
CTec3: 690 g
HTec3: 184 g
Pectinex Ultra: 46 g
Fermentation
30°C and 120 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Pretreatment
Temp - 205°C
Time - 0.225 h
Bagasse
Steam Explosion
Whole Slurry
Enzymatic Hydrolysis
Liquid
Fermentation
100 kg dry weight
39.5 kg glucan
25.2 kg xylan
19.4 kg lignin
15.9 kg others
63.1 kg WIS dry weight
36.8 kg glucan
3.07 kg xylan
18.6 kg lignin
4.63 kg others
36.9 kg PT Liquor dry weight
0.64 kg glucose
1.74 kg gluc-olig
6.74 kg xylose
7.49 kg xyl-olig
21.06 kg others
Solids
32.8 kg dry weight
9.44 kg glucan
3.07 kg xylan
18.6 kg lignin
3.05 kg others
67.2 kg Hydrolysate
31.01 kg glucose
1.74 kg gluc-olig
13.85 kg xylose
1.05 kg xyl-olig
24.3 kg others
16.23 kg Ethanol
0.56 kg glucose
1.73 kg gluc-olig
8.71 kg xylose
1.05 kg xyl-olig
Yp/s = 0.362 g EtOH/g sugars
Yx/s = 0.0218 g CDW/g sugars
EtOH titre - 34.62 g/L
Glucose consumption - 98.20%
Xylose consumption. - 37.14%
Productivity = 0.283 g EtOH/L/h
Glucan-to-Glucose - 70.99%
Xylan-to-Xylose 59.25%


AFEX™-CLM
E.
Pretreatment
137 °C and 0.5 h
100 kg Ammonia
70.0 kg Water
Hydrolysis
50°C and 96 h
Biomass: 37.5 kg glucan
CTec3: 581 g
HTec3: 263 g
Pectinex Ultra: 93.5 g
Fermentation
30°C and 96 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Cane Leaf Matter
AFEX™ Pretreatment
Solids
Enzymatic Hydrolysis
Liquid
Fermentation
100 kg dry weight
37.5 kg glucan
24.8 kg xylan
17.2 kg lignin
20.5 kg others
Ammonia for recovery
102 kg dry weight
37.5 kg glucan
24.8 kg xylan
12.4 kg lignin
27.3 kg others
Solids
42 kg dry weight
5.29 kg glucan
4.24 kg xylan
12.4 kg lignin
20.5 kg others
60 kg Hydrolysate
33.9 kg glucose
1.67 kg gluc-olig
20.8 kg xylose
2.25 kg xyl-olig
6.8 kg others
24.90 kg Ethanol
0.0 kg glucose
1.67 kg gluc-olig
0.87 kg xylose
2.25 kg xyl-olig
6.8 kg others
Yp/s = 0.455 g EtOH/g sugars
Yx/s = 0.0538 g CDW/g sugars
EtOH conc. - 41.20 g/L
Glucose cons. - 100%
Xylose cons. - 95%
Productivity = 0.343 g EtOH/L/h


CLM-
WIS & C5-LIQUOR
F.
Three-Stage Washing
50°C and 1 h per stage
Total Water use: 10kg per kg pressed solids
Hydrolysis
50°C and 96 h
Biomass: 32.5 kg glucan
CTec3: 609 g
HTec3: 162.5 g
Pectinex Ultra: 41 g
Fermentation
30°C and 96 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Pretreatment
Temp - 200°C
Time - 0.2 h
Cane Leaf Matter
Steam Explosion
Pressed Solids
Solids Washing & Separation
Washed Solids
Enzymatic Hydrolysis
Liquid
Fermentation
100 kg dry weight
37.5 kg glucan
24.8 kg xylan
16.2 kg lignin
21.5 kg others
PT Liquor
34.5 kg dry weight
0.19 kg glucose
1.76 kg gluc-olig
4.0 kg xylose
10.5 kg xyl-olig
18.1 kg others
59.9 kg WIS dry weight
32.5 kg glucan
3.97 kg xylan
15.7 kg lignin
7.73 kg others
5.6 kg WSS dry weight
0.2 kg glucose
0.34 kg gluc-olig
1.03 kg xylose
2.05 kg xyl-olig
1.98 kg others
Wash water
5.07 kg dry weight
0.20 kg glucose
0.34 kg gluc-olig
0.93 kg xylose
1.85 kg xyl-olig
1.76 kg others
59.9 kg WIS dry weight
32.5 kg glucan
3.97 kg xylan
15.7 kg lignin
7.73 kg others
0.53 kg WSS dry weight
0.10 kg xylose
0.21 kg xyl-olig
0.22 kg others
Solids
24.4 kg dry weight
2.69 kg glucan
0.57 kg xylan
15.7 kg lignin
5.43 kg others
36.0 kg Hydrolysate
32.0 kg glucose
0.98 kg gluc-olig
4.04 kg xylose
0.14 kg xyl-olig
2.52 kg others
16.58 kg Ethanol
0.0 kg glucose
1.83 kg gluc-olig
0.16 kg xylose
0.07 kg xyl-olig
2.52 kg others
Yp/s = 0.460 g EtOH/g sugars
Yx/s = 0.0443 g CDW/g sugars
EtOH conc. - 50.21 g/L
Glucose cons. - 100%
Xylose cons. - 96%
Productivity = 0.689 g EtOH/L/h
Glucan-to-Glucose - 88.66%
Xylan-to-xylose - 82.96%
Acid Hydrolysis
120°C and 1 h
XOS conc. - 20.1 g/L
GOS conc. - 3.45 g/L
Acid Loading: 0.75 %w/w
Fermentation
30°C and 120 h
S. cerevisiae 424A: 0.96 gCDW/L
CSL: 0.25% (w/w)
Cane Leaf Matter
C5-Liquor
Acid Hydrolysis
Fermentation
34.5 kg dry weight
0.19 kg glucose
1.76 kg gluc-olig
4.0 kg xylose
10.5 kg xyl-olig
18.1 kg others
34.5 kg dry weight
1.01 kg glucose
0.93 kg gluc-olig
15.37 kg xylose
0.42 kg xyl-olig
18.4 kg others
3.75 kg Ethanol
0.00 kg glucose
0.93 kg gluc-olig
6.08 kg xylose
0.42 kg xyl-olig
21.1 kg others
Yp/s = 0.229 g EtOH/g sugars
Yx/s = 0.0144g CDW/g sugars
EtOH conc. - 6.48 g/L
Glucose cons. - 100%
Xylose cons. - 60.41%
Productivity = 0.054 g EtOH/L/h
Gluc-oligomer-to-Glucose - 47.4%
Xyl-oligomer-to-xylose - 95.3%

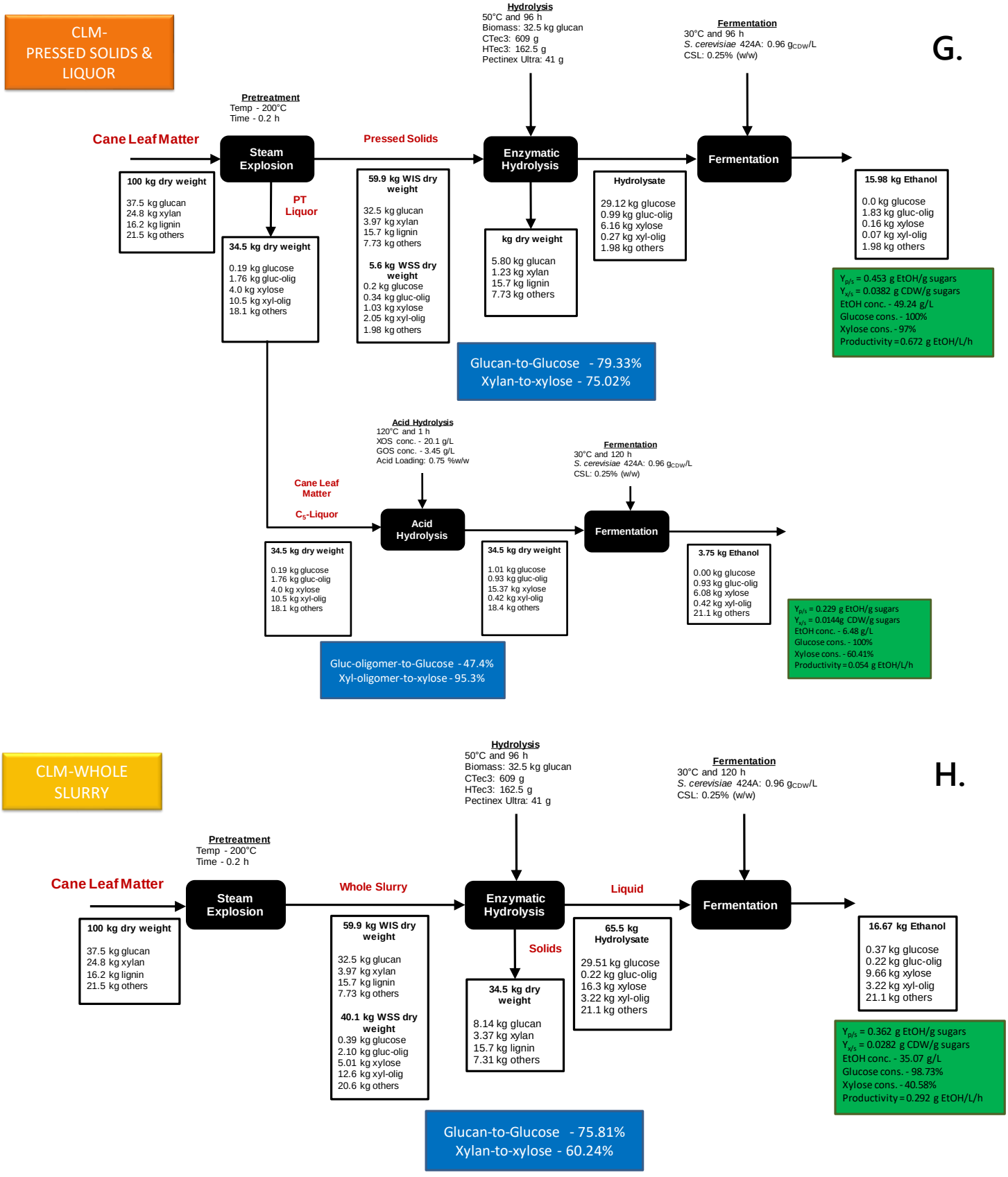



**Figure S4.4:** Material balances during pretreatment, washing, hydrolysis and fermentation for Processes I – IV. **A** – AFEX-bagasse (Process I), **B** – StEx-bagasse (washed) (Process II), **C** – StEx-bagasse (unwashed) (Process III), **D** – StEx-bagasse (whole slurry) (Process IV), **E** – AFEX-CLM (Process I), **F** – StEx-CLM (washed) (Process II), **G** – StEx (unwashed) (Process III), **H** – StEx (whole slurry) (Process IV). Abbreviations: WIS – water insoluble solids, WSS – water soluble solids.

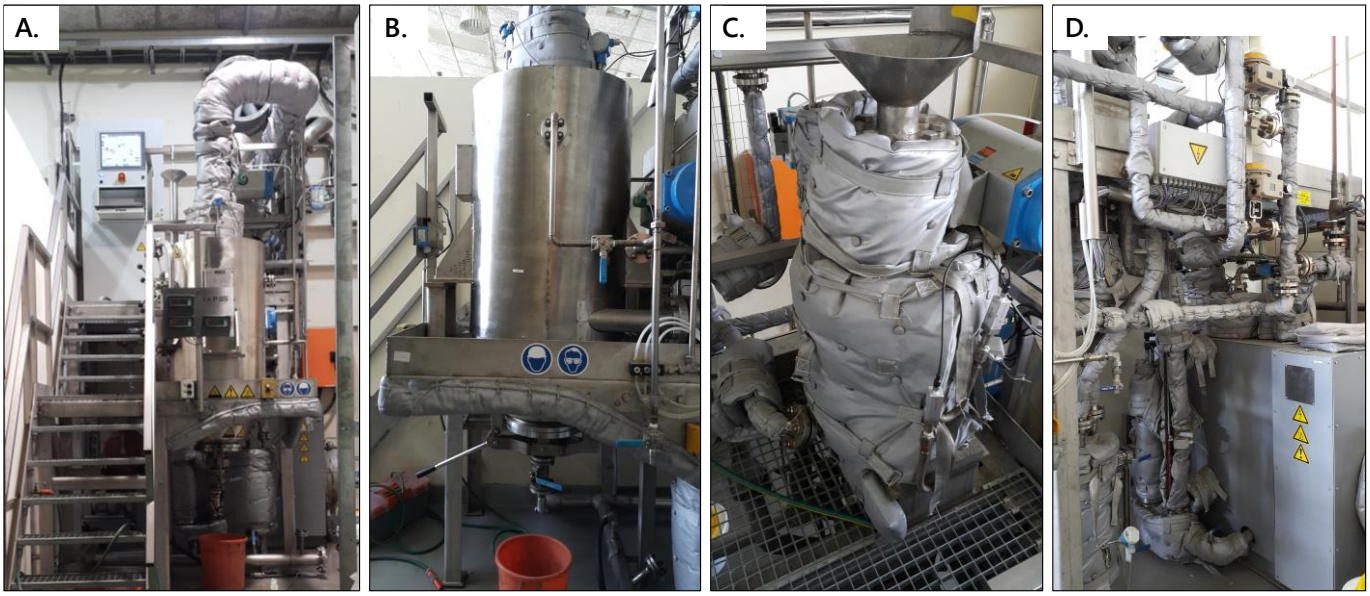



**Figure S4.5:** Illustration of **(A)** automated pilot-scale steam explosion unit with 19-L reactor, 100L-discharge tank, and 40 bar steam boiler **(B)** 100-L discharge tank, **(C)** 19-L reactor equipped with insulation jacket, steam delivery pipelines and 40-bar steam boiler

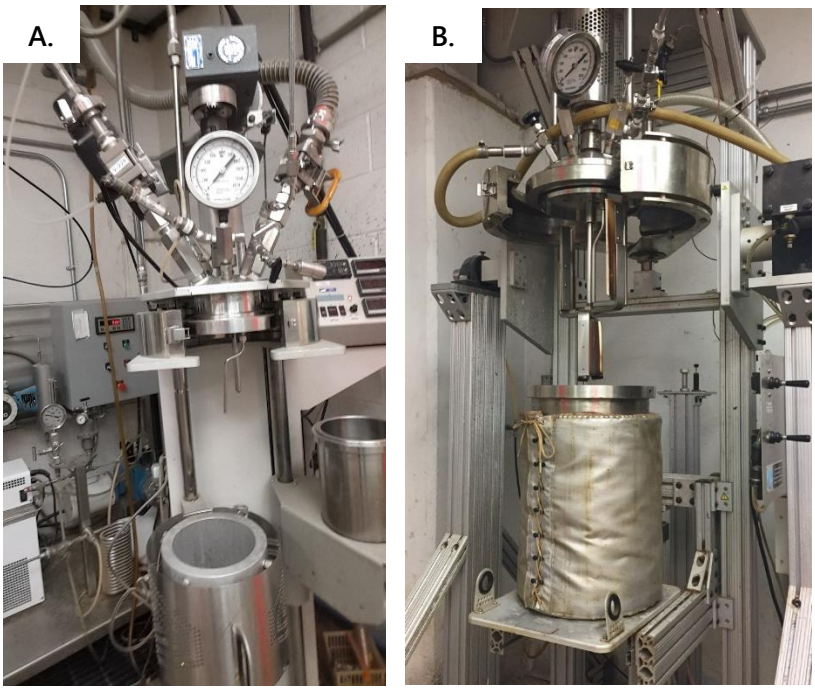



**Figure S4.6:** Illustration bench-scale Parr reactors used for AFEX™ pretreatment studies. (**A**) pre-biomass loading, (**B**) after loading biomass with reactor insulation jacket.

# CHAPTER FIVE:

# CONTRIBUTION 2

**Chapter published in:** Biofuels, Bioprocessing & Biorefining (BioFPR). ISI 5-year Impact Factor = 3.83

**Title:** Using steam explosion and AFEX™ to produce animal feeds and biofuel feedstocks in a biorefinery based on sugarcane residues

**Authors: Thapelo Mokomele**, Leonardo da Costa Sousa, Venkatesh Balan, Neill Goosen, Bryan Bals, Bruce E. Dale, and Johann F. Görgens

**Declaration:** This paper has been reproduced with permission of John Wiley & Sons, without textual amendments.

## Objective of dissertation and summary of findings in chapter

This chapter addresses the research objectives highlighted in **contribution 2** and extends upon the biorefinery concept described in **Chapter 4** to explore the potential use of StEx and AFEX™ to simultaneously generate enhanced animal feeds and digestible 2G ethanol feedstocks from SCB and CLM. In this chapter, SCB and CLM were pretreated at three distinct AFEX™-pretreatment conditions (including at pilot-scale) to evaluate the effect of pretreatment severity on the SCB and CLM animal feed value as described by the *in-vitro* true digestibility (IVTD) and metabolizable energy (ME), Maillard reaction product formation; and the ethanol production potential. The animal feed value and ethanol production potential of SCB and CLM steam exploded at the pretreatment conditions defined in **Chapter 4** were compared with the results obtained from the AFEX™-treated substrates.

The results demonstrated that both AFEX™ and StEx significantly enhance the animal feed value and the ethanol production potential of both SCB and CLM relative to untreated controls. However, higher non-protein nitrogen, IVTD and ME were obtained from AFEX™ treated SCB and CLM relative to both StEx-treated and untreated controls, suggesting that they might have a higher animal feed value. Whereas ethanol yields for StEx-treated sugarcane residues were estimated at 3368 litres per hectare sugarcane cultivation area, AFEX™ pretreatment facilitated ethanol yields up to 4360 litres

per hectare. The results from this work suggest that both StEx and AFEX™ are candidate pretreatment technologies for the integrated production of animal feeds and ethanol from sugarcane residues.

## Candidate declaration

With regards to **Chapter 5**, pages 147 – 188 of this dissertation, the nature and scope of my contributions were as follows:

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental planning | 70 |
| Executing experiments | 90 |
| Intepretation of experiments | 70 |
| Writing of chapter | 100 |

The following co-authors have contributed to **Chapter 5**, pages 147 – 188 of this dissertation:

| Name | e-mail address | Name of contribution | Extent of contribution (%) |
|---|---|---|---|
| Leonardo da Costa Sousa | sousaleo@egr.msu.edu | ✓Experimental planning<br>✓Intepretation of experiments<br>✓Review of chapter | 20<br>10<br>10 |
| Venkatesh Balan | vbalan@uh.edu | ✓Review of chapter | 20 |
| Bryan Bals | bals@mbi.org | ✓Experimental planning<br>✓Review of chapter | 10<br>10 |
| Neil Goosen | njgoosen@sun.ac.za | ✓Intepretation of experiments<br>✓Review of chapter | 10<br>20 |
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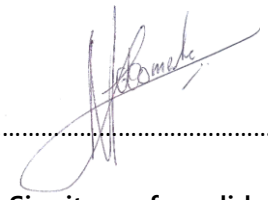

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**Signiture of candidate** **Date**

### Declaration by co-authors

All authors read and approved the final manuscript and hereby confirm that:

I. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to **Chapter 5**, page numbers 147-188 in the dissertation,

II. no other authors contributed to **Chapter 5**, page numbers 147-188 in the dissertation beside those specified above, and potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in **Chapter 5** of the dissertation.

# Using Steam Explosion or AFEX™ to Produce Animal Feeds and Biofuel Feedstocks in a Biorefinery Based on Sugarcane Residues

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## Abstract

The sustainable integration of sugarcane for biofuel (primarily bioethanol) and highly digestible livestock feed production systems has been touted as a potential avenue to increase the economic returns to agriculture and simultaneously promote energy security, particularly in developing countries. In this work we evaluated the efficacy of steam explosion (StEx) and ammonia fiber expansion (AFEX™) as potential processes for improving the *in-vitro* rumen digestibility, metabolizable energy and ethanol yields from sugarcane crop residues (bagasse and cane leaf matter (CLM)). AFEX™ pretreatment enhanced the *in-vitro* true digestibility and metabolizable energy content of sugarcane crop residues by 69% and 26%, respectively when compared to untreated controls. On the other hand, StEx increased the true digestibility and metabolizable energy content of the sugarcane residues by 54% and 7%, respectively. AFEX™ also increased the total nitrogen content of both sugarcane bagasse and CLM to more than 20.2 g/kg dry forage, more than 230% improvement relative to untreated controls. High solids loading enzymatic hydrolysis and fermentation of StEx- and AFEX™-pretreated sugarcane crop residues generated ethanol yields up to 3368 and 4360 litres of ethanol per hectare



of sugarcane cultivated, respectively, at a biomass-degrading enzyme dosage of 20 mg protein per gram glucan. This research strongly suggests that the use of suitably pretreated sugarcane crop residues in integrated sugarcane biofuel-livestock production systems can increase the total per hectare agricultural output without increasing the area of sugarcane cultivated. In effect, this integrated approach promotes more sustainable biofuel production and increased food production while simultaneously avoiding the potential for indirect land use change (ILUC).

**Key words:** Sugarcane, Bagasse, Cane Leaf Matter, Livestock production, Biofuels, Ethanol, Animal feeds, Indirect land use change



## 5.1 Introduction

Integrating existing crop residues or non-edible by-products from arable land production into animal feed diets (particularly ruminants) has been touted as a potential avenue for improving land-use efficiency for livestock production systems [17]. In the context of the sugar industry, approximately 280 kg dry weight of sugarcane crop residues (*i.e.* sugarcane bagasse and cane leaf matter (CLM)) are generated per tonne of wet cane harvested [22]. The sustainable use of these crop residues as feedstocks for domestic bioenergy production is currently being explored, particularly in major sugarcane-producing regions such as Brazil and South Africa. The use of sugarcane crop residues as sources of roughage in cattle diets has become prominent in Brazil, particularly in semi-arid regions, primarily due to prolonged periods of drought and low year-round availability of traditional forages[175]. However, the inclusion of sugarcane crop residues as roughage sources in animal feeds is limited by their low nutritional value due to factors such as low digestibility, low palatability and intake rates, low crude protein content and high lignin content [177,266].

Efficient and well-studied biomass pretreatment technologies such as ammonia fiber expansion (AFEX™, trademark of MBI International, USA) and steam explosion (StEx) have been demonstrated to significantly enhance the digestibility of low quality agricultural forages, including sugarcane residues, for biofuel production [34,267]. Moreover, the same pretreatment technologies also have the potential to increase the digestibility and energy value of agricultural residues for animal feeds by enhancing the accessibility of the carbohydrates and proteins embedded in the plant cell wall matrix to rumen microbes and enzymes [8]. Although the adoption of pretreatment technologies for upgrading low quality lignocellulosic biomass for animal feed has been slow, literature suggests that small increases in forage digestibility could result in significant gains in livestock productivity. For example, 3-5 % increases in forage *in-vitro* digestibility due to crop breeding, cultivar selection or pretreatment have been previously associated with 17-24% differences in livestock productivity and 25% increase in the economic value of the forage [180,181,268].



Steam explosion has been shown to enhance the *in-vitro* dry matter digestibility and *in-vivo* beef cattle performance as indicated by increased the voluntary dry matter intake (DMI), feed conversion efficiency and average daily gains relative to untreated controls [184,185,269]. Similarly, bench-scale AFEX™ pretreatment increased the *in-vitro* neutral detergent fiber digestibility of eleven different forages and perennial energy crops, including sugarcane bagasse [34]. Moreover, the inclusion of AFEX™ treated rice straw at modest levels (7%) in dairy cattle diets resulted in higher milk yields relative to untreated controls [270].

However, cattle fed basal diets containing ammonia-treated forages have previously displayed symptoms of hyper-excitability, possibly due to the formation of imidazole-derived toxins (particularly 4-methylimidazole) through Maillard-type reactions involving ammonia, free soluble sugars and high temperatures (usually > 100 °C) [125,159,186,271]. As a result, the removal of free soluble sugars from the forage or carrying out AFEX™ pretreatment at lower severity conditions (low temperature and lower ammonia to biomass loading) has been suggested as strategies to minimize the formation of imidazole-derived compounds [272]. However, reduction in either the pretreatment temperature or ammonia loading is likely to negatively impact the extent of improvement in digestibility of the pretreated forage [267]. On the other hand, reducing the ammonia loading during AFEX™ pretreatment minimizes capital and operating costs associated with ammonia recovery [130].

Since both StEx and AFEX™ can generate enhanced cattle feeds and improved feedstocks for cellulosic ethanol production, they have the potential to diversify future sugarcane crop residue biorefineries to co-produce ethanol and highly digestible animal feed at facilities integrated into regional sugar mills, or in a centralized biorefinery. In this work, for the first time, we evaluated and compared the nutritive quality of StEx and pilot-scale AFEX™ treated sugarcane bagasse and CLM in terms of nutritional composition, *in- vitro* true digestibility (IVTD) and metabolizable energy (ME) relative to untreated controls. We also quantified the major AFEX™ and StEx generated cell wall decomposition products and nitrogenous compounds using a Gas Chromatography-Mass

Spectrometry (GC-MS) based analytical methods. Lastly, we evaluated and compared the ethanol yields that can be achieved from high-solids loading enzymatic hydrolysis and fermentation of StEx and AFEX™ pretreated residues under industrially-relevant conditions. The results from this work could be used in future studies evaluating the impact of sustainable intensification of pasture land and sugarcane land for feeding ruminants and bioenergy production, particularly in expanding biofuel markets.

## 5.2 Materials and methods

### 5.2.1 *Biomass*

Sugarcane bagasse and fresh CLM (including green leaves, tops and trash) were collected from two industrial South African sugarcane sources located in Malelane (TSB Sugar, Mpumalanga) and Mount Edgecombe (SASRI, Kwazulu Natal). To prevent biomass spoilage, the bagasse and CLM were air-dried in separate greenhouses until the equilibrium moisture content was approximately 7% (dry weight basis, dwb). The biomass glucan, xylan, lignin and ash contents were determined according to National Renewable Energy Laboratory (NREL, Golden, CO, USA) protocols NREL/TP-510-42618 and NREL/TP-510-42620. In monocots, glucans are polysaccharides with a structural backbone consisting β-1,4-linked glucose units, whereas xylans are carbohydrate polymers consisting of a structural backbone made up β-1,4-linked xylose units. Sugarcane bagasse was composed of 39.5 ± 0.4% glucan, 25.2 ± 0.1% xylan, 19.4 ± 0.1% lignin, and 2.9 ± 0.7% ash content, whereas CLM was composed of 37.5 ± 0.6% glucan, 24.8 ± 0.4% xylan, 16.2 ± 0.8% lignin, and 7.3 ± 0.7 ash content.

### 5.2.2 *Pretreatment*

#### 5.2.2.1 *AFEX™ pretreatment*

AFEX™ pretreatment was evaluated at three distinct conditions to evaluate the effect of pretreatment severity on the IVTD, the extent of nitrogenous compound formation, and the ethanol yield for sugarcane bagasse and CLM. Pilot-scale operation presented the most mature mode of AFEX™ operation but required a significant amount of biomass per run. Given the limited availability

of biomass, pilot-scale AFEX™ was performed at an intermediate severity whereas two more runs at vastly different operating conditions were performed at bench-scale (Table 5.1).

Low- and high-severity AFEX™ pretreatment conditions were performed in a 3.8-L Parr reaction vessel (Parr Instrument Co, IL) equipped with temperature and pressure sensors based on previously published protocol [122,267]. The bench-scale AFEX™ pretreatments (Conditions 1 and 3 in Table 1) were performed with external heating to maintain the reaction temperature and the ammonia cycle consisted of a three-step sequence, viz. $NH_3$ charging, soaking, and $NH_3$ vaporization. Low-severity AFEX™ pretreatment was conducted at 100 °C, 60% biomass moisture content, 0.5:1 $NH_3$ to biomass loading (w/w) and 30 minutes treatment time. High-severity AFEX™ pretreatment was carried out at 130 °C, 60% biomass moisture content, 1.5:1 $NH_3$ to biomass loading and 30 minutes.

**Table 5.1:** Pretreatment conditions selected to evaluate the impact of steam explosion and AFEX™ pretreatment on the animal feed quality and ethanol production potential of sugarcane bagasse and cane leaf matter (CLM).

| Biomass sample | Pretreatment conditions | | | |
|---|---|---|---|---|
| | Water Loading (g $H_2O$/g DM) | Temperature (°C) | Residence Time (min) | $NH_3$ Loading (g $NH_3$/g DM) |
| **Sugarcane Bagasse** | | | | |
| Untreated | - | - | - | - |
| AFEX™ - condition 1† | 0.60 | 100 | 30.0 | 0.5 |
| AFEX™ - condition 2‡ | 0.60 | 120 - 80 | 60.0 | 0.7 |
| AFEX™ - condition 3† | 0.60 | 130 | 30.0 | 1.5 |
| Steam explosionΨ | 0.65 | 205 | 13.5 | - |
| **Sugarcane Cane Leaf Matter** | | | | |
| Untreated | - | - | - | - |
| AFEX™ - condition 1† | 0.60 | 100 | 30.0 | 0.5 |
| AFEX™ - condition 2‡ | 0.60 | 120 - 80 | 60.0 | 0.7 |
| AFEX™ - condition 3† | 0.60 | 130 | 30.0 | 1.5 |
| Steam explosionΨ | 0.65 | 200 | 10.0 | - |

† - AFEX™ pretreatment performed at bench scale using 3.8 L Parr reactor (Michigan State University)

‡ - AFEX™ pretreatment performed at pilot scale using 1-ton per day packed-bed AFEX reactor (MBI International)

Ψ - Steam explosion performed at in 19 L pilot scale reactor (Stellenbosch University)

AFEX™ pretreatment at an intermediate-severity (Condition 2) was performed in a pair of vertical 450-L packed-bed pilot-scale reactors as previously discussed by Sarks and co-workers [273]. Briefly, biomass at approximately 20% moisture (dwb) was packed into perforated stainless-steel

baskets at a density of approximately 80–100 kg dry weight/$m^3$ and these packed baskets were subsequently placed in the vertical reactors. Once assembled, the biomass was AFEX™-treated using a cycle of five sequential steps: pre-steaming, $NH_3$ charging, soaking, $NH_3$ vaporization, and steam stripping (Figure 5.1) [274]. During pretreatment, an ammonia to biomass loading of 0.7 kg $NH_3$/kg dry biomass was used with a total pretreatment time of 60 min. Due to the absence of reactor insulation, the average reactor temperature fluctuated between 120 and 115 °C for the first 20 min before steadily descending towards 80 °C for the duration of the pretreatment. The temperature and pressure profiles during the AFEX™ pilot scale pretreatment cycle are available in Figure S5.1 (Supplementary Information). After steam stripping, the ammonia was recovered and recycled to the next cycle (lag reactor). Upon completion of the pretreatment, the pretreated biomass was removed from the baskets, transferred to burlap sacks, and dried at 45 °C in a convection oven (Grieve Corporation, IL) until the moisture was brought down below 15% (dwb).

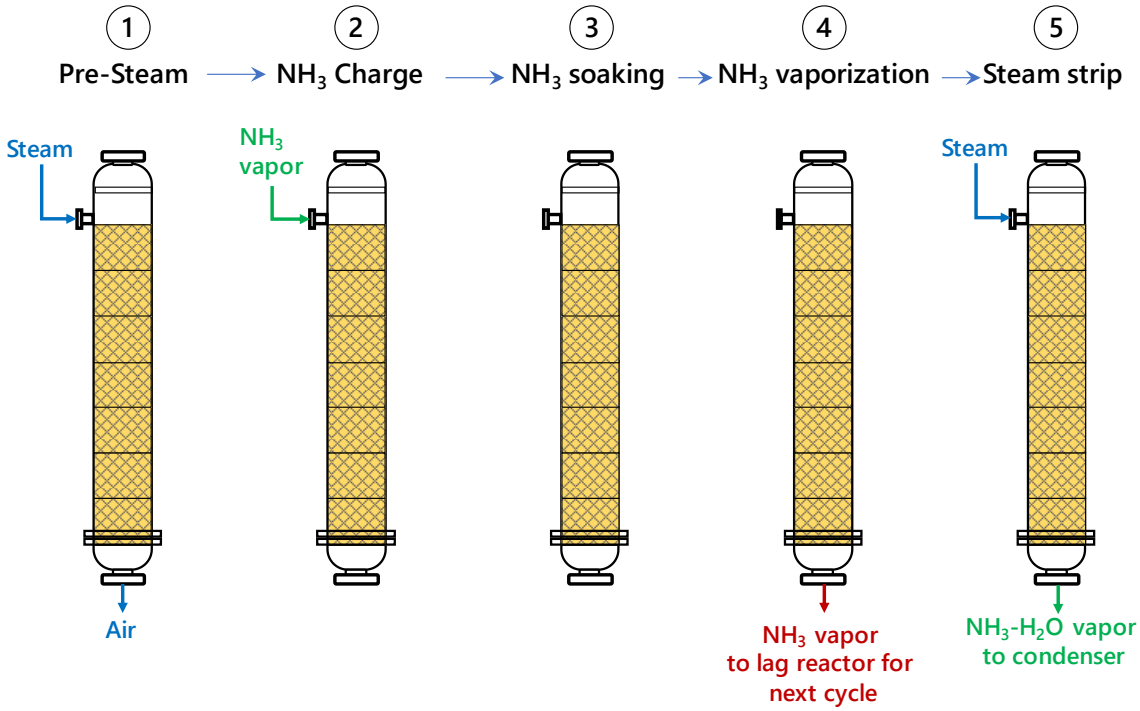



***Figure 5.1:*** Five-step pilot-scale packed-bed AFEX™ pretreatment sequence based on the design developed by MBI International, Lansing, MI [23]. Yellow boxes indicate perforated stainless-steel baskets packed with biomass.



#### 5.2.2.2 *Steam explosion*

Steam explosion (StEx) was performed at Stellenbosch University in an automated batch pilot-scale unit (IAP GmBH, Graz, Austria) equipped with a 19-L StEx reaction vessel, a 100-L blow-tank and a steam generator as previously described in **CHAPTER 4**. Briefly, 500 g (dwb) of water-impregnated biomass (bagasse or CLM) was top-loaded into the preheated StEx reaction vessel and heated to the desired temperature using 30 bar (absolute pressure) saturated steam. After the pretreatment time had elapsed, the reactor contents were discharged into the blow tank for sample collection. The pretreatment temperature and residence time used for StEx pretreatment of sugarcane bagasse and CLM are presented in Table 5.1. The collected biomass slurry was separated into solid and liquid fractions using a Eurotool TY5001 pneumatic press (Nesco Engineering, Cape Town, RSA). The solid fraction was washed in three stages with distilled water at 50 °C using a total of 10 litres water per kg wet pressed solids. After washing, the remaining solids were dried in a convection oven (Series 2000, Scientific, Cape Town, RSA) at 35 °C until the moisture was brought below 15% (dwb).

### 5.2.3 *Animal feed quality*

#### 5.2.2.3 *Nutritional composition*

In preparation for compositional analysis, untreated, AFEX™- and StEx pretreated samples were milled through a 1.0-mm screen using a Wiley mill and stored in sealed bags. The nutritional composition was determined using wet chemistry techniques at Dairy One Forage Lab (Ithaca, NY, USA). In brief, the milled samples were analyzed for total nitrogen content according to the Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Corporative Inc., Ithaca, NY, USA). Rumen degradable nitrogen was determined using the Cornell *Streptomyces griseus* protease (SGP) enzyme digestion protocol [275]. Consistent with animal science and nutrition methods, the structural component content of forages was quantified in terms of neutral detergent fiber and acid detergent fiber contents. Sequential neutral detergent fiber (aNDF) and acid detergent fiber (ADF) analyses (inclusive of ash) were performed using heat-stable α-amylase and sodium sulphite in an Ankom 200

Fiber Analyser unit (ANKOM Technology Corporation, Macedon, NY, USA) according to the method of Van Soest *et al.,* [276]. Acid detergent insoluble crude protein (ADIN) was analyzed by subjecting ADF residues to Kjeldahl nitrogen analysis. The mineral content was quantified using a Thermo iCAP 6300 Inductively Coupled Plasma Radial Spectrometer (Thermo Fischer scientific, Waltham, MA, USA). Each analysis was performed in triplicate for each forage.

#### 5.2.2.4 *In-vitro true digestibility and metabolizable energy*

IVTD is a standard laboratory anaerobic fermentation assay used simulate forage digestion in the rumen. IVTD assays, carried out at Dairy One Forage Lab (Ithaca, NY, USA), were used to compare fiber digestion and to estimate the energy content of the various pretreated forages. Rumen fluid was collected from the ruminal contents of high-producing dairy cows consuming a typical total mixed ration diet. The forage samples were incubated in a mixture of Van Soest buffer solution and rumen fluid at 39 °C under anaerobic conditions in a Daisy$^{II}$ incubator (ANKOM Technology Corporation) [277]. After 24 or 48 h, the samples were extracted using the above-mentioned aNDF procedure to eliminate bacterial contamination. The metabolizable energy (ME), net energy for lactation ($NE_l$) and total digestible nutrients (TDN) were estimated according to NRC methods (2001) [278]. The IVTD assays were performed in triplicate for each forage.

#### 5.2.2.5 *Cell wall decomposition product analysis*

Untreated, StEx and AFEX™-pretreated biomass samples were milled through a 2.0-mm screen in a Wiley mill and extracted with pure acetone (J.T Baker Inc., Phillipsburg, NJ, USA) or Milli-Q plus water (Millipore, Billerica, MA, USA) using a Dionex™ Accelerated Solvent Extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) as described by earlier authors [123]. For the quantification of nitrogenous compounds, furan derivatives, carboxylic acids, and aromatic compounds, one gram of each biomass sample was loaded into a 11 ml ASE cell and extracted with acetone at 70 °C for two cycles, at 1500 psi (or 103 bar) cell pressure, for 10 minutes. After extraction, the solvent extracts were stored at 4 °C before being injected directly into the GC-MS for analysis without derivatization.



Instrumentation for GC-MS analysis consisted of an Agilent 5973 689N GC-MS, equipped with an Agilent 7683 auto sampler and a split less injector (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed using a capillary 30 m J&W DB-WAX capillary column (0.25 mm ID, 0.25 µm film thickness). The temperature gradient was modified from Chundawat *et al.,* [123] as follows: Initial column temperature at 40 °C (1 min hold), from 15 °C/min to 75 °C (3 min hold), 20 °C/min to 165 °C (3 min hold), 30 °C/min to 260 °C (3 min hold). The spectra of eluted compounds were generated using 70 eV electron ionization in full scan mode, and the NIST05 database was used to help identify major imidazole and pyrazine derivatives. External standards of pyrazine, 2,6-dimethylpyrazine, 1-methylimidazole, 4-methylimidazole, acetic acid, acetamide, levulinic acid, formic acid, *p*-coumaric acid, ferulic acid, HMF and furfural were prepared for compound quantification in pure acetone at 0.5–50 mg/L. Calibration curves were calculated based on the peak area of the selected ion chromatogram (molecular ion, $M^{\bullet+}$) of each compound relative to the area of the external standards. Due to the absence of authentic standards, the calibration curves of 2,5-dimethylpyrazine and 4-methylimidazole were assumed valid to quantify all the other pyrazines and imidazoles, respectively [123].

To quantify soluble sugars, one-gram of biomass was extracted with MilliQ-Plus water using the ASE protocol and conditions (as described above). The aqueous extracts were prepared for water-soluble monomeric and oligomeric sugar analysis using the standard NREL LAP procedure (NREL/TP-510-42623) [279]. For measuring fructose, sucrose, glucose, xylose, arabinose, galactose and mannose content in biomass, aqueous extracts were quantified in an HPLC system equipped with an Aminex HPX-87P column (Biorad, Hercules, CA, USA) as previously described by earlier authors [135].

## 5.2.4 *Ethanol Production*

### 5.2.4.1 *Enzymes*

Commercially relevant fungal enzyme preparations Cellic® CTec3, Cellic® HTec3, and Pectinex Ultra-SP were used in enzymatic hydrolysis and ethanol production studies. These enzymes were



generously donated by Novozymes Inc. (Franklinton, NC, USA). Previously optimized ratios of CTec3, HTec3 and Pectinex Ultra-SP were used for low and high solids loading hydrolysis of untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM [267]. A summary of the enzyme combinations used is presented in Table 5.2.

**Table 5.2:** Optimal enzyme combinations used for enzymatic hydrolysis of untreated, AFEX™- and StEx-treated sugarcane bagasse and sugarcane cane leaf matter

| Biomass sample | Enzyme Loading (mg/g glucan) | | | |
|---|---|---|---|---|
| | Cellic® CTec3 | Cellic® HTec3 | Pectinex Ultra-SP | Total |
| ***Sugarcane Bagasse*** | | | | |
| Untreated | 13.3 | 6.7 | 0.0 | 20.0 |
| AFEX™ - condition 1 | 13.6 | 5.4 | 1.0 | 20.0 |
| AFEX™ - condition 2 | 13.6 | 5.4 | 1.0 | 20.0 |
| AFEX™ - condition 3 | 13.6 | 5.4 | 1.0 | 20.0 |
| Steam explosion | 18.0 | 2.0 | 0.0 | 20.0 |
| ***Sugarcane Cane Leaf Matter*** | | | | |
| Untreated | 13.3 | 6.7 | 0.0 | 20.0 |
| AFEX™ - condition 1 | 12.4 | 5.6 | 2.0 | 20.0 |
| AFEX™ - condition 2 | 12.4 | 5.6 | 2.0 | 20.0 |
| AFEX™ - condition 3 | 12.4 | 5.6 | 2.0 | 20.0 |
| Steam explosion | 14.0 | 4.0 | 2.0 | 20.0 |

### 5.2.4.2 *Enzymatic hydrolysis*

#### *5.2.4.2.1 Low solids loading enzymatic hydrolysis*

Low solids loading enzymatic hydrolysis was used to compare sugar release by combinations of commercial fungal enzymes to corresponding results obtained by fiber digestion using rumen fluid. Enzymatic hydrolysis was performed in 20 mL screw-cap scintillation vials at 1% glucan loading using 20 mg enzyme mixture per gram of glucan, incubated at 50 °C, pH 4.8 for 48 h in an orbital shaker (New Brunswick, Scientific, NJ, USA). The reaction mixture was supplemented with 50 mM citrate buffer and 0.02 % (w/v) sodium azide to maintain the hydrolysis pH and to prevent microbial contamination, respectively. After 48 h of enzymatic hydrolysis, the hydrolysates were filtered through a 0.2 µm filter, and soluble sugars, mainly glucose and xylose, were determined by HPLC (described below).

#### *5.2.4.2.2 High-Solids loading enzymatic hydrolysis*

High solids loading enzymatic hydrolysis (HSL-EH) and fermentation were performed to evaluate the potential ethanol yield from the various pretreated samples that can be obtained at industrially- relevant conditions. HSL-EH was performed for 96 h in 250 mL baffled Erlenmeyer flasks with 100 mL working volume, incubated at 50 °C, pH 4.8, in an orbital shaker at 250 rpm (New Brunswick Scientific, USA). The enzyme loading was fixed at 20 mg protein/g glucan using the optimal combinations of CTec3, HTec3, and Pectinex Ultra-SP described in Table 5.2. The solids loading was maintained at 10% (w/w) carbohydrate loading, defined as the sum of the insoluble glucan and xylan, soluble xylo-oligosaccharides and gluco-oligosaccharides, and soluble monomeric glucose and xylose in the pretreated material. Biomass was added in fed-batch mode (half at $t$ = 0 h and the rest at $t$ = 3 h) to facilitate easy mixing. After 96 h of hydrolysis, the flask contents were centrifuged at 10,000 $x$ $g$ for 30 min. The supernatant was supplemented with 0.25% (w/w) corn steep liquor, the pH was adjusted to 5.5 before being filter sterilized through a 0.22 µm PES filter and then refrigerated at 4 °C until used in fermentation studies. An overall mass balance protocol previously published by Gunawan *et al.,* [255] was employed.

### 5.2.4.3 *Fermentation*

The sterile HSL-EH hydrolysates were fermented using a recombinant *Saccharomyces cerevisiae* 424A (LNH-ST), kindly provided by Prof. Nancy Ho (retired from Purdue University, West Lafayette, IN, USA), with glucose and xylose-metabolizing capability. Seed cultures of this strain were prepared by inoculating frozen stock cultures to 100 mL YPDX media (75 $g.L^{-1}$ glucose, 25 $g.L^{-1}$ xylose, 10 $g.L^{-1}$ yeast extract, and 20 $g.L^{-1}$ tryptone) and growing the cultures micro-aerobically for 18 h. The grown cultures were harvested and inoculated into HSL-EH hydrolysate-containing shake flasks at an initial optical density ($OD_{600}$) of 2. Each fermentation was performed in triplicate in 125 mL Erlenmeyer flasks with 50 mL working volume and incubated at 30 °C for 120 h. Samples were removed after 120 h and prepared for sugar and ethanol quantification.



#### 5.2.4.4 *Analytical methods*

Glucose, xylose, ethanol, acetate, lactate, glycerol and xylitol concentrations from enzymatic hydrolysis and fermentation were analyzed using a Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD, USA) equipped with an Aminex Biorad HPX-87H column as described previously [135]. The column temperature was maintained at 50 °C, with sulfuric acid (5 mM) used as the mobile phase at a flowrate of 0.6 mL/min.

### 5.2.5 *Statistical analysis*

Statistical significance of experimental results was determined through a one-way analysis of variance (ANOVA) in combination with Tukey's *post hoc* test for multiple comparisons (Minitab Inc., State College, PA, USA). The null hypothesis was accepted or rejected at 95% confidence interval ($P < 0.05$). Linear regression was performed to correlate the enhancement of biomass digestibility by fungal enzymes to fiber digestion by rumen fluid. Linear regression was carried out in Minitab software (Minitab Inc., State College, PA, USA), and the accuracy and significance of the regression equations were assessed using the coefficient of determination ($R^2$) and the regression model *P* value, respectively.

## 5.3 Results and discussion

### 5.3.1 *Characteristics of untreated, AFEX™- and StEx-treated forages*

AFEX™ pretreatment results in darkening of all the forages and the degree of darkening increased with increased pretreatment severity (Figure S5.2, Supplementary Information). Since AFEX™ is a dry to dry process, no soluble liquid streams are removed during AFEX™ pretreatment. Thus the resulting changes in the chemical composition of the forage are occurring mainly due to ammonolysis, hydrolysis and Maillard-type reactions, and the deposition of unreacted ammonia or ammonium ions onto the biomass [271]. On the other hand, StEx solubilized much of the hemicellulose fraction and partially solubilized lignin into a separate liquid stream, thus changing the chemical composition of the residual solids [267,280]. The hemicellulose-rich StEx liquid fraction was



separated from the pretreatment slurry and was not included in this work. StEx pretreatment of sugarcane bagasse and CLM resulted in approximately 30-40% dry matter losses into the pentose ($C_5$) rich liquid stream [267]. In a biorefinery context, the solubilized $C_5$-liquid stream from StEx might be fermented to ethanol or anaerobically digested to produce methane whilst the pretreated solids would serve as fodder for cattle [281].

The nutritional characteristics of the untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM forage samples are presented in Table 5.3. AFEX™-treatment increased the total nitrogen content of sugarcane bagasse and CLM forages to at least 20.2 g/kg dry forage (>230% improvement relative to untreated controls), with the highest levels of nitrogen found in forage samples pretreated at an ammonia loading of 1.5 kg $NH_3$/kg dry forage (Condition 3). This observed increase in total nitrogen is primarily due to non-protein nitrogen (NPN) that is chemically linked to the biomass during pretreatment. Because there is a very high microbial population in the rumen, this NPN can be a valuable nitrogen source for ruminants since it can be converted to microbial protein in the rumen and subsequently used by the animal [282].

***Table 5.3:*** Nutritional composition of untreated, AFEX™- and StEx-treated sugarcane bagasse and sugarcane CLM. Mean values from replicates (n = 3) are presented in the table. Components presented on a dry basis.

| Components | Sugarcane Bagasse | | | | | Sugarcane Cane Leaf Matter | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Untreated | AFEX™ Condition 1 | AFEX™ Condition 2 | AFEX™ Condition 3 | Steam Explosion | Untreated | AFEX™ Condition 1 | AFEX™ Condition 2 | AFEX™ Condition 3 | Steam Explosion |
| DM (g/100g forage) | 97.3 $^{A}$ | 94.7 $^{C}$ | 94.0 $^{C,D}$ | 94.5 $^{C}$ | 95.6 $^{A,B}$ | 93.4 $^{C}$ | 92.9 $^{D}$ | 90.6 $^{E}$ | 91.2 $^{E}$ | 94.8 $^{B,C}$ |
| Total Nitrogen (g/kg DM) | 4.8 $^{F}$ | 21.6 $^{C}$ | 20.2 $^{D}$ | 25.1 $^{B}$ | 6.2 $^{E,F}$ | 6.4 $^{E}$ | 23.0 $^{B}$ | 21.4 $^{C}$ | 26.4 $^{A}$ | 6.4 $^{E}$ |
| ADIN (g/kg DM) | 2.1 $^{A,B}$ | 2.2 $^{A}$ | 2.2 $^{A}$ | 2.4 $^{A}$ | 2.4 $^{A}$ | 1.4 $^{B}$ | 1.8 $^{A,B}$ | 1.9 $^{A,B}$ | 1.9 $^{A}$ | 2.6 $^{A}$ |
| % Soluble nitrogen, % of N | 55.0 $^{E}$ | 67.0 $^{C}$ | 68.0 $^{B,C}$ | 71.0 $^{B}$ | 59.0 $^{D}$ | 57.0 $^{D,E}$ | 72.0 $^{A}$ | 66.0 $^{C}$ | 70.0 $^{A,B}$ | 52.0 $^{F}$ |
| % Rumen degradable nitrogen, % of N | 57.0 $^{D}$ | 70.0 $^{B}$ | 71.0 $^{B}$ | 75.0 $^{A}$ | 60.0 $^{C}$ | 69.0 $^{B}$ | 77.0 $^{A}$ | 71.0 $^{B}$ | 75.0 $^{A}$ | 54.0 $^{E}$ |
| Available Nitrogen (g/kg DM) | 2.7 $^{E,F}$ | 19.4 $^{C}$ | 17.9 $^{D}$ | 22.7 $^{B}$ | 3.8 $^{E,F}$ | 5.0 $^{E}$ | 21.3 $^{B}$ | 19.5 $^{C}$ | 24.5 $^{A}$ | 3.8 $^{E,F}$ |
| ADF (g/kg DM) | 594.0 $^{C,D}$ | 618.0 $^{C}$ | 633.0 $^{C}$ | 559.0 $^{D,E}$ | 785.0 $^{A}$ | 528.0 $^{E,F}$ | 531.0 $^{E,F}$ | 536.0 $^{E}$ | 498.0 $^{F}$ | 708.0 $^{B}$ |
| aNDF (g/kg DM) | 888.0 $^{A}$ | 799.0 $^{B}$ | 780.0 $^{B,C}$ | 685.0 $^{D}$ | 787.0 $^{B,C}$ | 809.0 $^{B}$ | 633.0 $^{E}$ | 638.0 $^{E}$ | 548.0 $^{F}$ | 758.0 $^{C}$ |
| NFC (g/kg DM) | 41.0 $^{F}$ | 25.0 $^{F}$ | 53.0 $^{F}$ | 117.0 $^{D,E}$ | 126.0 $^{C,D,E}$ | 102.0 $^{E}$ | 153.0 $^{B,C,D}$ | 178.0 $^{B}$ | 226.0 $^{A}$ | 160.1 $^{B,C}$ |
| % Calcium | 0.12 $^{D}$ | 0.11 $^{D}$ | 0.15 $^{C}$ | 0.11.0 $^{D}$ | 0.09 $^{D}$ | 0.33 $^{A}$ | 0.33 $^{A}$ | 0.36 $^{A}$ | 0.35 $^{A}$ | 0.22 $^{B}$ |
| % Phosphorus | 0.06 $^{A}$ | 0.05 $^{A,B}$ | 0.07 $^{A}$ | 0.05 $^{A,B}$ | 0.01 $^{C}$ | 0.03 | 0.04 $^{B}$ | 0.04 $^{B}$ | 0.04 $^{B}$ | 0.01 $^{C}$ |
| % Magnesium | 0.06 $^{B}$ | 0.05 $^{B}$ | 0.07 $^{B}$ | 0.06 $^{B}$ | 0.02 $^{C}$ | 0.15 $^{A}$ | 0.15 $^{A}$ | 0.15 $^{A}$ | 0.15 $^{A}$ | 0.01 $^{C}$ |
| % Potassium | 0.13 $^{B}$ | 0.13 $^{B}$ | 0.09 $^{B,C}$ | 0.06 $^{C}$ | 0.01 $^{D}$ | 0.59 $^{A}$ | 0.6 $^{A}$ | 0.63 $^{A}$ | 0.61 $^{A}$ | 0.01 $^{D}$ |

DM: dry matter; ADIN: Acid detergent insoluble nitrogen; ADF: Acid detergent fiber; aNDF: amylase and sodium sulphite treated neutral detergent fibre; NFC – non-fibrous carbohydrates

ADF (*i.e.,* cellulose and lignin), aNDF (*i.e.,* hemicellulose, cellulose and lignin)

Different superscripts within each row indicate significant differences as determined using one-way ANOVA with post-hoc Turkey's test ($P < 0.05$)



In fact, more than 70% of the total nitrogen in the AFEX™-forage samples was quantified as degradable by the rumen microbes, approximately 20% higher (absolute) than for untreated controls ($P < 0.05$). This suggests that the NPN that is chemically bound to biomass can largely be utilized by the rumen bacteria for the synthesis of amino acids required to support their growth [282]. Furthermore, there were relatively small increases (< 0.15%) in the ADIN found on the AFEX™-treated forages, showing that there was comparatively little heat-damaged nitrogen resulting from the moderate-to-high temperature AFEX™ pretreatment conditions. Moreover, the total nitrogen contents of these AFEX™-treated forages were higher than those reported for traditional forages such as corn silage, but lower than alfalfa hay [34].

Unlike AFEX™ in which controlled amounts of NPN can be chemically bound to biomass during pretreatment, StEx only uses water and biomass to release organic acids that catalyse forage pretreatment [104]. Hence, the total nitrogen content of steam exploded forages did not significantly change relative to the untreated controls ($P > 0.05$). Furthermore, the rumen-degradable nitrogen content of the StEx-treated forages was lower than 59%. However, depending on their intestinal-digestibility, nitrogen in these forages that cannot be degraded in the rumen could benefit lactating dairy cows as supplemental nitrogen (and amino acids) that could be absorbed in the small intestine [283]. Otherwise, StEx-treated forages can be supplemented with a well-known, low-cost ruminant feed NPN supplement such as urea and combined with molasses (readily available from sugar refineries) to satisfy their nitrogen deficiency and to increase their palatability in ruminant diets [266].

The major structural carbohydrate contents (*i.e.* aNDF) of AFEX™-treated forages was 10 -32% lower for both sugarcane bagasse and CLM forages relative to untreated controls ($P < 0.05$). The aNDF reduced with increasing AFEX™ pretreatment severity, demonstrating greater cleavage of cell wall ester-linkages by hydrolysis and ammonolysis-type reactions with increasing AFEX™ pretreatment severity, as previously reported for other agricultural grasses [34,123,253,270]. Similarly, StEx-treated sugarcane bagasse and CLM forages were characterized by lower aNDF and higher ADF relative to the untreated controls ($P < 0.05$), primarily due to the removal of a substantial portion of the hemicellulose

carbohydrates and organic acids into a separated liquid stream and increase in the lignin content in the pretreated solids. Similar reductions in the NDF contents of steam pretreated sugarcane bagasse have been reported in literature [269].

### 5.3.2 *In- vitro true digestibility and forage energy value*

Both AFEX™ and StEx significantly improved the aNDF digestibility and the *in-vitro* true digestibility of sugarcane bagasse and CLM forages relative to untreated controls ($P < 0.05$), as seen in Table 5.4. For sugarcane bagasse, StEx-treated bagasse (508 g/kg dry forage) gave the most fiber digested after 24 h and 48 h rumen fluid incubation time, relative to the AFEX™-treated and untreated bagasse samples ($P < 0.05$). In contrast, low severity AFEX™-treatment (511 g/kg dry forage) and StEx-treated (526 g/kg dry forage) resulted in the highest fiber digestion of the sugarcane CLM forages after 48 h incubation time. The difference between the latter two forages was statistically insignificant ($P > 0.05$).

Sugarcane CLM forages (untreated and StEx/AFEX™ pretreated) generally displayed higher extents and rates of IVTD relative sugarcane bagasse forages ($P < 0.05$). AFEX™ pretreatment of sugarcane CLM increased the $IVTD_{48}$ (after 48 h of rumen fluid incubation) from 490 g/kg dry forage to 787-830 g/kg dry forage, depending on the pretreatment conditions employed. This is equivalent to an increase of 60–69% relative to untreated controls. Similarly, StEx pretreatment of sugarcane CLM forages increased the $IVTD_{48}$ by an equivalent of 267 g/kg dry forage (or 54%), however this ultimate digestibility was obtained more slowly than for the AFEX™-treated CLM forages. Considering sugarcane bagasse, AFEX™- (at high severity, Condition 3) and StEx-pretreatment increased the $IVTD_{48}$ from 400 g/kg dry forage to 707 and 710 g/kg dry pretreated forage relative to untreated controls (approximately 77% increase), respectively. Increases in IVTD are generally desirable as they suggest that the potential intake of these pretreated forages would be less likely be limited by gut fill relative to their untreated controls [284]. Furthermore, given that feed is unlikely to remain in the rumen for 48 h, forages with faster rates of fiber digestion or $IVTD_{24}$ (*e.g.* AFEX™-treated CLM) will probably improve voluntary DMI in high-producing ruminants more than forages with a lower rate of fiber digestion [285].

**Table 5.4:** Comparison of the neutral detergent fibre (aNDF) digestibility, in-vitro true rumen digestibility and energy content of untreated, AFEX- and StEx-treated sugarcane bagasse and sugarcane CLM. Mean values from replicates (n = 3) are presented in the table.

| Biomass | Pretreatment | aNDF Digested (g/kg dry forage) | | *In-vitro* True Digestibility (g/kg dry forage) | | TDN [a] (g/kg dry forage) | $NE_L$ [b] (MJ/kg dry forage) | ME [c] (MJ/kg dry forage) |
|---|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 24 h | 48 h | | | |
| Bagasse | Untreated/Control | 192.2 $^{H}$ | 289.4 $^{F}$ | 310.0 $^{E}$ | 400.0 $^{H}$ | 460.0 $^{G}$ | 1.48 $^{F}$ | 7.24 $^{E}$ |
| Bagasse | AFEX™ - Condition 1 | 194.9 $^{H}$ | 397.8 $^{E}$ | 430.0 $^{D}$ | 590.0 $^{F}$ | 520.0 $^{F}$ | 2.40 $^{D,E}$ | 8.26 $^{C}$ |
| Bagasse | AFEX™ - Condition 2 | 218.1 $^{G}$ | 418.0 $^{D}$ | 480.0 $^{C}$ | 626.7 $^{E}$ | 555.0 $^{E}$ | 2.91 $^{C,D}$ | 8.39 $^{C}$ |
| Bagasse | AFEX™ - Condition 3 | 207.3 $^{G}$ | 412.3 $^{D,E}$ | 500.0 $^{C}$ | 710.0 $^{D}$ | 615.0 $^{B,C}$ | 4.43 $^{B}$ | 9.13 $^{B}$ |
| Bagasse | Steam Explosion | 277.6 $^{D}$ | 507.5$^{B}$ | 540.0 $^{B}$ | 706.7 $^{D}$ | 580.0 $^{D,E}$ | 2.91 $^{C,D}$ | 7.75 $^{D}$ |
| Cane Leaf Matter | Untreated/Control | 264.6 $^{E}$ | 311.5 $^{G}$ | 440.0 $^{D}$ | 490.0 $^{G}$ | 480.0 $^{G}$ | 1.85 $^{E,F}$ | 7.70 $^{D}$ |
| Cane Leaf Matter | AFEX™ - Condition 1 | 366.9 $^{A}$ | 510.5 $^{A,B}$ | 650.0 $^{A}$ | 806.7 $^{A,B}$ | 625.0 $^{A,B,C}$ | 4.61 $^{B}$ | 9.09 $^{B}$ |
| Cane Leaf Matter | AFEX™ - Condition 2 | 322.4 $^{B}$ | 454.0 $^{C}$ | 660.0 $^{A}$ | 786.7 $^{B,C}$ | 630.0 $^{A,B}$ | 4.90 $^{A,B}$ | 9.27 $^{B}$ |
| Cane Leaf Matter | AFEX™ - Condition 3 | 241.9 $^{F}$ | 411.0 $^{D,E}$ | 650.0 $^{A}$ | 830.0 $^{A}$ | 655.0 $^{A}$ | 5.63 $^{A}$ | 9.73 $^{A}$ |
| Cane Leaf Matter | Steam Explosion | 301.3 $^{C}$ | 526.0 $^{A}$ | 490.0 $^{C}$ | 756.7 $^{C}$ | 585.0 $^{C,D}$ | 3.52 $^{C}$ | 8.18 $^{C}$ |
| | ANOVA *p*-value | < 0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

a – TDN: Total digestible nutrients

b – $NE_l$: Net energy for lactation

c – ME: Metabolizable Energy content. Represents the estimated difference between the digestible energy in the feed and the energy lost in waste [38].

Different superscripts within each column indicate significant differences as determined using one-way ANOVA with post-hoc Turkey's test ($P < 0.05$)



StEx and AFEX™-treatment of the sugarcane residues also significantly increased the metabolizable energy (ME), the total digestible nutrients (TDN), and the net energy for lactation ($NE_l$) relative to the untreated controls ($P < 0.05$). In general, AFEX™-treated sugarcane CLM forages had greater ME, TDN, and $NE_l$ than AFEX™-treated sugarcane bagasse forages when pretreated at similar conditions. AFEX™-treatment increased the ME of sugarcane bagasse and CLM by an equivalent of 14–26% (1.01–1.89 MJ/kg) and 18–26% (1.38–2.03 MJ/kg) relative to untreated controls, respectively, depending on the pretreatment conditions ($P < 0.05$). In comparison, StEx-treated sugarcane bagasse and CLM forages only increased the ME by 7% and 6%, respectively ($P < 0.05$), relative to untreated controls.

The NDF concentration of forages in ruminant diets is closely related to stimulation of chewing activity, influencing of the ruminal pH, and promoting steady energy supply to dairy cows [268]. Still, increases in forage cell wall digestibility by rumen microorganisms and metabolizable energy through AFEX™ or StEx pretreatment could potentially benefit voluntary DMI and performance of high-producing dairy or beef cattle relative to untreated controls, as previously reported [185,268,270]. However, previous animal feed trials have demonstrated that the inclusion of steam-pretreated bagasse at elevated levels (>32%) in complete rations significantly reduces the feed palatability and voluntary DMI of beef cattle, thereby limiting its inclusion in cattle diets to intermediate levels [185]. Similarly, cattle hyper excitability has been observed when ammoniated roughages have comprised more than 50% of the ruminants ration [186]. Hence, *in-vivo* animal trial studies are required to ascertain the true effect of including AFEX™ or StEx pretreated sugarcane crop residues in ruminant diets on the level of forage inclusion in complete ration, DMI, digestibility, metabolic efficiency and ruminant performance.

### 5.3.3 *Quantification of Nitrogenous compounds*

In addition to evaluating the digestibility and energy value of pretreated forages, we evaluated the impact of pretreatment type and pretreatment conditions on the formation of Maillard-type anti-nutritional compounds [125,186]. A summary of the quantified major Maillard-type compounds (imidazole and pyrazines) and the major nitrogenous compound, acetamide, is presented in Figure 5.2. A

more complete table including nitrogenous compounds, soluble sugars, furan derivatives, carboxylic acids, aromatic compounds is available in Table S5.1 (Supplementary Information).

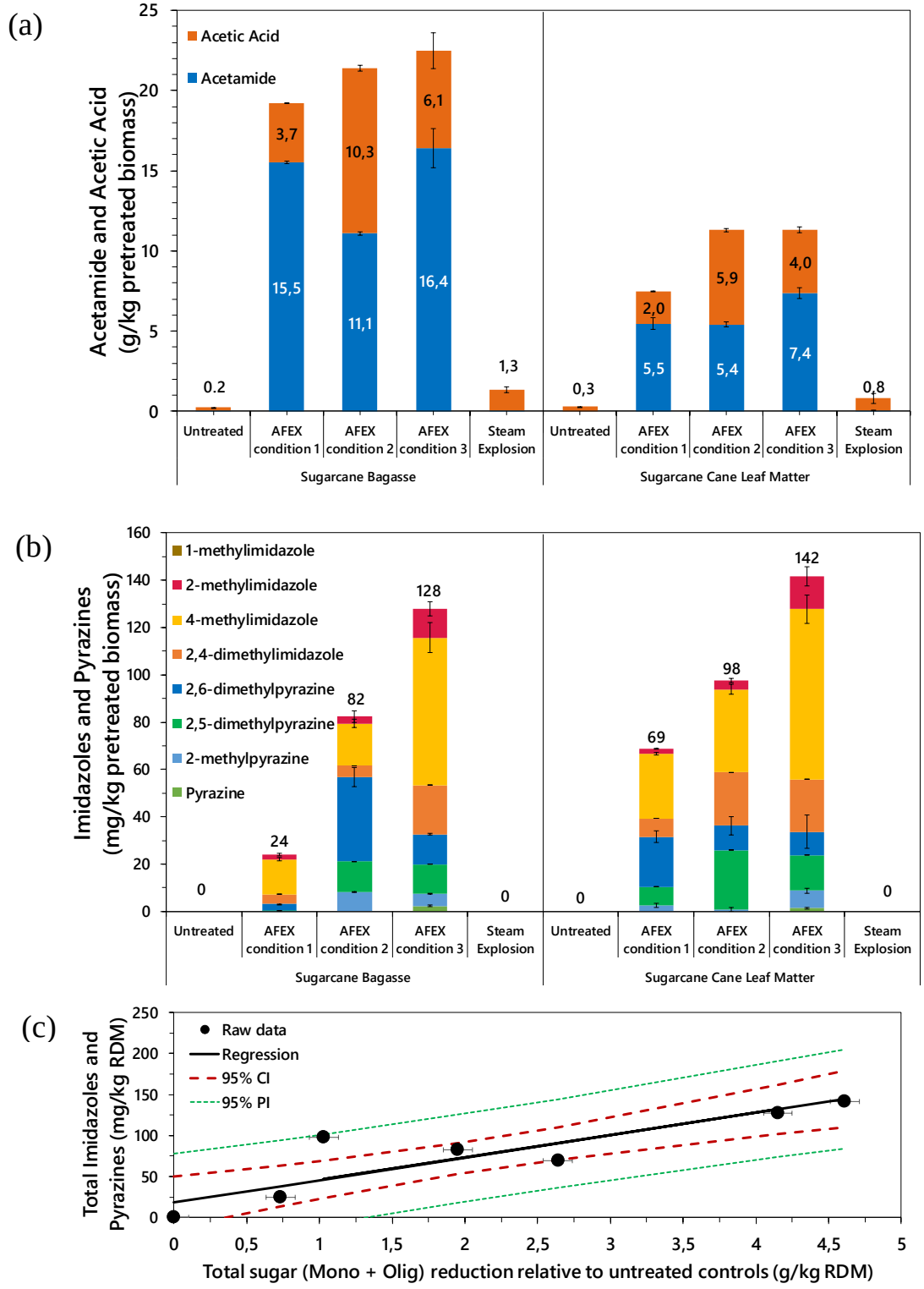



**Figure 5.2:** Quantification of nitrogenous compounds for untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM. (**a**) - acetamide and Acetic acid yields, (**b**) - Imidazole and pyrazine yields, (**c**) - correlation between total imidazole and pyrazine production as a function of the reduction in the soluble sugar (glucose, xylose, arabinose, galactose, fructose, sucrose) content in AFEX™ pretreated bagasse and CLM. The regression (black line), 95% confidence interval (red line), and 95% prediction interval (green line) are shown in the figure.

Most of the additional nitrogen quantified from AFEX™-treated forage extracts was in the form of acetamide, consistent with previous findings for other AFEX™-treated agricultural grasses [78,271]. Whilst, it is expected that some residual ammonia may have remained bound to the biomass even after drying, acetamide accounted for more than 11.1 g/kg dry forage and 5.4 g/kg dry forage for AFEX™-sugarcane bagasse and sugarcane CLM, respectively (Figure 5.2a). In AFEX™ pretreatment, acetyl side chain groups are cleaved by competing hydrolysis and ammonolysis reactions, which result in the formation of acetic acid and acetamide, respectively [136]. AFEX™-treated sugarcane bagasse extracts consisted of higher yields of acetic acid and acetamide relative to CLM. This was primarily due to untreated sugarcane bagasse being composed of higher acetyl group content relative to untreated CLM [267]. Furthermore, extracts from AFEX™ bagasse and CLM pretreated at pilot-scale (Condition 2) consisted of larger amounts of acetic acid and lower acetamide relative to extracts from AFEX™ forages at Conditions 1 and 3. Pilot-scale AFEX™ pretreatment is typically performed with an initial pre-steaming step which is absent from the bench-scale scale AFEX™ pretreatment set-up (Conditions 1 and 3). Pre-steaming the forages can contribute to the hydrolysis of acetyl-linkages at the biomass-moisture contact layer, thereby forming acetic acid, prior to the activation of competing acetamide-forming ammonolysis reactions after the addition of ammonia. In contrast, since the StEx forages were washed after pretreatment, likely removing acetic acid and acetamide, only relatively small increases in acetic acid (< 1.3 g/kg dry forage) and acetamide (< 0.032 g/kg dry forage) were found in both StEx-pretreatment bagasse and CLM extracts.

Eight substituted Maillard-derived imidazoles and pyrazines were also quantified from acetone extracts of untreated, AFEX™-treated and StEx treated bagasse and CLM forages. Major imidazole derivatives quantified were 4-methylimidazole, 2,4-dimethylimidazole and 2-methylimidazole, whilst 2,5-methylpyrazine and 2,6-methyl pyrazine were among the major pyrazine derivatives (Figure 5.2b). Imidazole and pyrazine derivatives form via the temperature



dependent condensation of ammonia with reducing sugars present in untreated forages [286]. An increasing total amount of imidazoles and pyrazines was measured with an increase in AFEX™ pretreatment severity (high temperature and high ammonia to biomass loading), with AFEX™-CLM forages (69–142 mg/kg dry forage) producing more total Maillard products in their extracts relative to AFEX™-bagasse forages (24–128 mg/kg dry forage). In particular, 4-methylimidazole was found at 15-71 mg/kg dry forage, depending on the AFEX™-treatment condition and the treated sugarcane residue. Even though only eight substituted Maillard compounds were quantified in this work, previous work has reported the presence of phenolic amides and a wider array of imidazoles and pyrazines [123].

In comparison, the total imidazole and pyrazine derivatives yields previously reported for AFEX™-corn stover was 945 mg/kg dry forage when pretreated at 1 g $NH_3$ per g dry forage and 130 °C [123]. The most likely reason for this variation can be explained by the higher initial soluble sugar content in corn stover, particularly fructose, relative to the sugarcane forages used in this work. Besides, we found a significant correlation ($R^2$ = 0.878, $P$ < 0.002) between the total yield of imidazoles and pyrazine derivatives and the difference in the soluble sugar (glucose, fructose, and sucrose) content in AFEX™-treated sugarcane bagasse and CLM forages relative to untreated controls (Figure 5.2c). Given the reported anti-nutritional nature of Maillard compounds on cattle, the quantification of cell wall nitrogenous compounds demonstrates that reduced AFEX™ pretreatment severity can reduce the yield of Maillard-type compounds formed during pretreatment. However, the true effect of these Maillard-type products will depend on their concentration on the forage, the level of AFEX™-treated forage inclusion in complete cattle rations, and their potential presence in animal products (*e.g.* dairy milk).



## 5.3.4 *Ethanol Production*

### 5.2.4.5 *Low solids loading enzymatic hydrolysis*

The efficacy of pretreatment conditions on the degree of forage digestibility for ethanol production has been traditionally determined using a standard assay based on fungal enzymes. It has been postulated that the same pretreatment conditions optimized for ethanol production would be suitable for maximizing fiber digestion by rumen microbes [34]. Within the investigated pretreatment ranges, AFEX™ pretreatment at Condition 3 and StEx resulted in the highest sugar (monomeric and oligomeric) release from low solids loading enzymatic hydrolysis for both sugarcane bagasse and CLM (Figure 5.3a) ($P > 0.05$). When the release of major structural carbohydrates (monomeric and oligomeric glucose and xylose) from a 48-h low solid loading enzymatic hydrolysis assay was correlated with the fiber digestion by rumen fluid (48-h incubation period), the enzymatic hydrolysis assay could explain 70.5% of the variability in ruminant fiber digestion with changing forage pretreatment conditions (Figure 5.3-b, $P < 0.001$).

In general, low solids loading enzymatic hydrolysis assays do not include solubilized lignin and other minor cell wall decomposition products that are hydrolyzed during the assay (including arabinan oligomers, amides, organic acids and hydroxycinnamic acids) [78,287]. Hence, the inclusion of these products can potentially increase the enzymatic hydrolysis and rumen digestion correlation. Nevertheless, these results suggest that the standard enzymatic hydrolysis assay may be a practical tool for further screening of pretreatment conditions to maximize sugar yields for ethanol production and fiber digestion by rumen fluid.

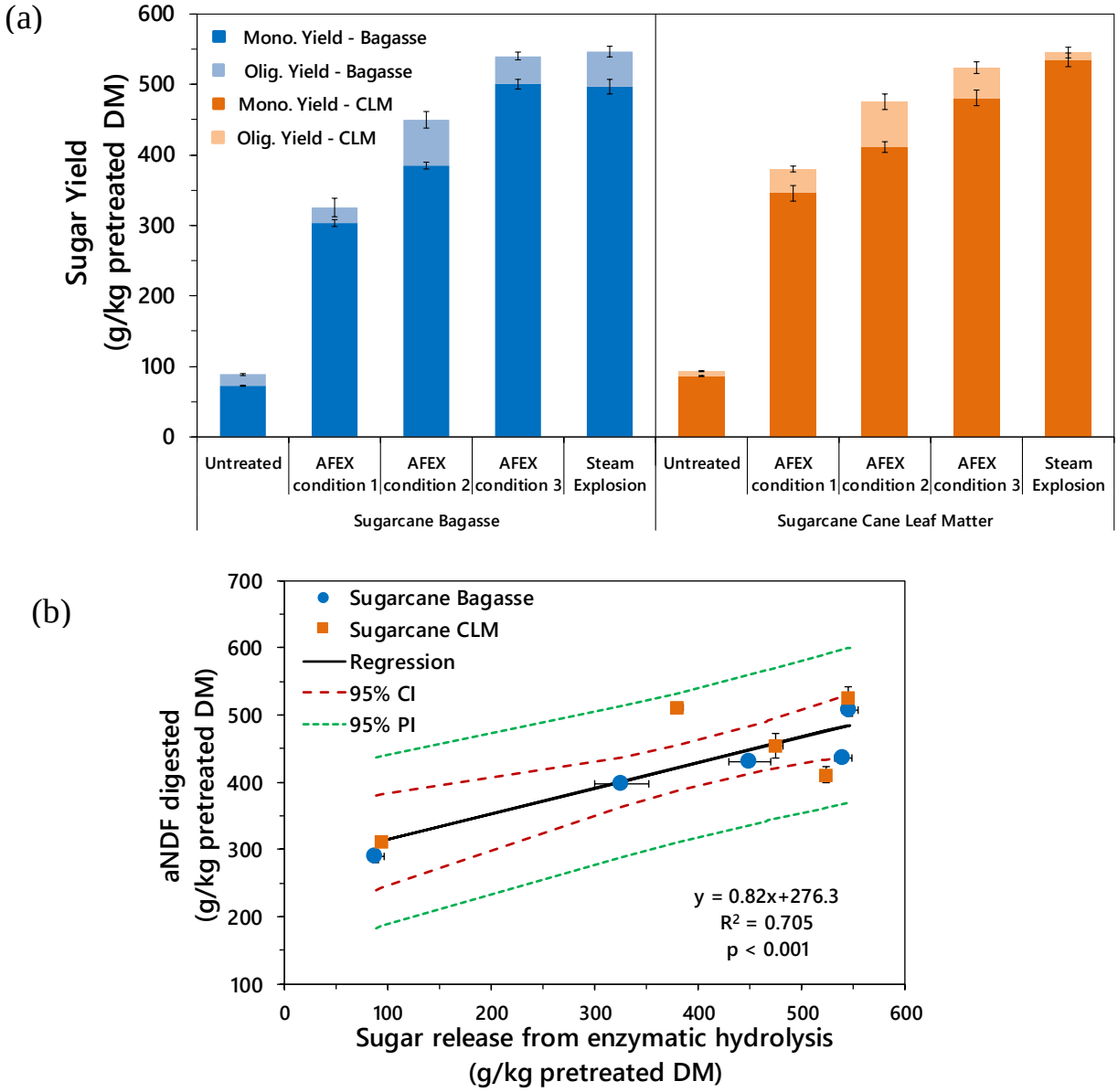



**Figure 5.3: a** – Total sugar released (monomeric and oligomeric glucose and xylose) from low solids loading enzymatic hydrolysis with commercial fungal enzymes at various pretreatment conditions. **b** – Correlation between sugar release from fungal enzymes and the neutral detergent fiber digestion by rumen fluid from untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM. The regression (black solid line), 95% confidence interval (red dotted line), and 95% prediction interval (green dotted line) are presented in the figure.

### 5.2.4.6 *High solids loading enzymatic hydrolysis and fermentation*

The potential ethanol yields from the various pretreated forages were evaluated in a separate hydrolysis and fermentation configuration with a 10% carbohydrate loading (polymeric glucan + xylan), 20 mg protein per g glucan enzyme dosage and an enzymatic hydrolysis residence time of 96 h (Figure 5.4).

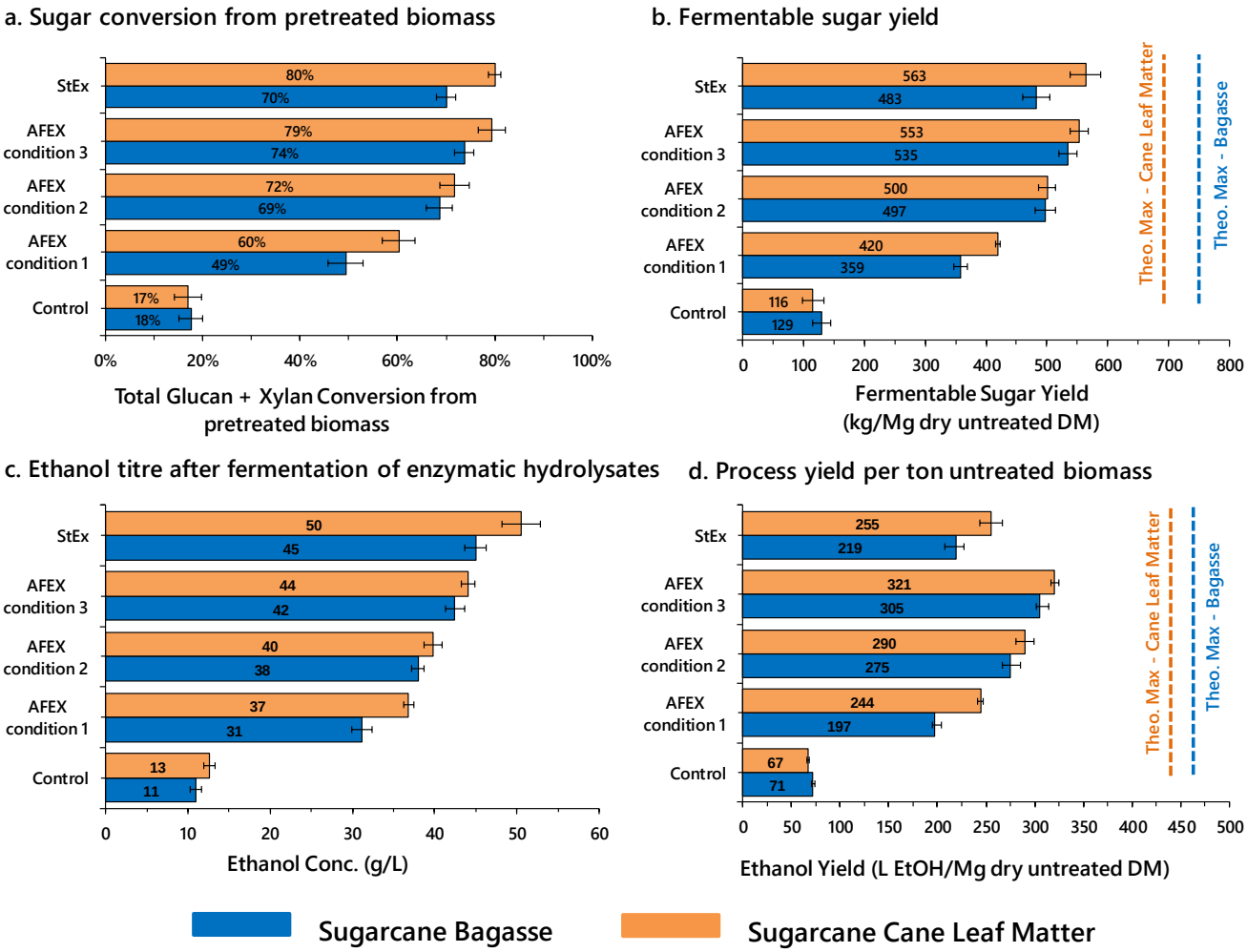



**Figure 5.4:** Comparison of ethanol production from untreated, AFEX™- and StEx sugarcane bagasse and sugarcane CLM. **a** – Total glucan and xylan conversion of pretreated biomass to monomeric glucose and xylose. **b** – Fermentable sugar yield based on one mega gram ton of untreated biomass. The fermentable sugar yield for StEx bagasse and CLM includes the sugars recovered in the pretreatment $C_5$-liquor **c** - The ethanol titre recovered after fermentation of enzymatic hydrolysates using recombinant *S. cerevisiae* 424A (LNH-ST). **d** – Total process yield for the production of ethanol per mega gram of untreated biomass input. The ethanol yield for the StEx $C_5$-liquor was adopted from Mokomele et al., [7] (**CHAPTER 4**).

Ultimately, AFEX™ pretreatment at condition 3 produced the greatest ethanol yield (305 – 321 L ethanol per ton untreated DM) for both sugarcane bagasse and CLM. This result was due a combination of: (a) higher enzymatic hydrolysis efficiency, (b) comparable carbohydrate recovery after pretreatment and high solids loading enzymatic hydrolysis with StEx, and (c) superior fermentability of the enzymatic hydrolysates of AFEX™-treated biomass by *S. cerevisiae 424A* (LNH-ST) without detoxification. Whilst the carbohydrate recovery for the StEx process was similar to

AFEX™ (Condition 3), we recently reported that the fermentability of the pretreatment liquor stream by *S. cerevisiae* 424A (LNH-ST) was strongly limited because of microbial inhibition due to pretreatment degradation products [267]. As a result, the ethanol yields for StEx were significantly lower than AFEX™ pretreatment at conditions 2 and 3 for both sugarcane bagasse and CLM ($P < 0.05$). Nonetheless, the fermentation of enzymatic hydrolysates from StEx, AFEX™ (condition 2) and AFEX™ (condition 3) each achieved ethanol titres greater than the minimum concentration of 4% (w/w) required for minimizing downstream ethanol recovery costs [288]. Recent techno-economic analysis studies have suggested that 16 – 36% of the bagasse recovered at the end of the sugar production process can be used to supplement the lignin-rich enzymatic residues to provide steam and energy for the sugar mill and the 2G ethanol production process, with the remainder being available for biofuel production [32,265,289]. Assuming an average sugarcane crop yield of 80 wet ton per hectare of cultivation area, 75% (dwb) of the available bagasse is allocated to ethanol production, and that 50% (dwb) of the CLM would be left on the field during harvesting to maintain soil fertility, we estimate that AFEX™-treated sugarcane crop residues would produce approximately 3934 and 4360 litres of ethanol per hectare of cultivation area for Condition 2 and 3, respectively (Table 5.5). In comparison, StEx-treated sugarcane crop residues would generate 3368 litres of ethanol per hectare of sugarcane cultivation area.

In addition to screening AFEX™ pretreatment conditions for high sugar and subsequent biofuel yields, energy balances and process economics are additional key considerations when evaluating AFEX™ pretreatment efficacy at various severities. Sugarcane bagasse is typically recovered from sugar mills at moisture contents greater than 50% (dwb) [264]. However, current pilot-scale AFEX™ operation is designed to receive biomass at an initial moisture content of approximately 30% (dwb) before being pre-steamed towards an optimized water loading of 0.6 to 0.7 g $H_2O$/g DM. Whilst StEx pretreatment efficiency is unlikely to be limited by the moisture content of sugar mill bagasse, previous work showed that AFEX™ treatment of high moisture bagasse required

ammonia loadings of approximately 2.0 g $NH_3$/g DM to achieve high carbohydrate enzymatic digestibility [46]. For AFEX™, the ammonia loading is known to have the greatest impact on the process economics, primarily due to costs associated with ammonia recovery and the capital cost of the AFEX™ reactor [130]. Hence, techno-economic analysis may be required to determine whether the ethanol yield or ruminant digestible energy increase achieved by increasing the AFEX™ pretreatment severity from the Condition 2 to Condition 3 offsets the additional operational and capital costs required to dry the high moisture bagasse and/or to handle higher ammonia loadings.

**Table 5.5:** Potential ethanol yields from StEx- and AFEX™-treated sugarcane bagasse and CLM forages

| **Sugarcane Crop Yield** | |
|---|---|
| Cane Yield (ton wet cane/ha) | 80.0 |
| Bagasse (kg dry fiber/ton wet cane) † | 140.0 |
| Available Bagasse (ton dry fiber/ha) ‡ | 8.4 |
| Cane Leaf Matter (kg dry fiber/ton wet cane) † | 140.0 |
| Available Cane Leaf Matter (ton dry fiber/ha) ψ | 5.6 |
| **Cellulosic Ethanol Yield (litres of Ethanol per ha cultivation area)** | |
| StEx (solids + $C_5$-liquor) ϕ | 3368 |
| AFEX™ - condition 1 | 3021 |
| AFEX™ - condition 2 | 3934 |
| AFEX™ - condition 3 | 4360 |

† - Assuming 140 kg dry fiber per ton wet cane [45]

‡ - Assuming 75% of bagasse collected from the sugar mill is allocated to biofuel production and the remainder will supplement lignin-rich enzymatic residues for energy cogeneration [32,265]

ψ - Assuming 50% of CLM left on the field to retain soil fertility

ϕ - Ethanol yield from $C_5$-liquor adopted from section 4.3.5, **CHAPTER 4**.

### 5.3.5 *Opportunities for integrated biofuel-livestock production systems*

In the future, increased agricultural production will be met through improved land productivity as technology advances and pasture and herd management practices are improved [15]. The sustainable intensification of the available pasture land and sugarcane land towards the co-production of livestock feeds and biofuels presents an example of an integrated system that can potentially take advantage of the synergies that exist between food and bioenergy production [5]. In Brazil, it has been suggested that intensification of the livestock industry could help reduce pressure



on Amazon deforestation and liberate land for expanded sugarcane and/or soybean production, while meeting the beef demand up to at least 2040 [37,290,291].

In this work, we demonstrated that well-studied technologies, AFEX™ and StEx, can significantly increase the digestibility of sugarcane crop residues for animal and biofuel production. Sugarcane crop residues represent nearly two-thirds of the mass of the sugarcane crop. Hence, establishing biorefineries for upgrading of sugarcane crop residues for the co-production of ruminant feeds and biofuels presents an opportunity for increasing sugarcane cultivation area use efficiency and enhancing the economic value of crop residues, all whilst contributing to the existing sugar and expanding bioenergy markets. Integrating and expanding food and biofuel production without the need for additional crop acreage avoids the indirect land use change (ILUC) effect with its potential for increased emission of greenhouse gases.

The cost and year-round availability of traditional forages such as corn silage, alfalfa hay and orchard grass hay are a real challenge for livestock farmers, particularly during dry seasons or during winter when fresh forages are scarce. The use of pretreated sugarcane crop residues as fodder sources in developing countries, where cereal grain supplies are limited or utilized primarily for human food, could potentially lead to higher food security and decelerate future increases in the price of meat/dairy products [292]. In addition, preliminary reports have indicated that if AFEX™-treated crop residues can replace a significant portion of high-value forages such as alfalfa, they could be sold for $50-100 per ton, significantly improving the economics of the sugarcane crop residue bio-economy [33].

A preliminary *in-vivo* animal feed study suggested that AFEX™-treated corn stover pellets could substitute for 30% of corn grain in Holstein beef steer diets without significant loss in productivity as measured by weight gain and gain efficiency [174]. Considering that corn grain is a major economic and energy constituent of ruminant diets in developing countries such as Brazil and South Africa, reducing their inclusion in animal diets could make more corn available for feeding



monogastric organisms (*e.g.* poultry) or even for human food. Moreover, since corn grain production has a higher water usage footprint than sugarcane crop production, displacing a portion of the corn grain in livestock feeds with pretreated sugarcane crop residues may have a positive overall impact on water usage footprint of livestock production [293]. On-going 12-week *in-vivo* trials substituting 30% of concentrate and 20% of green fodder with AFEX™-treated wheat straw pellets in Murrah buffalo diets have showed no detrimental health effects and no significant reduction in milk yields (unpublished data, MBI International). These results suggest that the concentrations of nitrogenous compounds produced during AFEX™ pretreatment of both corn stover and wheat straw may have no health effects on the investigated ruminants at their respective inclusion levels.

The emergence of an animal feed market based on crop residues may also help de-risk the biomass supply chain and logistics challenges facing the nascent cellulosic biofuels industry. Biomass supply chain systems for large-scale cellulosic biorefineries are mostly in their infancy. In the context of the sugarcane industry, the integration of biomass pretreatment and densification units within existing sugar mills would facilitate sharing of biomass logistics, handling infrastructure and processing utilities whilst reducing capital investment and operational overhead required for pretreatment, blending and storage of the sugarcane crop residues [57]. Upon start-up, the eventual allocation and distribution of the available pretreated sugarcane residues would be chosen by sugarcane grower participation, the sugar market, local traditional fodder availability and animal feed demand, biofuel production cost and demand, and biomass electricity market-related factors [265]. As a result, in addition to capital investment, these integrated systems would require a regulatory environment and supporting policies that would encourage sustainable and socially responsible livestock and biofuel production.

Additional integrated livestock feed-biofuel system opportunities can be realized when manure from the animal feeding operations are anaerobically digested to produce biogas at the livestock farm or at the sugar mill [294]. The generated biogas can be used as an on-site energy source



and the anaerobic digestion solids residues can be collected, combined with ash generated from the sugar mill combustion operations and incorporated into the soil in sugarcane crop fields to enhance soil fertility [224]. This strategy can potentially reduce input fertilizer costs for the sugarcane grower and generate further income and/or reduce costs for the livestock farmer. Furthermore, spent yeast generated from the biofuel fermentation process could be recovered and sold to farmers to improve the nutritive value of animal feeds by providing a source of protein having high proportions of essential amino acids [97,157]. Hence, the co-production of AFEX™ and StEx-treated forages presents a potential scenario for exploring more efficient land use for sustainably producing food (from animal products) and bioenergy.

However, the environmental and agronomic impacts of intensification on soil carbon sequestration, water usage and availability, and biodiversity should be assessed using location-specific life-cycle analyses. Nonetheless, the results presented in this work provide a basis for further technical assessments (*in-vivo* animal feed trials) with AFEX™ or StEx pretreated sugarcane crop residues and the subsequent optimization of the economic, environmental, and social impacts of integrated sugarcane-biofuels-livestock production systems to determine their overall potential for meeting local and national needs for food and energy.

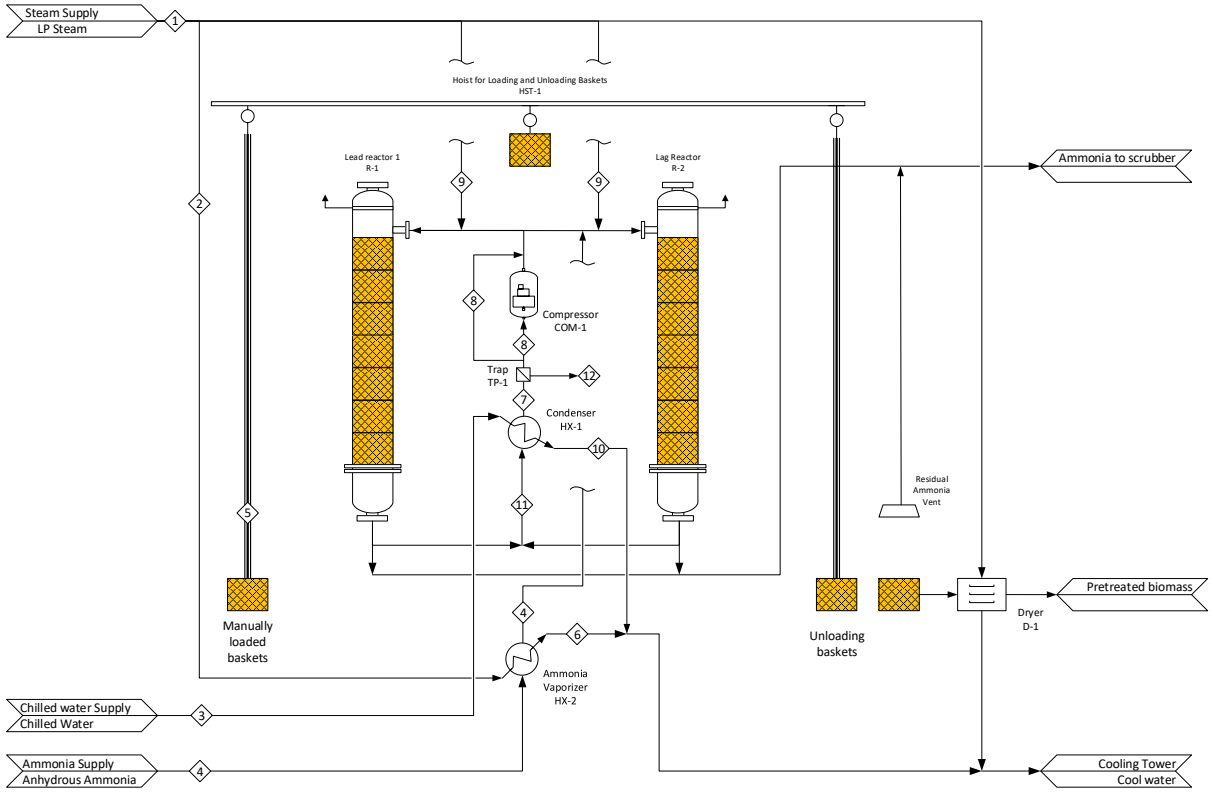
## 5.4 Conclusions

In this work we demonstrated that both AFEX™ and StEx pretreatment of sugarcane crop residues resulted in improved ruminant feeds, as measured by the *in-vitro* true digestibility and metabolizable energy, relative to their untreated controls. Further, the total nitrogen content of AFEX™-treated sugarcane bagasse and CLM forages increased to more than 20.2 g/kg dry forage, with acetamide quantified as the major nitrogenous compound generated by the pretreatment. In addition, StEx and AFEX™ pretreatment significantly increased the enzymatic digestibility of both sugarcane crop residues, resulting in an estimated 3881 and 5214 litres of cellulosic ethanol per hectare of sugarcane cultivation area at industrially relevant conditions, respectively.

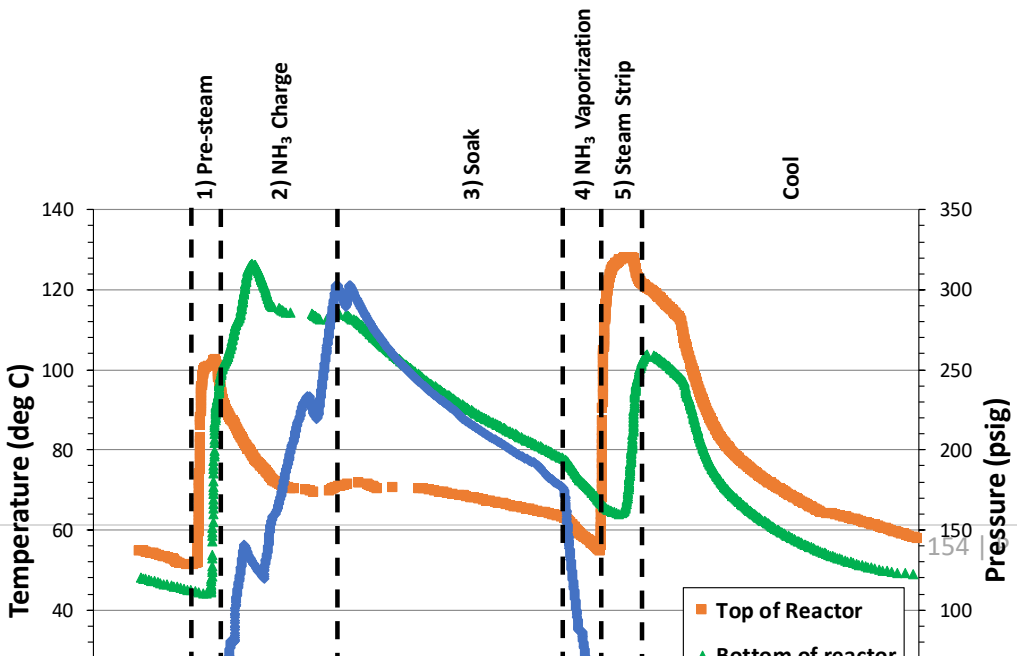
Given the synergies between the sugarcane production chains for biofuels and livestock production, simultaneously enhancing the ruminant digestible energy content and ethanol yields of AFEX™ and StEx treated forages presents an opportunity for more efficient land use for sustainably producing food (animal feed) and bioenergy. However, future research dedicated to *in-vivo* animal feed trials and economic and life-cycle assessments are required to establish or perhaps maximize the full potential of integrated sugarcane livestock-biofuel systems.

## 5.5 Supplementary Information – CHAPTER 5

(a)




(b)



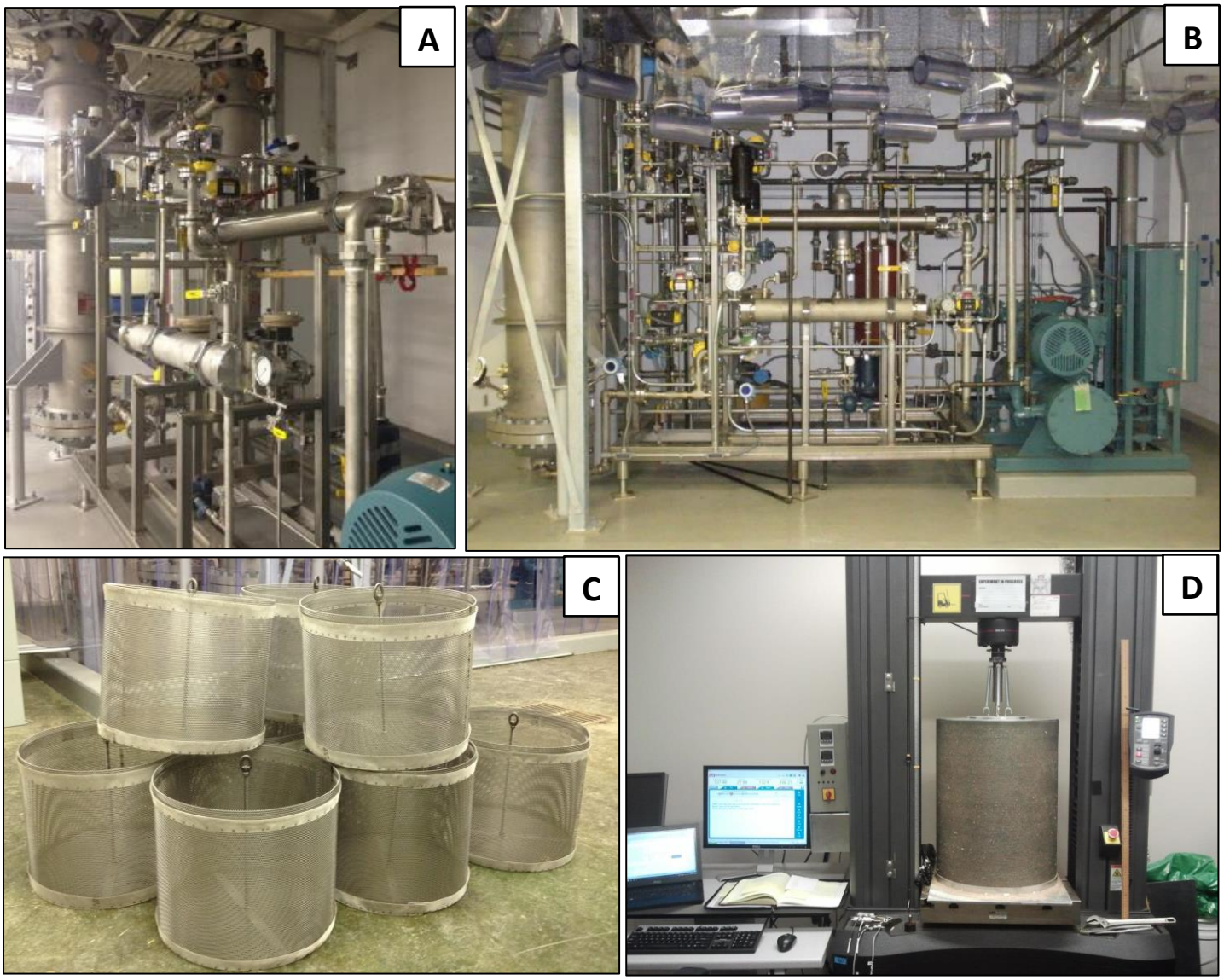



***Figure S5.2: (A)*** Side-view of MBI's two vertical 450-L packed-bed AFEX pilot-scale reactor system with seven basket holding capacity and a one-ton per day throughput; **(B)** Front-view of AFEX™ reactor system; **(C)** stainless steel baskets for loading biomass; **(D)** Instron®5900 Universal testing system for evaluating the maximum biomass packing density into the stainless-steel



***Table S5.1-A:*** Quantification of nitrogenous compounds, furan derivatives, organic acids, aromatic compounds and soluble sugars from untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM. Data in the table represent the mean values (n = 3).

| Cell-wall decomposition products | Sugarcane Bagasse (mg analyte/kg dry forage) | | | | | Sugarcane Cane Leaf Matter (mg analyte/kg dry forage) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Untreated | AFEX™ condition 1 | AFEX™ condition 2 | AFEX™ condition 3 | Steam Explosion | Untreated | AFEX™ condition 1 | AFEX™ condition 2 | AFEX™ condition 3 | Steam Explosion |
| ***Nitrogenous compounds (total)*** | **27** | **15,554** | **11,186** | **16,531** | **32** | **7** | **5,545** | **5,517** | **7,498** | **47** |
| Pyrazine | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 |
| 2-methylpyrazine | 0 | 0 | 8 | 5 | 0 | 0 | 2 | 1 | 7 | 0 |
| 2,5-dimethylpyrazine | 0 | 0 | 22 | 12 | 0 | 0 | 8 | 25 | 15 | 0 |
| 2,6-dimethylpyrazine | 0 | 3 | 35 | 13 | 0 | 0 | 21 | 10 | 10 | 0 |
| 1-methylimidazole | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2,4-dimethylimidazole | 0 | 4 | 5 | 21 | 0 | 0 | 8 | 23 | 22 | 0 |
| 4-methylimidazole | 0 | 15 | 18 | 62 | 0 | 0 | 27 | 35 | 71 | 0 |
| 2-methylimidazole | 0 | 2 | 3 | 12 | 0 | 0 | 2 | 4 | 14 | 0 |
| Acetamide | 27 | 15,529 | 11,094 | 16,404 | 32 | 7 | 5,476 | 5,419 | 7,358 | 47 |
| ***Furans Derivatives (total)*** | **8** | **1** | **4** | **4** | **462** | **16** | **6** | **6** | **11** | **403** |
| 5-Hydroxymethylfurfural | 2 | 1 | 4 | 4 | 209 | 9 | 6 | 6 | 11 | 184 |
| Furfural | 6 | 0 | 0 | 0 | 252 | 8 | 0 | 0 | 0 | 220 |
| ***Carboxylic Acids (total)*** | **221** | **3,691** | **10,310** | **6,103** | **1,335** | **300** | **2,035** | **5,915** | **3,972** | **781** |
| Acetic Acid | 211 | 3,680 | 10,302 | 6,096 | 1,317 | 291 | 2,022 | 5,898 | 3,964 | 769 |
| Levulinic Acid | 10 | 11 | 8 | 7 | 18 | 9 | 13 | 18 | 8 | 12 |
| Lactic Acid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ***Aromatic Compounds (total)*** | **9** | **277** | **884** | **454** | **738** | **8** | **344** | **678** | **344** | **490** |
| Vanillin | 9 | 68 | 57 | 48 | 316 | 8 | 15 | 22 | 25 | 289 |
| Vanillic Acid | 0 | 11 | 77 | 79 | 422 | 0 | 5 | 77 | 88 | 201 |
| Ferulic Acid | 0 | 91 | 273 | 183 | 0 | 0 | 127 | 62 | 94 | 0 |
| *p*-Coumaric Acid | 0 | 107 | 477 | 144 | 0 | 0 | 197 | 517 | 136 | 0 |

***Table S5.1-B:*** Quantification of nitrogenous compounds, furan derivatives, organic acids, aromatic compounds and soluble sugars from untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM. Data in the table represent the mean values (n = 3).

| Cell-wall decomposition products | Sugarcane Bagasse (mg analyte/kg dry forage) | | | | | Sugarcane Cane Leaf Matter (mg analyte/kg dry forage) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Untreated | AFEX™ condition 1 | AFEX™ condition 2 | AFEX™ condition 3 | Steam Explosion | Untreated | AFEX™ condition 1 | AFEX™ condition 2 | AFEX™ condition 3 | Steam Explosion |
| ***Soluble Sugars (total)*** | **11,201** | **10,940** | **9,660** | **8,060** | **9,820** | **20,600** | **18,870** | **20,020** | **17,300** | **12,620** |
| Glucose (monomers + oligomers) | 3,380 | 3,340 | 3,320 | 1,870 | 4,510 | 6,410 | 5,050 | 5,520 | 4,630 | 5,210 |
| Xylose (monomers + oligomers) | 3,650 | 3,210 | 1,910 | 1,420 | 2,780 | 4,040 | 3,610 | 3,720 | 3,630 | 3,160 |
| Arabinose (monomers + oligomers) | 0 | 270 | 410 | 1,010 | 0 | 0 | 1,010 | 1,210 | 1,750 | 0 |
| Fructose + sucrose | 4,171 | 3,920 | 4,000 | 3,760 | 2,530 | 10,150 | 9,200 | 9,570 | 7,690 | 4,250 |
| ***Total soluble compounds (µg/g DM)*** | **11,201** | **12,865** | **9,660** | **11,840** | **9,820** | **22,106** | **18,670** | **21,975** | **16,300** | **12,620** |

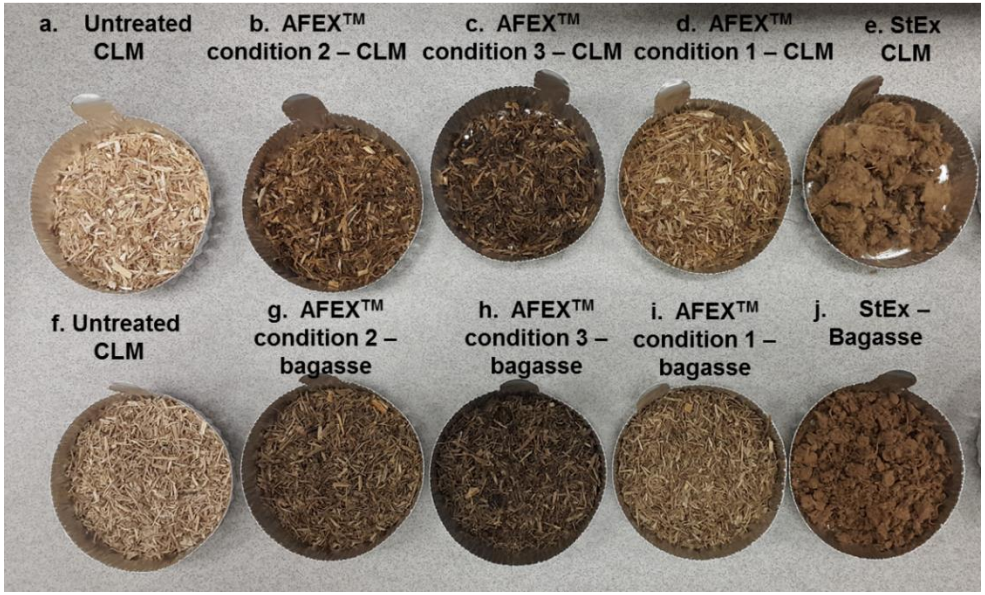



**Figure S5.3:** Physical appearance of untreated, AFEX™- and StEx-treated sugarcane bagasse and CLM.

# CHAPTER SIX:

# CONTRIBUTION 3

**Published in:** *Bioresource Technology,* 272: 326-336 (2019). ISI 5-year Impact Factor = 5.978

**Title:** Incorporating anaerobic co-digestion of StEx or ammonia fiber expansion pretreated sugarcane residues with manure into sugarcane-based bioenergy-livestock nexus.

**Authors:** Thapelo Mokomele, Leonardo da Costa Sousa, Venkatesh Balan, Eugéne van Rensburg, Bruce E. Dale, and Johann F. Görgens



## Objective of dissertation and summary of findings in chapter

This chapter addresses the research objectives highlighted in **contribution 3. CHAPTER 5** revealed that both AFEX™ and StEx are candidate processes for simultaneously producing biofuel feedstocks and animal feeds from sugarcane residues. For sugarcane and livestock dense regions, the use of both technologies has the potential to enhance agricultural output from sugarcane cultivation, particularly in intensive livestock feeding systems. This chapter expands on the integrated biofuel-livestock production system to investigate the potential benefit of co-digesting StEx- or AFEX™-pretreated sugarcane residues with livestock manure as a bioenergy production and manure management strategy for livestock farms located in sugarcane dense regions.

The structural characterization of AFEX™ pretreated SCB and CLM showed significant cleavage of ether-linked hemicelluloses and ester-linked lignin carbohydrate crosslinks. In addition to these structural alterations, AFEX™-treated sugarcane residues were characterised by near optimal C/N, resulting in enhanced anaerobic biodegradation rates and methane yields 299 L $CH_4$/kg VS, with or without co-digestion with manure. In contrast, high methane yields from StEx or untreated SCB and CLM could only be accomplished by co-digesting with manure to achieve C/N ratios in the range 18-35. Furthermore, solids digestates recovered after the co-digestion of untreated, AFEX™- or StEx-

treated sugarcane residues with animal manure were significantly enriched in macronutrients, suggesting that they could be used as partial replacements for the CLM that is typically left on the field during green harvesting.

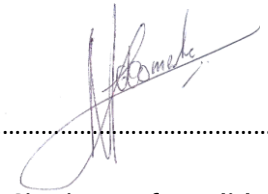
## Candidate declaration

With regards to **Chapter 6**, pages 189 – 219 of this dissertation, the nature and scope of my contributions were as follows:

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental planning | 100 |
| Executing experiments | 100 |
| Intepretation of experiments | 70 |
| Writing of chapter | 100 |

The following co-authors have contributed to **Chapter 6**, pages 189 – 219 of this dissertation:

| Name | e-mail address | Name of contribution | Extent of contribution (%) |
|---|---|---|---|
| Leonardo da Costa Sousa | sousaleo@egr.msu.edu | ✓Intepretation of experiments<br>✓Review of chapter | 10<br>20 |
| Venkatesh Balan | vbalan@uh.edu | ✓Review of chapter | 20 |
| Eugéne van Rensburg | eugenevrb@sun.ac.za | ✓Review of chapter | 20 |
| Bruce E. Dale | bdale@egr.msu.edu | ✓Review of chapter<br>✓Co-ordination of collaboration | 20<br>50 |
| Johann F. Görgens | jgorgens@sun.ac.za | ✓Intepretation of experiments<br>✓Review of chapter<br>✓Co-ordination of collaboration | 20<br>20<br>50 |



…… October 2018 ……

**Signiture of candidate** **Date**

### Declaration by co-authors

All authors read and approved the final manuscript and hereby confirm that:

III. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to **Chapter 6,** pages 189 – 219 in the dissertation,

IV. no other authors contributed to **Chapter 6,** pages 189 – 220 in the dissertation beside those specified above, and

V. potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in **Chapter 6,** pages 189 – 219 of the dissertation.

# Incorporating anaerobic co-digestion of steam exploded or AFEX™ pretreated sugarcane residues with manure into a sugarcane-based bioenergy-food nexus

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## Abstract

The aim of this study was to evaluate and compare the potential benefits of co-digesting pretreated sugarcane lignocelluloses with dairy cow manure (DCM) as a bioenergy production and waste management strategy for intensive livestock farms located in sugarcane dense regions. Ammonia fiber expansion (AFEX™) increased the nitrogen content and accelerated the biodegradability of sugarcane bagasse (SCB) and cane leaf matter (CLM) through the cleavage of lignin carbohydrate crosslinks, resulting in the highest specific methane yields (292 – 299 L $CH_4$/kg $VS_{added}$), biogas methane content (57 – 59% v/v) and biodegradation rates, with or without co-digestion with DCM. To obtain comparable methane yields, untreated and steam exploded (StEx) SCB and CLM had to be co-digested with DCM, at mass ratios providing initial C/N ratios in the range of 18 to 35. Moreover, co-digestion with DCM improved the nutrient content of the solid digestates, suggesting that these digestates could potentially be used as biofertilizer to replace a fraction of CLM that is typically left on sugarcane fields during green harvesting.

**Keywords:** AFEX™, Steam explosion, Anaerobic Co-digestion, Sugarcane residues, Dairy cow manure; Methane yield

## 6.1 Introduction

Current and future trends demonstrate that the increasing world population, dwindling arable land and increased demand for renewable energy present an opportunity to relook the way land is used to meet the future food, feed and bioenergy demands [15]. With livestock production representing the largest anthropic use of global agricultural land, the adoption of intensified of livestock farming practices (increased livestock per unit area) has been touted as a strategy for improving land use efficiency for food and bioenergy production, reducing deforestation, and enhancing economic returns for livestock farmers [36,204,223].

Recent studies have introduced a biorefinery concept whereby biomass pretreatment technologies are integrated into existing industrial sites (*e.g.* sugar/ethanol mills) for the production of conversion-ready biofuel feedstocks and highly digestible ruminant animal feeds from sugarcane crop residues [11,36]. Our recent work showed that pilot-scale ammonia fiber expansion (AFEX™) and steam explosion (StEx) were effective treatments for simultaneously enhancing the *in-vitro* true digestibility and fungal enzyme degradability of sugarcane bagasse (SCB) and cane leaf matter (CLM) for ruminant feeding and ethanol production at industrially relevant conditions, respectively [35]. This concept is of particular interest to sugarcane and livestock dense regions such as Brazil, where an estimated 210 million cattle head are distributed over 167 million hectares of pasture and concurrently producing more than 300 million tons of sugarcane residues per annum [5]. While the prospective adoption of intensified livestock production in these areas can increase land use efficiency, intensive livestock farming systems are typically accompanied by the production of surplus animal manure, which represents a significant pollution risk with potential negative environmental impacts [204].

Poor cattle manure management practices, particularly for intensified livestock production systems, can significantly contribute to manure odor nuisance, manure disposal challenges, pollution of ground water, spreading of pathogens, and greenhouse gas emissions [192,295]. It is well reported

that anaerobic digestion (AD) of manure alone can lead to low biogas yields due to nutrient imbalance and ammonia toxicity to methanogens [198,296]. Anaerobic co-digestion of cow manure with agricultural residues can potentially harness the synergies between the two substrates by enhancing the digestion nutrient balance (*e.g.* C/N ratio, macro- and micronutrients), improving the digestion buffer capacity and potentially mitigating inhibition challenges encountered in mono-digestion [192,218,296,297]. Furthermore, co-digestion with regionally available residues produces farmer-owned renewable energy for on-farm use, increases the total biogas production capacity, reduces pathogens in digestate, improves the fertilizer value of the digestate, and ultimately provides a more sustainable manure management strategy [192,298]. Alternatively, depending on the proximity of the intensive animal farms to cellulosic biorefineries, the animal manure from these farms could be transported and co-digested with sugarcane residues in AD-based wastewater treatment plants that will form part of the water circuit of prospective cellulosic biorefineries.

With substrate hydrolysis considered as one of the rate-limiting steps during AD of lignocellulosic biomass, numerous pretreatment technologies (including StEx, ultrasound, NaOH pretreatment) have been employed as strategies for unlocking the recalcitrance of lignocellulosic biomass and subsequently accelerating its anaerobic biodegradability for enhanced biogas production [206,211,299–302]. Among these pretreatments, De Paoli and co-workers (2011) reported the highest methane yields for SCB by using StEx pretreatment at 200 °C to achieve modest methane yields of 258 L $CH_4$/kg VS [205]. In contrast, the combination of mechanical milling and 12% (w/w) NaOH pretreatment of CLM achieved methane yields of 291 L $CH_4$/kg VS, the highest methane yields reported in literature for CLM [207]. However, the use of high NaOH concentrations limited the potential applicability of the digestates as fertilizer or soil application due to potential long-term salinization effects.

To date, there are no literature studies evaluating the methane production potential from AFEX treated lignocelluloses, neither in mono-digestion nor co-digestion with animal manure. AFEX™,

demonstrated at pilot-scale with high ammonia recovery, has the unique characteristic of enhancing biomass biodegradability through the cleavage of ester-linked lignin carbohydrate complexes of monocots, particularly ferulates, diferulates and coumarates, whilst fixing controlled amounts of degradable nitrogen onto the biomass to achieve C/N ratios in the range 25–35 [35,140,303]. Coincidently, these C/N ratios lay within the optimum range recommended for efficient AD [304].

With the availability of SCB and CLM in sugarcane producing areas, we extend upon the integrated biofuel and livestock production concept to incorporate the anaerobic co-digestion of dairy cow manure from intensified animal feeding systems with sugarcane residues for decentralized (farm-based) or centralized biogas production (Figure 6.1).

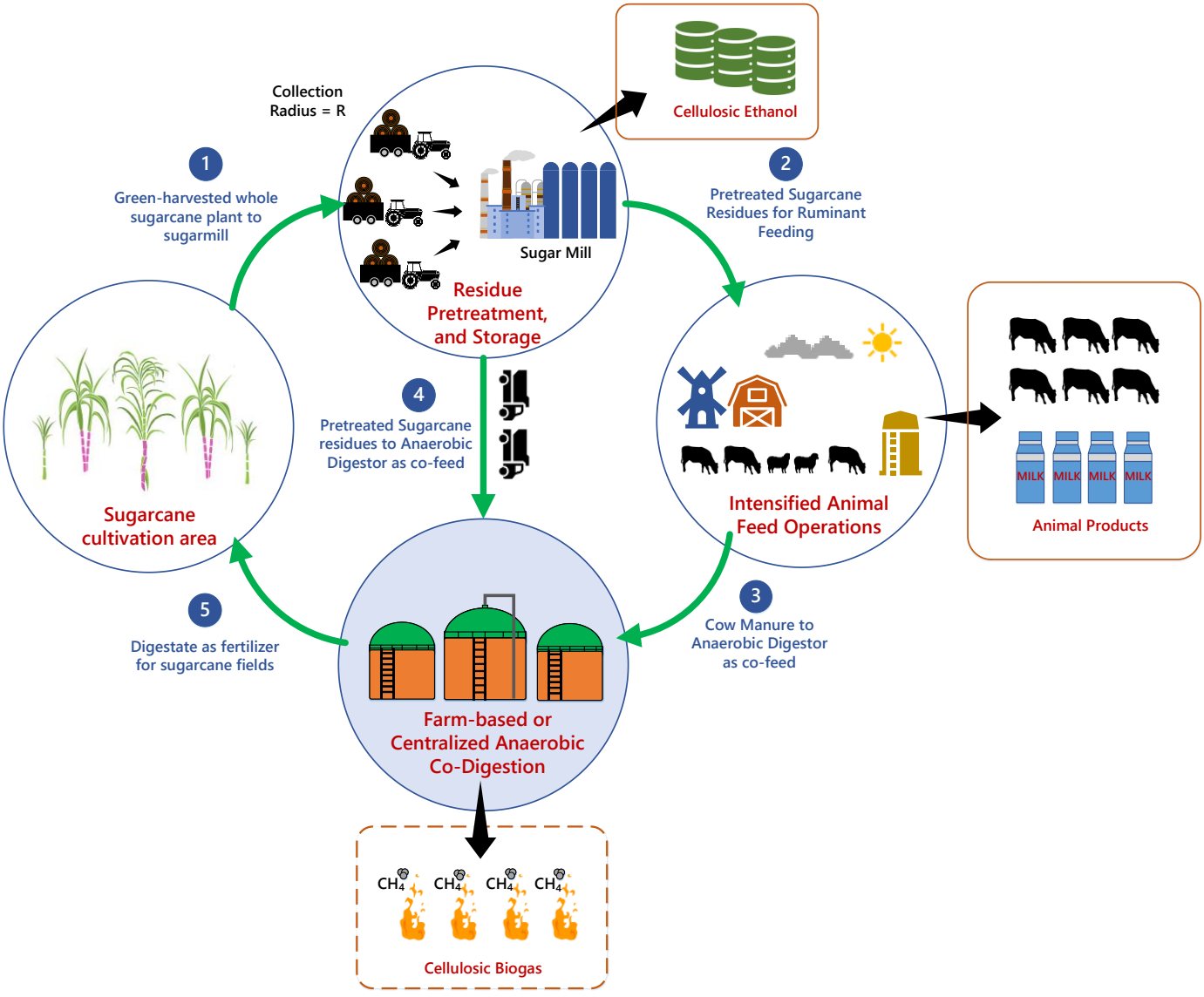



***Figure 6.1:*** Incorporating farm-based or centralized anaerobic co-digestion of sugarcane residues with livestock manure into integrated biofuel and livestock production systems for sugarcane and livestock dense regions

The aim of the present study was to experimentally assess the potential use of untreated, StEx- or AFEX™-treated of SCB and CLM as co-substrates with dairy cow manure (DCM) for high biogas production in batch anaerobic co-digestion. To achieve this, the impact of the two pretreatment technologies in mono- and co-digestion were compared in terms of cumulative methane yield, methane content, biodegradation rate and total volatile fatty acid (VFA) production. Further, an energy conversion assessment and solid digestate nutrient value was quantified for mono- or co-digestion substrates yielding specific methane yields greater than the mono-digestion of DCM, untreated SCB and untreated CLM. The results of this work provide insights into the potential use of either untreated or pretreated sugarcane residues as co-feeds with DCM for integrated livestock and biofuel production systems for bioenergy production and sustainable manure management.

## 6.2 Material and Methods

### 6.2.1 *Substrate, inoculum and cow manure*

SCB and CLM were collected in the spring season of 2014 from two sugar mills located in Malelane (TSB Sugar, South Africa) and Mount Edgecombe (SASRI, South Africa) and prepared as previously described [267]. Inoculum was collected from an active farm-based anaerobic digester (Durbanville, WC, South Africa) that readily treats swine and cow manure under mesophilic conditions (~ 37 °C). The inoculum was degassed in a 30 L continuously stirred tank reactor (CSTR) under mesophilic conditions for five days to minimize endogenous methane production from any residual biodegradable organic material collected from the active digester. Fresh DCM was collected from lactating dairy cows consuming a typical total mixed ration diet at the Stellenbosch University Dairy Farm (Stellenbosch, South Africa) and refrigerated at 4 °C until required for use.

### 6.2.2 *Steam explosion and AFEX™ pretreatment*

StEx pretreatment of SCB and CLM was carried out in an automated pilot scale unit equipped with a 19-L reaction vessel, 100-L discharge vessel and a 40-bar steam boiler (IAP GmBH, Graz, Austria). Further details on the StEx pretreatment protocol, experimental conditions and chemical composition

adopted for these materials can be found in **CHAPTER 4**. The unwashed solid fraction after StEx was used in anaerobic biodegradability assays to evaluate the biomethane potential of StEx treated sugarcane residues. AFEX™ pretreatment was performed at pilot-scale using a pair of 450-L vertical packed bed reactors at MBI International (Lansing, MI, USA) [273]. Pretreatment conditions applied included an ammonia-to-biomass loading of 0.7 g $NH_3$/g DM, 0.6 g $H_2O$/g DM moisture content, non-uniform temperature range of 120 – 80 °C, and residence time of 60 min [35]. Both SCB and CLM were pretreated at the same AFEX™ conditions.

### 6.2.3 *Batch anaerobic digestion assays*

Batch assays were conducted to evaluate the effect of StEx or AFEX™ pretreatment on the anaerobic biodegradability of SCB and CLM in mono-digestion and in co-digestion with DCM. Biomethane potential (BMP) assays were carried out in 100 mL serum bottles closed with a butyl rubber stoppers and sealed with aluminum crimps using a previously reported protocol [193]. Each assay was conducted at 6% total solids loading with an inoculum to substrate ratio (ISR) of 0.4 ($VS_{inoculum}/VS_{substrate}$). In preparation of BMP assays, the untreated and pretreated SCB and CLM samples were milled separately and passed through a 2-mm Wiley mill. The milled samples were added to the assay bottles with appropriate amounts of DCM, distilled water and inoculum to a final working volume of 70 mL. After inoculation, each assay bottle was sealed without pH adjustment, purged with $N_2$ gas for 2 minutes, and incubated at mesophilic conditions (37 ± 1 °C) for 55 days. Gas production was measured daily by volume displacement of a graduated syringe pierced through the butyl stopper, with the biogas composition quantified by gas chromatography (described below). The pH of each assay was measured before and after the BMP tests. For statistical inference, all assays were performed in triplicate.

To evaluate the effect of pretreatment and the effect of biomass-to-DCM mixing ratios, two sets of BMP assays were performed. The first set of BMP assays were performed to evaluate and compare the effect of StEx and AFEX™ pretreatment on the mono- and co-digestion methane yield, biogas

methane content, biodegradation rate, and VFA production for both SCB and CLM. For this set of assays, co-digestion of untreated and pretreated SCB and CLM were performed at a fixed biomass-to-DCM ratio of 50:50 (VS basis), with mono-digestion of the DCM, untreated and pretreated SCB and CLM samples performed in parallel. A second set of BMP assays were performed to evaluate the effect of the ratio of biomass-to-DCM ratio on the specific methane production during anaerobic co-digestion. For these assays, untreated, StEx-treated and AFEX™-treated CLM were used as the co-feeds at biomass-to-DCM mixture ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (VS basis). For both sets of BMP assays, blank and positive control assays with no substrate and microcrystalline cellulose (Avicel PH-101) were included as reference assays to determine the background methane production and inoculum methanogenic activity, respectively.

### 6.2.4 *Kinetic model analysis*

A kinetic assessment of the batch BMPs was performed to compare the extent and rate of biodegradability of the various pretreated and co-digestion assays relative to untreated mono-digestion controls. The empirical Cone model was used to fit the measured specific methane yields for the domain t ≥ 0 days as described by Equation 6.1.

$$\beta(t) = \frac{\beta_0}{1+(kt)^{-n}} \qquad \text{(Equation 6.1)}$$

In Equation 6.1, $\beta$ (L $CH_4$/kg $VS_{added}$) is the accumulated methane yield at time t; $\beta_0$ (L $CH_4$/kg $VS_{added}$) represents the maximal cumulative methane yield, $k$ ($day^{-1}$) is the biodegradation rate constant, and $n$ is the dimensionless Cone model shape constant [305].

### 6.2.5 *Analytical Techniques*

Structural carbohydrates and Klason lignin were determined according to National Renewable Energy Laboratory (NREL) protocols NREL/TP-510-42618 and NREL/TP -510-42620. The total carbon, nitrogen, hydrogen, and sulfur in biomass samples was measured by elemental analysis conducted using a Vario EL Cube elemental analyser (Elementar GmBH, Germany). The macro-mineral content (Ca, Na, Mg, P,K, Fe) in biomass samples was quantified using a Thermo iCAP 6200 ICP-AES (Thermo

Fischer Scientific, MA, USA). The biomass higher heating value (HHV) was measured using a bomb calorimeter (Cal2k Eco Calorimeter, RSA), which was previously calibrated with benzoic acid, in accordance with the ASTM standard D5865-11a. The lower heating value (LHV) was estimated from the measured HHV according to the European Standard (EN) 14918.

To qualitatively monitor functional group changes in pretreated biomass, Fourier Transform Infrared (FTIR) spectroscopic analysis of untreated, StEx- and AFEX™-treated SCB and CLM samples was performed using a Thermo-Nicolet iS10 spectrometer operating in ATR mode with a diamond crystal. Spectra were obtained with an average of 64 scans for each sample at a resolution of 4 $cm^{-1}$ in the range 650 – 4000 $cm^{-1}$ using OMNIC® software.

Crystallinity of the cellulose fibers was evaluated using a D8 Advance X-Ray diffractometer equipped with a Lynxeye detector with its beam parallelized by a Gobel mirror (Bruker AXS Inc., Madison, USA). CuKα radiation was generated at an accelerating voltage of 40 kV voltage and an electric current of 40 mA. Scans were obtained from 2θ of 8.00° to 30.03° in increments of 0.02° and a scan rate of 5°/min. The crystallinity index (CrI) was calculated according to Equation 2:

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

where $I_{002}$ is the intensity of the diffraction from the 002-lattice plane at 2θ = 22.5°, and $I_{am}$ is the intensity of diffraction at 2θ = 18.0°.

The biogas composition from BMP assays was quantified using a gas chromatograph (CompactGC4.0, Global Analyzer Solutions, Netherlands) equipped with two thermal conductivity detectors (TCD) for $CO_2$, $CH_4$, $N_2$, $O_2$, and $H_2$ quantification. Helium gas was used as the carrier gas at 5.0 mL/min and the operating temperatures of the injector, detector and column were 60 °C, 110 °C, and 65 °C, respectively. For analysis of VFAs, samples after AD were centrifuged at 10,000 rpm for 5 minutes before the supernatants filtered through a 0.22 µm filter and subjected to HPLC quantification. The quantity of each VFA was measured using a Dionex UltiMate 3000 HPLC system equipped with UV detector (Thermo Fischer Scientific, UK). The column was a Bio-Rad Aminex HPX-

87H ion exclusion column operating at 65 °C with 0.005 M $H_2SO_4$ as the mobile phase at a flowrate of 0.6 mL/min. The total VFA was calculated as the sum of the measured acetic acid, n-butyric acid, n-valeric acid, propanoic acid, and n-caproic acid.

### 6.2.6 *Energy conversion assessment*

A gross energy conversion assessment was carried out to evaluate the AD efficiency in converting the heat of combustion energy in the inlet feedstocks into biogas equivalent energy using Equation 3.

$$EC_{mixture}(\%) = \frac{V_{methane_{STP}} \times \rho_{methane_{STP}} \times LHV_{methane}}{\sum(m_i \times LHV_i)} \times 100 \quad (3)$$

where $EC_{mixture}$, $V_{methaneSTP}$, $\rho_{methaneSTP}$, and $LHV_{methane}$ represent the energy conversion factor for biogas relative to the inlet substrate mixture heat of combustion, the specific methane yield at standard temperature and pressure (STP: 273 K and 101.325 kPa), the methane density at STP (0.717 kg/m$^3$) and the net calorific value of methane at STP (50.4 MJ/kg). Similarly, $m_i$ and $LHV_i$ denotes the mass and net calorific value of dry SCB, CLM, or DCM added to the BMP assays.

### 6.2.7 *Statistical analysis*

Statistical significance of experimental results was determined through a one-way ANOVA in combination with Tukey's HSD *post hoc* test for multiple comparisons (Minitab Inc., State College, PA, USA). The null hypothesis was accepted or rejected at 95% confidence interval ($P < 0.05$). Linear regression was performed to correlate the C/N ratio of various mono-and co-digestion experiments to the specific methane yields obtained from the BMP assays in Minitab software. The accuracy and significance of the regression equation was assessed using the coefficient of determination ($R^2$) and the regression model *P* value, respectively. The estimation of the parameters of the Cone model was performed with the least squares method using the Solver Function in Microsoft® Excel and the degree of fit was quantified using the Root Mean Square Error (RMSE) and Akaike's Information Criterion (AIC) as previously described [306]. To establish parameter estimation certainty, 95% confidence intervals

of the Cone model parameters were computed using a Monte Carlo simulation approach in Microsoft® Excel as previously described [307].

## 6.3 Results and Discussion

### 6.3.1 *Substrate characteristics*

The chemical composition, macro-nutrient content and gross calorific value of the DCM, inoculum, untreated, StEx- and AFEX™-treated SCB and CLM are presented Table 6.1.

***Table 6.1:*** Total solids, volatile solids, chemical composition, macro-nutrient content, and calorific value of substrates used during anaerobic digestion assays

| Parameter | Sugarcane Bagasse | | | Cane Leaf Matter | | | Cattle Dairy Manure | Inoculum |
|---|---|---|---|---|---|---|---|---|
| | Untreated | AFEX | StEx | Untreated | AFEX | StEx | | |
| % TS (% FM) | 90.8 ± 0.8 | 90.8 ± 0.6 | 94.2 ± 0.5 | 93.3 ± 0.4 | 92.3 ± 0.8 | 92.9 ± 0.4 | 15.8 ± 1.1 | 2.7 ± 0.1 |
| % VS (% TS) | 96.1 ± 0.4 | 97.9 ± 0.3 | 96.1 ± 0.3 | 92.4 ± 0.3 | 91.9 ± 0.8 | 91.0 ± 1.1 | 83.7 ± 0.9 | 67.3 ± 1.5 |
| pH | - | - | - | - | - | - | 6.93 ± | 7.61 ± 0.0 |
| Cellulose (% TS) † | 39.5 ± 0.4 | 39.5 ± 0.4 | 59.4 ± 0.5 | 37.5 ± 0.6 | 37.5 ± 0.6 | 55.3 ± 0.4 | N/D | N/D |
| Arabinoxylan (% TS) † | 26.4 ± 0.6 | 23.4 ± 0.6 | 7.1 ± 0.7 | 27.5 ± 0.7 | 25.5 ± 0.8 | 11.0 ± 0.4 | N/D | N/D |
| Klason Lignin (% TS) † | 19.3 ± 0.1 | 16.9 ± 0.1 | 29.5 ± 0.4 | 16.2 ± 0.8 | 14.4 ± 1.1 | 27.3 ± 0.3 | N/D | N/D |
| % Carbon (C) † | 45.8 ± 0.7 | 46.3 ± 0.8 | 48.0 ± 0.2 | 43.5 ± 0.2 | 43.9 ± 0.2 | 46.1 ± 0.3 | 42.0 ± 0.5 | 33.3 ± 0.6 |
| % Nitrogen (N) | 0.30 ± 0.0 | 1.46 ± 0.1 | 0.28 ± 0.0 | 0.41 ± 0.0 | 1.55 ± 0.1 | 0.38 ± 0.0 | 2.53 ± 0.1 | 2.39 ± 0.1 |
| % Calcium (Ca) † | 0.12 ± 0.0 | 0.11 ± 0.0 | 0.05 ± 0.0 | 0.33 ± 0.0 | 0.33 ± 0.0 | 0.22 ± 0.0 | 2.47 ± 0.1 | 3.63 ± 0.1 |
| % Magnesium (Mg) † | 0.06 ± 0.0 | 0.07 ± 0.0 | 0.06 ± 0.0 | 0.15 ± 0.0 | 0.15 ± 0.0 | 0.15 ± 0.0 | 0.64 ± 0.0 | 1.81 ± 0.0 |
| % Phosphorus (P) † | 0.06 ± 0.0 | 0.07 ± 0.0 | 0.01 ± 0.0 | 0.04 ± 0.0 | 0.03 ± 0.0 | 0.02 ± 0.0 | 0.47 ± 0.0 | 2.79 ± 0.1 |
| % Potassium (K) † | 0.13 ± 0.0 | 0.13 ± 0.0 | 0.01 ± 0.0 | 0.60 ± 0.1 | 0.59 ± 0.1 | 0.01 ± 0.0 | 0.70 ± 0.1 | 2.22 ± 0.2 |
| % Sodium (Na) † | 0.01 ± 0.0 | 0.01 ± 0.0 | 0.01 ± 0.0 | 0.04 ± 0.0 | 0.04 ± 0.0 | 0.04 ± 0.0 | 0.13 ± 0.0 | 1.48 ± 0.3 |
| Sulphur (S) † | 0.05 ± 0.0 | 0.06 ± 0.0 | 0.07 ± 0.0 | 0.27 ± 0.0 | 0.21 ± 0.0 | 0.04 ± 0.0 | 0.26 ± 0.0 | 0.95 ± 0.0 |
| C/N ratio | 153 ± 3 | 32 ± 1 | 172 ± 2 | 107 ± 1 | 28 ± 2 | 121 ± 1 | 17 ± 1 | 14 ± 1 |
| HHV (MJ/kg) † | 18.5 ± 0.1 | 19.1 ± 0.0 | 19.9 ± 0.0 | 17.7 ± 0.1 | 18.3 ± 0.1 | 18.9 ± 0.1 | 17.9 ± 0.0 | 13.8 ± 0.1 |
| LHV (MJ/kg) † | 17.1 ± 0.1 | 17.7 ± 0.0 | 18.6 ± 0.0 | 16.4 ± 0.1 | 17.0 ± 0.0 | 17.6 ± 0.0 | 16.6 ± 0.0 | 12.7 ± 0.0 |

N/D – not determined; TS – Total Solids; VS – Volatile solids; FM – fresh matter;

† - % TS basis

AFEX™-pretreatment significantly increased the nitrogen content of both SCB and CLM, resulting in substrates with C/N ratios of 32 and 28, respectively. The nitrogen chemically linked onto the biomass via ammonolysis, hydrolysis and Maillard-type reactions predominantly exists as acetamide and phenolic amides, which are readily degradable by dairy cattle rumen microbes for bacterial protein synthesis [35,308]. In contrast, the high temperature StEx pretreatment resulted in

the solubilization of 30-40% of the initial dry matter (mostly hemicelluloses and water/acid soluble extractives) into a liquor stream that was removed prior to AD [267]. Accordingly, the StEx-SCB and StEx-CLM substrates were enriched in cellulose (> 55%) and Klason lignin (> 27%) contents, and subsequently characterized by C/N ratios in the range 120-170. Further, unlike the untreated and AFEX™-treated substrates, StEx-treated SCB and CLM demonstrated lower S, Ca, K and P contents, suggesting that these macro-nutrients were water soluble and therefore partially extracted into the liquid phase during StEx pretreatment.

### 6.3.2 *Structural characterization of StEx/AFEX™-treated SCB and CLM*

To further investigate the structural modifications to SCB and CLM after StEx/AFEX™ pretreatment, comparison of the changes in the characteristic functional groups and crystallinity index relative to untreated controls were performed by ATR-FTIR and XRD analyses, respectively (Figure 6.2).

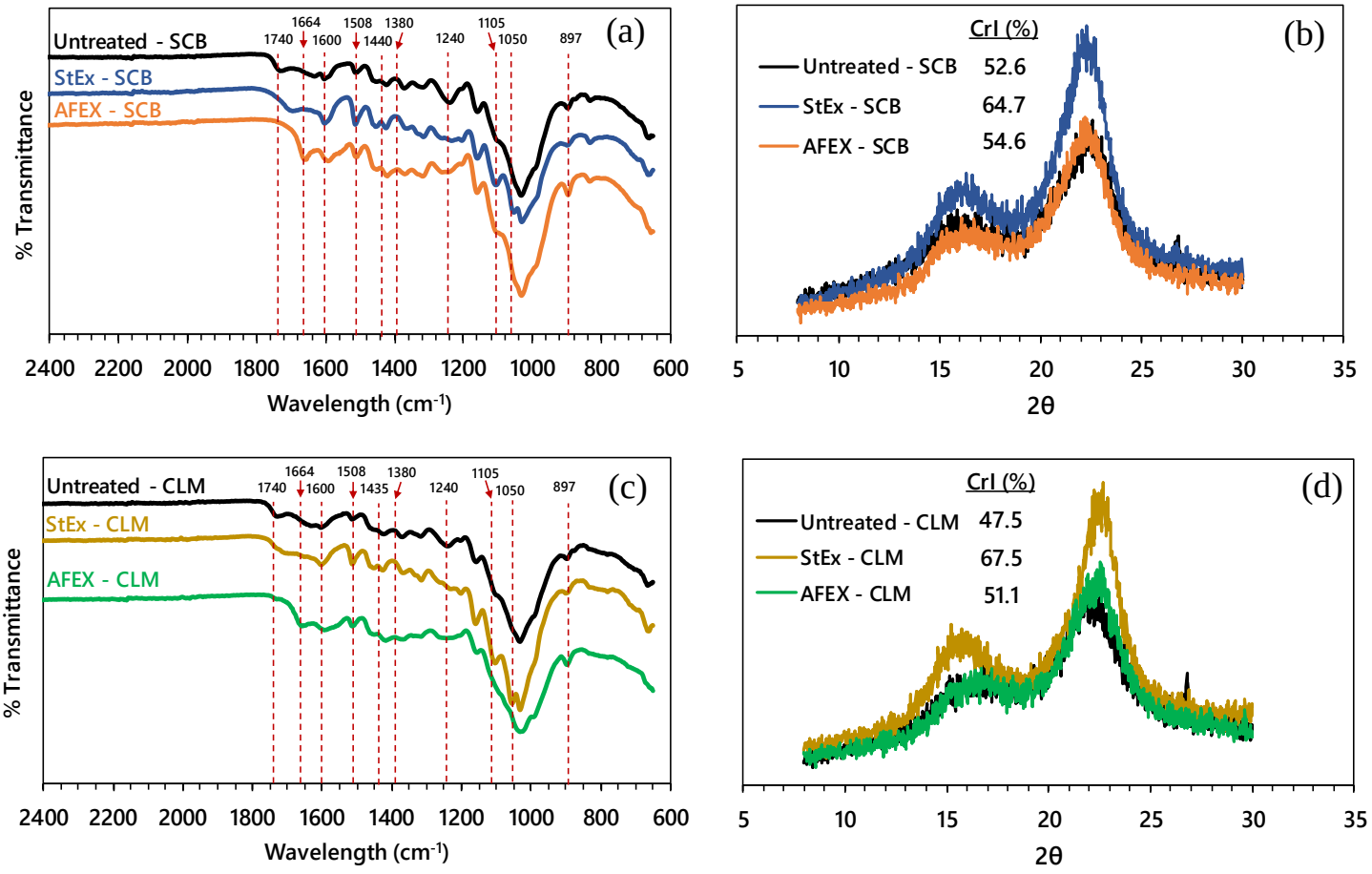



**Figure 6.2:** Comparing the structural characteristics of untreated, AFEX™, and StEx pretreated SCB and CLM. ATR-FTIR data for untreated and pretreated **(a)** SCB, and **(c)** CLM. XRD spectra of untreated and pretreated SCB **(b)** and CLM **(d)**.

In the fingerprint region (600 – 1800 $cm^{-1}$), FTIR spectra of AFEX™-treated SCB and CLM demonstrated a significant decrease in intensity of the 1240 $cm^{-1}$ (ether linkages in hemicellulose and lignin), 1380 $cm^{-1}$ (C-H deformation in hemicellulose and cellulose) and 1740 $cm^{-1}$ (ester carbonyl C=O stretching) peaks relative to untreated controls, suggesting significant cleavage of ester linkages in lignin-hemicellulose complexes, acetyl groups and lignin side chains [309,310]. The appearance of the peak at 1664 $cm^{-1}$ confirms the formation of acetyl and phenolic amides, which are derived from the de-esterification of hemicellulose-lignin complexes by ammonolysis reactions [136,140]. Similarly, the reduction in bands at 1740 $cm^{-1}$ and 1240 $cm^{-1}$ in StEx treated SCB and CLM, suggest significant removal of hemicelluloses and/or cleavage of acetyl groups, consistent with the chemical composition presented in Table 6.1. Further, the increase in peak intensity at 1440 $cm^{-1}$ (H-O-C bending in hemicelluloses, lignin and cellulose), 1508 $cm^{-1}$ (phenyl skeletal vibration of lignin), and 1600 $cm^{-1}$ (C=C and C=O stretching in aromatic lignin), reflects an enrichment of lignin content and potential presence of low molecular weight lignin fractions in StEx-treated SCB and CLM samples [245,311]. Also, the increase in intensity bands at 1035 $cm^{-1}$ (primary C-O/C-H groups stretching in cellulose) and 1160 $cm^{-1}$ (secondary C-O/C-H group stretching in cellulose) relative to untreated SCB and CLM could reflect the increase in cellulose content in biomass due to hemicellulose removal [312].

From the XRD spectra, it was evident that StEx increased the CrI of SCB and CLM from 53% and 48% to 65% and 67% ($P < 0.05$), respectively, consistent with previous work with $SO_2$-impregnated StEx pretreatment of SCB [311]. It is well documented that the partial removal of amorphous cellulose and hemicelluloses for low pH pretreatments such as StEx results in a material that is rich in crystalline cellulose and lignin. In contrast, slight increases in CrI were observed for AFEX™-treated SCB (55%) and CLM (51%) relative to untreated controls ($P < 0.05$). This result agrees with previous work that suggested that high pH pretreatments have lesser effect on cellulose crystallinity compared to lower pH pretreatments [310].

### 6.3.3 *Effect of StEx and AFEX™ pretreatment on methane yield and content after mono- and co-digestion*

#### 6.3.3.1 *Mono-digestion*

The effect of StEx and AFEX™-pretreatment of SCB and CLM on the cumulative methane yield, methane content, and total VFA concentration after mono- or co-digestion is presented in Figure 6.3. After a 55-day digestion period, the mono-digestion of untreated SCB and untreated CLM produced specific methane yields of 258 ± 8.3 and 231 ± 4.6 L $CH_4.kg^{-1}$ of $VS_{added}$, respectively. The methane yields for these substrates were consistent and within range of those reported in previous studies [205,313,314]. The methane yield for DCM (274 ± 5.9 L $CH_4$/kg $VS_{added}$) was slightly higher than the literature reported range of 130 – 255 L $CH_4$/kg $VS_{added}$, which can be attributed to the potential differences in the DCM chemical composition (influenced by the cow diet), near optimum C/N ratio of the DCM used in this work and the potential acclimation of the inoculum used in this work to swine and cow manure digestion [197,221].

AFEX™ pretreatment of SCB and CLM facilitated the generation of the highest specific methane yields for anaerobic mono-digestion, enhancing the methane yields by 8% and 26% relative to the mono-digestion of untreated controls, respectively ($P < 0.05$). This could be explained by the structural changes that are attributed to AFEX™-pretreatment, favourable substrate C/N ratio and lower Klason lignin content of the AFEX™ treated substrates relative to the untreated and StEx-treated substrates [297]. Further, it is hypothesized that the additional nitrogen chemically-linked to the biomass from AFEX™ pretreatment did not lead to excess ammonia accumulation, which would otherwise result in VFA accumulation, drop in the digestion pH, and ultimately inhibit the methanogenic community [202,315]. Consequently, low total VFA concentrations (< 105 $mg.L^{-1}$) were detected after AFEX™-SCB and AFEX™-CLM mono-digestion (Fig. 2 G-H), significantly lower than the literature reported VFA inhibition concentration range of 1500 – 2000 $mg.L^{-1}$ [198]. However, time based VFA, $NH_4^+$-N, and $NH_3$-N quantification is required to confirm the absence of ammonia inhibition.

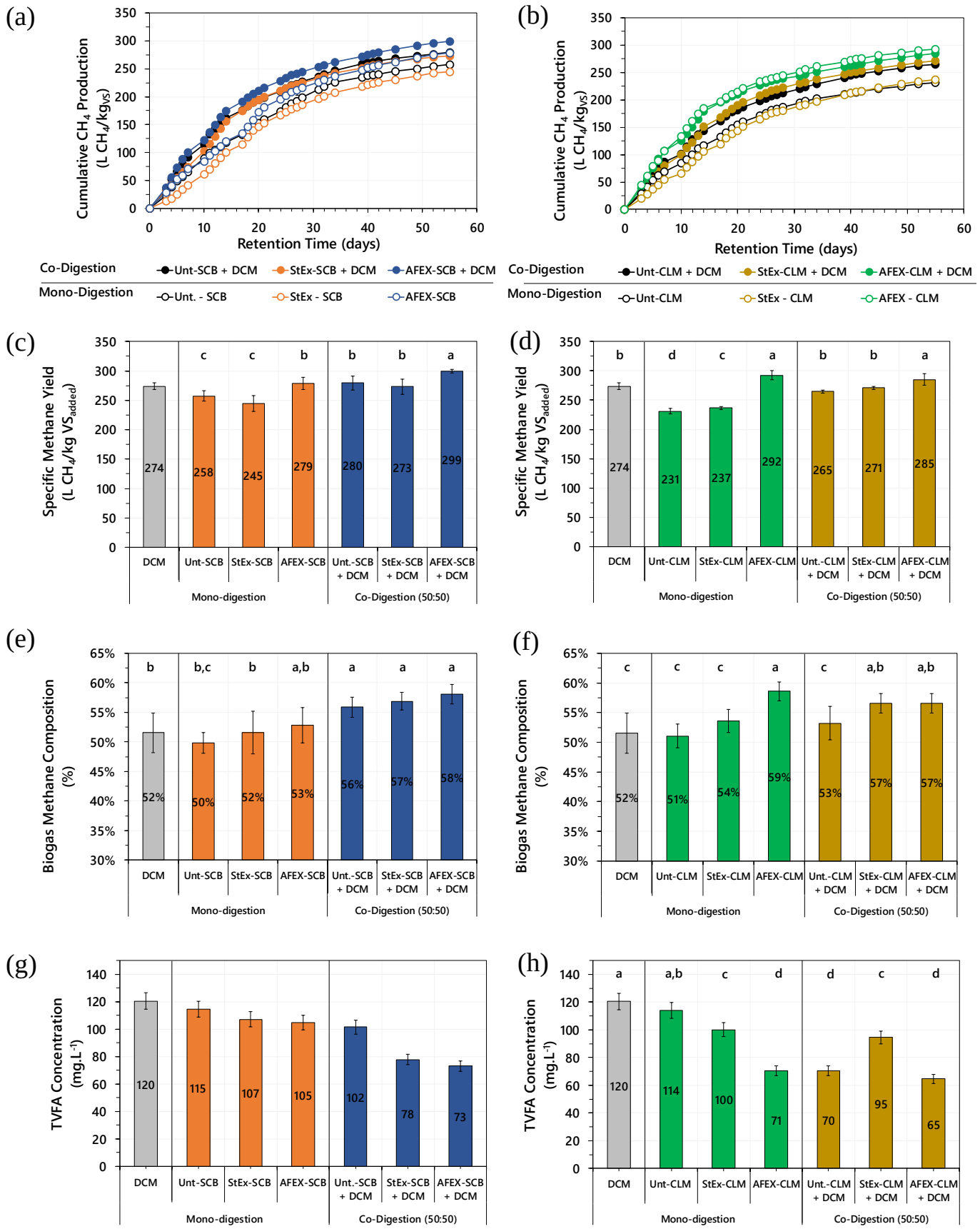



***Figure 6.3:*** Comparison of the cumulative methane production profiles **(a,b)**, specific methane production **(c,d)**, biogas methane content **(e,f)**, and total VFA production **(g,h)** from mono-digestion and co-digestion (50:50) of untreated, AFEX™-treated and StEx-treated SCB and CLM. Different alphabets above bar graph indicate significant differences as determined by one-way ANOVA with Tukey's *post-hoc* HSD test ($P < 0.05$)

Nonetheless, the specific methane yield and biogas methane content for the mono-digestion of AFEX™-CLM (292 ± 7.6 L $CH_4$/kg $VS_{added}$ and 59 ± 1.6%, respectively) were statistically higher than that of DCM mono-digestion ($P < 0.05$), suggesting that the AFEX™-CLM fibers were more biodegradable and/or the digestion nutrient balance was more suitable relative to DCM mono-digestion.

StEx pretreatment of SCB and CLM did not significantly improve or regress the specific methane yields during anaerobic mono-digestion relative to untreated controls ($P > 0.05$). The extent of StEx-SCB and StEx-CLM anaerobic biodegradation could have been limited by the substrate characteristics such as low biodegradable organic matter content, low digestion C/N ratio, high content of recalcitrant lignin, and the presence of toxic furan and phenolic moieties that are bound to the unwashed solids [301]. Risberg *et al.,* (2013) also reported insignificant difference for wheat straw steam exploded at 210 °C and 10 min relative to untreated controls, citing the removal of biodegradable organic matter (predominantly hemicelluloses) and potential microbial community inhibition by pretreatment-derived compounds as limiting factors for StEx-treated substrate mono-digestion [16]. Nevertheless, the cumulative methane yields for StEx-SCB and StEx-CLM mono-digestion achieved in this work (245 ± 13.2 and 237 ± 3.5 L $CH_4$/kg $VS_{added}$) were higher than those previously reported by Costa *et al.,* (2014) and De Paoli *et al.,* (2011) for hydrothermally pretreated SCB and steam exploded CLM, respectively [205,211].

### 6.3.3.2 *Co-digestion*

The co-digestion of untreated, StEx-treated and AFEX™-treated SCB and CLM with DCM at a mixture ratio of 50:50 (VS basis) significantly increased the specific methane yield for all the mixtures, except for the AFEX™-CLM + DCM mixture, relative time mono-digestion assays. Like the mono-digestion assays, the highest co-digestion methane yields were attained by the AFEX™-treated substrates, with AFEX™-SCB + DCM (299 ± 4.3 L $CH_4$/kg $VS_{added}$) enhancing methane yields by 16%, 7% and 9% relative to the mono-digestion of untreated-SCB, AFEX™-SCB, and DCM, respectively ($P < 0.05$). With AFEX™-treated SCB having high anaerobic biodegradability and a C/N ratio within the

recommended optimum range, the enhancement of the methane yield through co-digestion suggests that the DCM supplied the digesters with some essential macronutrients, micronutrients, and/or trace elements that may be required for maximizing the activity and synergy of the microbial population for degrading the AFEX™-SCB + DCM mixture. For instance, DCM can provide supplementary cations such as $Mg^{2+}$ $Ca^{2+}$, and $Fe^{2+}$ that are essential for the growth of methanogenic archaea and for stabilizing anaerobic digestion [197,316]. Accordingly, the increase of the methane yield for the AFEX™-SCB + DCM mixture beyond the additive contribution of the each substrate indicates some synergistic effect in mixing the two substrates [297]. For the nitrogen-limited untreated and StEx-treated SCB and CLM substrates, co-digestion with DCM potentially provides alkalinity for improving the digestion buffer capacity, nutrient balance and nitrogen to support microbial synthesis of amino acids, protein and nucleic acids [198]. As a result, co-digesting untreated and StEx-treated SCB and CLM with DCM increased methane yields by 8-15% relative to their mono-digestion counterparts ($P < 0.05$).

#### 6.3.3.3 *Kinetic analysis of methane production*

Kinetic analysis and data modelling of methane production from mono-digestion and co-digestion of untreated, StEx-treated and AFEX™-treated SCB and CLM was performed to evaluate the effect of pretreatment and co-digestion on biodegradation rate constant ($k$) and the maximum methane yield ($\beta_0$). The estimated Cone model parameters are presented in Table 6.2 and the model prediction plots are available in Figure 6.4.

Model simulation demonstrated that the Cone model adequately predicted the experimental mono- and co-digestion methane production profiles, as shown by the low RMSE and AIC and high $R^2_{Adj}$ ( > 0.995) for all the assays [305]. The Cone model parameters were characterized by a narrow range of lower and upper 95% confidence interval limits, with the best-fitted parameters placed within this range, indicating high probability and certainty of the estimated model parameters [306,307]. As evidenced by the increased substrate biodegradation rate constants, the combination of biomass pretreatment and co-digestion with DCM significantly improved the substrate biodegradation rate

relative to the mono-digestion for all the assays except for AFEX™-CLM, suggesting that co-digestion was beneficial for improving the overall AD productivity and extent of digestion efficiency. The similar substrate biodegradation rate constants for the mono-digestion of AFEX™-CLM and co-digestion of AFEX™-CLM + DCM could be explained by the relatively similar C/N ratios between the two digestion mixtures (23 vs 19), indicating that AFEX™-CLM mono-digestion may already have sufficient fiber biodegradability, nutrient balance and buffer capacity to negate the benefits of DCM supplementation.

**Table 6.2:** Estimated Cone model kinetic parameters with the corresponding 95% parameter confidence intervals and degree of model fit.

| Substrate | Estimated Cone Kinetic Parameters | | | Degree of Model fit | | |
|---|---|---|---|---|---|---|
| | $B_0$ (L $CH_4$.kg $VS_{added}$) | k ($day^{-1}$) | n | $R^2_{adj}$ | RMSE (L $CH_4$.kg $VS_{added}$) | AIC |
| DCM | 325 | 0.066 | 1.26 | 0.998 | 3.01 | 76.5 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (315 – 338) | (0.061 – 0.070) | (1.19 – 1.32) | | | |
| Untreated-SCB | 367 | 0.040 | 1.23 | 0.997 | 4.36 | 100.2 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (349 – 401) | (0.033 – 0.043) | (1.13 – 1.29) | | | |
| StEx – SCB | 287 | 0.050 | 1.77 | 0.998 | 3.32 | 82.8 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (280 – 295) | (0.048 – 0.052) | (1.70 – 1.84) | | | |
| AFEX™-SCB | 372 | 0.048 | 1.34 | 0.995 | 6.01 | 120.8 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (345 – 407) | (0.042 – 0.052) | (1.21 – 1.47) | | | |
| Untreated- CLM | 297 | 0.052 | 1.25 | 0.997 | 3.64 | 88.7 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (281 – 315) | (0.047 – 0.058) | (1.15 – 1.34) | | | |
| StEx – CLM | 296 | 0.048 | 1.46 | 0.998 | 3.32 | 82.7 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (284 – 312) | (0.044 – 0.051) | (1.37 – 1.54) | | | |
| AFEX™-CLM | 336 | 0.078 | 1.29 | 0.998 | 3.09 | 78.1 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (331 – 344) | (0.075 – 0.080) | (1.23 – 1.33) | | | |
| Untreated-SCB + DCM | 342 | 0.063 | 1.24 | 0.999 | 2.41 | 62.4 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (333 – 351) | (0.059 – 0.065) | (1.19 – 1.28) | | | |
| StEx - SCB + DCM | 310 | 0.069 | 1.51 | 0.998 | 3.20 | 80.5 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (303 – 318) | (0.066 – 0.072) | (1.45 – 1.59) | | | |
| AFEX™-SCB + DCM | 356 | 0.069 | 1.27 | 0.999 | 2.75 | 70.7 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (346 – 367) | (0.064 – 0.072) | (1.22 – 1.33) | | | |
| Untreated- CLM + DCM | 339 | 0.055 | 1.18 | 0.998 | 3.18 | 80.1 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (326 – 355) | (0.050 – 0.059) | (1.14 – 1.25) | | | |
| StEx - CLM + DCM | 314 | 0.065 | 1.44 | 0.998 | 3.35 | 83.4 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (307 – 324) | (0.062 – 0.068) | (1.38 – 1.49) | | | |
| AFEX™-CLM + DCM | 321 | 0.080 | 1.33 | 0.998 | 3.68 | 89.3 |
| (Lower $CI_{95\%}$ - Upper $CI_{95\%}$) | (314 – 334) | (0.074 - 0.083) | (1.25 - 1.39) | | | |

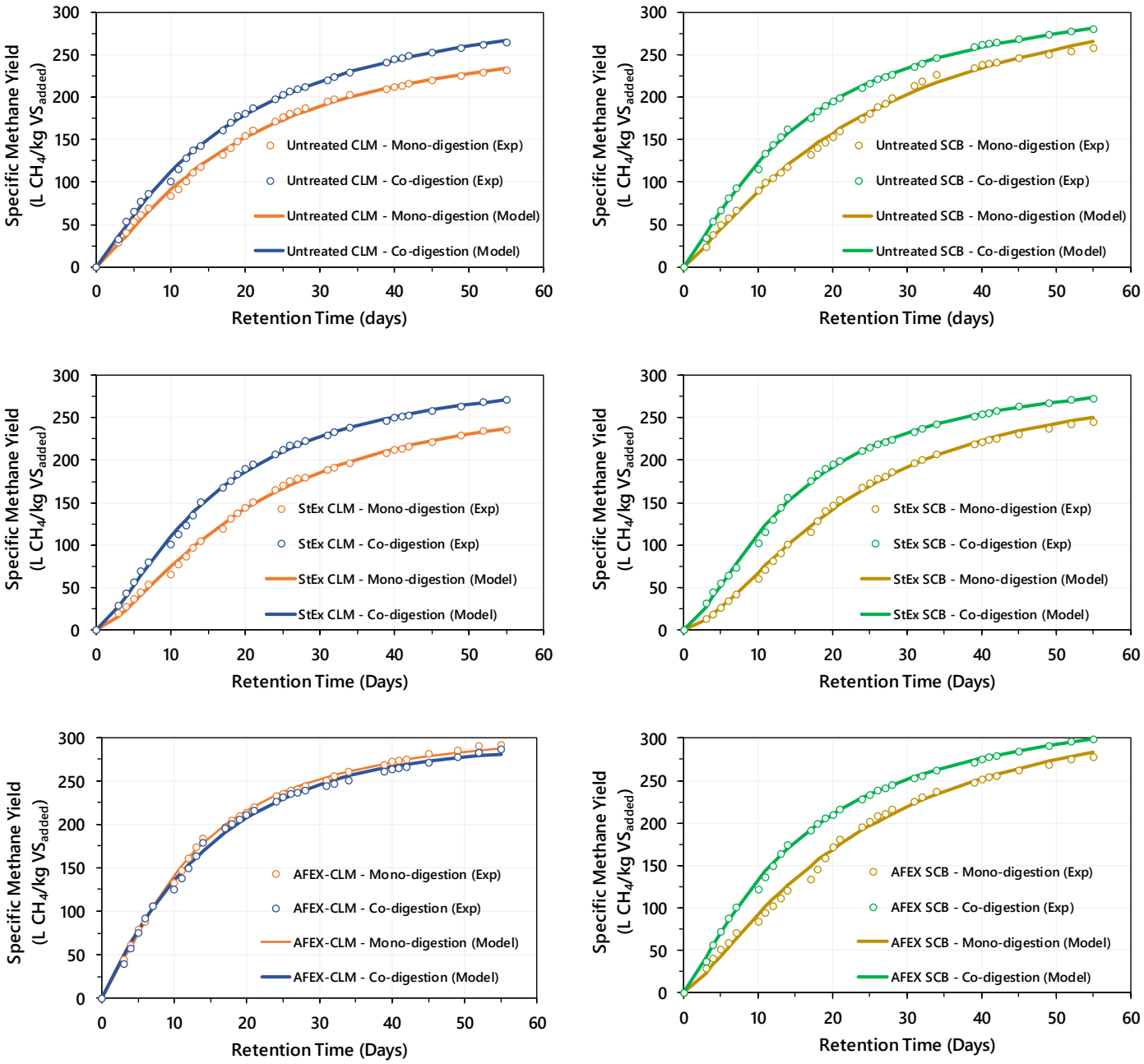



**Figure 6.4:** Experimentally measured and Cone model predicted methane yield as a function of digestion time for mono- and co-digestion of untreated, AFEX™-treated and StEx-treated SCB and CLM.

### 6.3.4 *Methane production from co-digestion of untreated, StEx- and AFEX™- treated CLM with DCM at different ratios*

Cumulative methane yields from the mono- or co-digestion of untreated, StEx-treated and AFEX™-treated CLM at biomass-to-DCM ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (VS basis) are presented in Figure 6.5. Co-digestion of AFEX™-CLM + DCM did not have a statistically significant

effect for all the ratios studied in this work ($P$ > 0.05), potentially due to the already high biodegradability of AFEX™-CLM and the narrow changes in the C/N ratio (18 – 23) for all the AFEX™-CLM + DCM mixtures. This result suggests that AFEX™-treated CLM can be a flexible substrate for AD plants with non-uniform DCM supply, where AFEX™-treated CLM can be digested at any mixture ratio without significantly reducing methane yields and biogas methane quality. In contrast, the co-digestion of untreated CLM + DCM at a biomass-to-DCM mixture ratio of 75:25 (C/N = 35) resulted in a significant increase in the cumulative methane yield relative to the mono-digestion of either DCM or untreated CLM ($P$ < 0.05), suggesting some degree of synergy when mixing the two substrates at this ratio. Moreover, the methane yield of 292 ± 6.7 L $CH_4$/kg $VS_{added}$ achieved with this substrate mixture was statistically comparable to AFEX™-treated-CLM in mono- and co-digestion with DCM ($P$ > 0.05). For StEx-treated CLM, reducing the biomass-to-DCM ratio below 50:50 resulted in an increase in the digestion C/N ratio (> 50) and a significant reduction in the cumulative methane yield (< 245 L $CH_4$/kg $VS_{added}$).

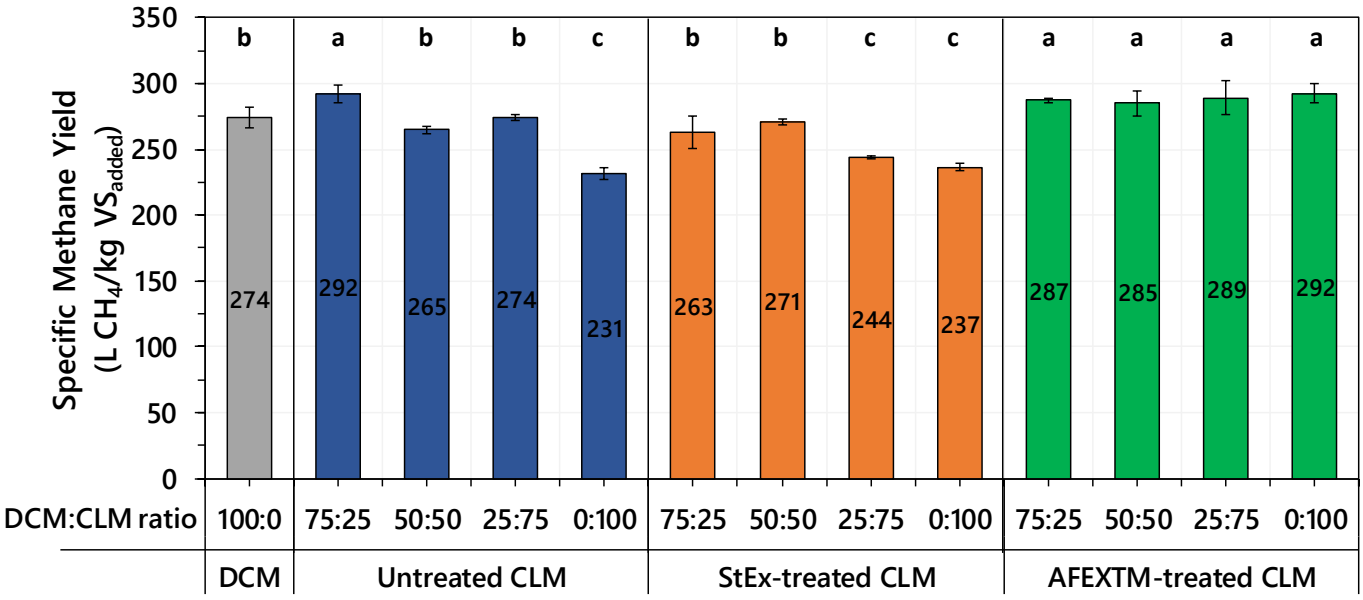



**Figure 6.5:** Evaluating the effect of DCM-to-CLM ratio on the specific methane yield after anaerobic co-digestion for 55 days. Different alphabets above bar graph indicate significant difference as determined by one-way ANOVA with Tukey's *post hoc* HSD test ($P$ < 0.05)

To support the hypothesis that blending DCM with untreated or pretreated CLM resulted in a significant shift in the mixture C/N ratio and the corresponding cumulative specific methane yield, the biomass + DCM C/N ratio for the afore-mentioned mixtures was correlated with the obtained specific methane yields (Figure 6.6).

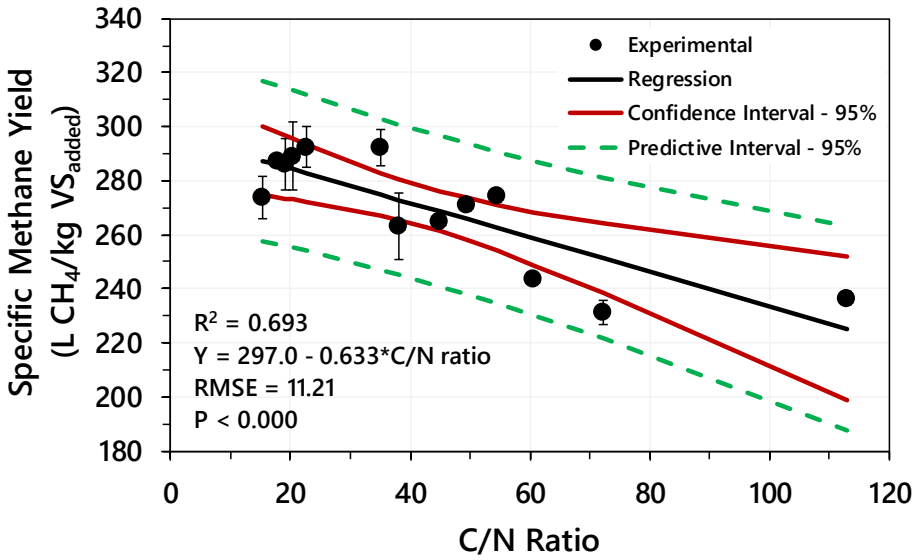



***Figure 6.6:*** Correlating the specific methane yields after anaerobic co-digestion to the inlet mixture C/N ratio

Within the wide-range of C/N ratios considered in this work, a statistically significant negative linear correlation was found ($P < 0.000$) between an increasing C/N ratio and the cumulative methane yield with $R^2$ and RMSE values of 69.3% and 11.21, respectively. Therefore, the linear correlation can explain 69.3% of the variation in the specific methane yield as a function of the C/N ratio within the wide C/N range of 15 - 113. This result can be elucidated by the fact that the C/N ratio is not the only factor contributing to the specific methane yield and that factors such as fiber biodegradability, buffer capacity, micro- and trace element balance, and the dilution of toxic compounds are simultaneously influenced by various co-digestion ratios and therefore also significantly contribute to the cumulative methane yield variation [197]. Nonetheless, based on the experimental data, the highest methane yields were achieved for mixture C/N ratios in the range of 18 – 35, comparable to the optimum range reported in literature (15 – 45) [192].

Janke and co-workers [20] combined mechanical milling and alkaline pretreatment (using 12g NaOH/g DM) of CLM and reported the highest specific methane yields reported in literature for CLM (291 L $CH_4$/kg VS) (see Table 6.3). However, although their AD process was not inhibited by the high $Na^+$ concentrations, it was also reported the high $Na^+$ concentrations in the digestate of the NaOH treated CLM could potentially limit the applicability of the digestate as fertilizer/soil conditioner due to its potential long-term soil salinization effect. In contrast, AFEX facilitated the methane yields that were greater than 290 L $CH_4$/kg VS (with or without co-digestion with cow manure), with high catalyst recovery (> 97%) and no negative impacts on the digestate. Similarly, the co-digestion of StEx treated SCB and CLM produced methane yields that were significantly higher than those reported for the mono-digestion of steam exploded sugarcane residues.

***Table 6.3:*** Comparing the effect of AFEX™ and StEx pretreatment of sugarcane residues on the specific methane yield with literature

| Manure Type | Lignocellulosic substrate | Operation conditions | Lignocellulose Pretreatment | Manure/Residue ratio | Methane Yield (L $CH_4$/kg VS) | References |
|---|---|---|---|---|---|---|
| - | Sugarcane CLM | Mesophilic C/N = 22.6 | AFEX™ pretreatment (120 °C) ǂ | 0/1 | 292 ± 7.8 | This Study |
| DCM | Sugarcane Bagasse | Mesophilic C/N = 21.3 | AFEX™ pretreatment (120 °C) ǂ | 1/1 | 299 ± 4.3 | This Study |
| DCM | Sugarcane CLM | Mesophilic C/N = 20.2 | AFEX™ pretreatment (120 °C) ǂ | 1/1 | 286 ± 9.6 | This Study |
| DCM | Sugarcane bagasse | Mesophilic C/N = 66.6 | Steam explosion (205 °C) | 1/1 | 273 ± 10.4 | This Study |
| DCM | Sugarcane CLM | Mesophilic C/N = 50.6 | Steam explosion (200 °C) | 1/1 | 271 ± 6.4 | This Study |
| DCM | Sugarcane CLM | Mesophilic C/N = 35.1 | No pretreatment | 3/1 | 292 ± 6.7 | This Study |
| Pig manure + DCM (1/1) | Sugarcane CLM | Mesophilic C/N = 29 | 6% (w/w) NaOH pretreatment | 1.6/1 | 225 | [216] |
| - | Sugarcane Bagasse | Mesophilic C/N = 63 - 89 | No pretreatment | 0/1 | 121 – 245 | [205] [209] [210] |
| - | Sugarcane Bagasse | Mesophilic C/N = 64.3 | Steam Explosion (200 °C) | 0/1 | 258 | [205] |
| - | Sugarcane Bagasse | Mesophilic C/N = N/A | Hydrothermal pretreatment (200 °C) | 0/1 | 197 | [211] |
| - | Sugarcane Bagasse | Mesophilic C/N = N/A | Hydrothermal pretreatment (180 °C) | 0/1 | 190 | [212] |
| - | Sugarcane Bagasse | Mesophilic C/N = N/A | 8.5% (w/w) $Ca(OH)_2$ pretreatment | 0/1 | 178 | [212] |
| - | Sugarcane CLM | Mesophilic C/N = 46-54 | No pretreatment | 0/1 | 79 – 231 | [205] [213] |
| - | Sugarcane CLM | Mesophilic C/N = 38.6 | Steam Explosion (200 °C) | 0/1 | 181 | [205] |
| - | Sugarcane CLM | Mesophilic C/N = N/A | Mechanical + 12 % (w/w) NaOH pretreatment | 0/1 | 291 | [207] |

Practical considerations for selecting the preferred co-digestion ratio will depend on several factors including the relative amounts of sugarcane residues and DCM available to the AD plant, the size and number of domestic intensive, extensive and feedlot animal feeding systems, biomass/DCM storage and transportation logistics, seasonal availability of the untreated and/or pretreated sugarcane residues, and livestock farming interaction with domestic food production [317]. Whilst AFEX™-CLM offers process flexibility by maintaining high methane yields irrespective of the blending ratio with DCM, simply blending untreated CLM with DCM at the appropriate ratio may be a cheaper manure management solution whilst also maximizing methane yields for AD plants with adequate and consistent supply of DCM and sugarcane CLM. However, AD plants located in areas with limited DCM supply may experience reduced methane yields due to the over-compensation of the untreated CLM/SCB in their digestion mixtures. Alternatively, for AD plants located near sugar mills supplying AFEX™-treated CLM and SCB as feedstock to the cellulosic ethanol industry or as feed for intensified animal feed market, results from this work suggest that these AFEX™-treated residues can perhaps be blended with untreated CLM/SCB and DCM to achieve appropriate C/N ratios to maximize cumulative methane yields and biogas methane content. Moreover, since AFEX™ facilitates easier crop residue pelletization, pelletized AFEX™-treated sugarcane residues can be stored on-site, securing stable and high biodegradable biomass for farm-based or centralized AD plants located in areas with inconsistent year-round DCM supply [273].

### 6.3.5 *Energy conversion assessment and solid digestate fertilizer value*

Anaerobic co-digestion of untreated CLM + DCM (25:75), AFEX™-SCB + DCM (50:50), AFEX™-CLM and AFEX™-CLM + DCM (all mixtures) led to co-digestion C/N ratio's that were within the range of 18 – 35 and subsequently resulted in methane yields that were statistically higher than the mono-digestion of untreated SCB, untreated CLM and DCM. An energy conversion assessment of these substrate mixtures was performed to estimate the ability of AD to convert the energy stored in the ingestates (non-digested substrate mixtures) into a methane-rich biogas stream (Table 6.4). In all the

AFEX™-pretreatment and/or co-digestion cases, the biogas energy recovery was in the range 50–53%, significantly higher than any of the mono-digestion cases ($P < 0.05$). These energy recoveries corresponded with volatile solids removal rates in the range of 56-60%, suggesting that a large portion of the ingestate energy remained in the recalcitrant solid digestate organic matter, which can be further valorised by conventional routes and used as soil amendments, biofertilizer, or dried and pelletized for thermochemical conversion in areas with domestic digestate oversupply [318].

It is common practice to separate the AD digestate into liquid and solid digestates fractions for easy handling and storage. Macro-nutrient analysis of the solid digestates from the co-digestion assays showed that nitrogen, phosphate, and potassium (N-P-K) contents were more concentrated in the digestate relative to that of undigested SCB, CLM, and DCM (see Table 6.1). NPK represented 5-6% of the total solids in the digestate, concentrated to more than 300% relative to the NPK in raw SCB and CLM. The increases in NPK in the solid digestate relative to the ingestate are typically attributed to the degradation of organic carbon to $CH_4$ and $CO_2$, microbial biomass, and the preservation and partial mineralization of N, P and K during AD [319]. Further, the highest NPK, Mg, Ca, Na, S and Fe values were achieved for the co-digestion assays, implying that DCM supplementation adds additional essential minerals which enhance the digestate fertilizer value.



**Table 6.4:** Methane production, energy conversion efficiency and solid digestate fertilizer value for selected mono-digestion and co-digestion substrates

| Substrate | DCM | Untreated-SCB | Untreated-CLM | Untreated-CLM + DCM | AFEX-SCB + DCM | AFEX-CLM | AFEX-CLM + DCM |
|---|---|---|---|---|---|---|---|
| **DCM/Biomass Ratio** | **100/0** | **0/100** | **0/100** | **75/25** | **50/50** | **0/100** | **50/50** |
| **Digestion C/N ratio** | **15** | **101** | **72** | **35** | **20** | **23** | **19** |
| VS degraded in digestion (%) | 51 ± 1.2% $^{C}$ | 47 ± 2.1% $^{D}$ | 42 ± 1.2 % $^{E}$ | 58 ± 1.3% $^{A,B}$ | 60 ± 0.1% $^{A}$ | 59 ± 0.7% $^{A}$ | 56 ± 1.6% $^{B}$ |
| $CH_4$ $Yield_{STP}$ ($Nm^3$ $CH_4$/Mg $VS_{added}$) | 262 ± 10.7 $^{C}$ | 246 ± 12.4 $^{D}$ | 221 ± 6.1 $^{E}$ | 286 ± 6.6 $^{A,B}$ | 286 ± 3.2 $^{A}$ | 280 ± 7.2 $^{A,B}$ | 274 ± 9.2 $^{B}$ |
| Biogas $CH_4$ content (% v/v) | 52 ± 3.4% $^{B}$ | 50 ± 1.8% $^{B}$ | 51 ± 2.0% $^{B}$ | 55 ± 5.0% $^{A}$ | 58 ± 1.6% $^{A}$ | 59 ± 1.6% $^{A}$ | 57 ± 1.2% $^{A}$ |
| Energy Conversion Efficiency (%) | 47 ± 1.4% $^{C}$ | 48 ± 1.7% $^{C}$ | 41 ± 0.9% $^{D}$ | 52 ± 0.5% $^{A,B}$ | 53 ± 0.6% $^{A}$ | 53 ± 1.5% $^{A,B}$ | 50 ± 1.8% $^{B}$ |
| **Solid digestate macro-nutrient value** | | | | | | | |
| Total N (kg/Mg dry digestate) | 33.6 ± 1.4 | 13.3 ± 0.3 | 14.4 ± 0.3 | 25.3 ± 1.2 | 24.4 ± 0.9 | 17.5 ± 0.7 | 26.1 ± 1.1 |
| Total P (kg/Mg dry digestate) | 23.1 ± 0.9 | 13.1 ± 0.5 | 15.3 ± 0.6 | 20.3 ± 0.8 | 21.2 ± 0.9 | 15.6 ± 0.5 | 19.1 ± 0.6 |
| Total K (kg/Mg dry digestate) | 13.0 ± 0.4 | 4.8 ± 0.1 | 12.5 ± 0.2 | 12.6 ± 0.3 | 11.5 ± 0.2 | 7.9 ± 0.1 | 11.1 ± 0.2 |
| Total Ca (kg/Mg dry digestate) | 66.0 ± 1.9 | 20.5 ± 0.6 | 26.0 ± 0.8 | 51.0 ± 1.5 | 44.0 ± 0.9 | 19.9 ± 0.4 | 45.6 ± 0.9 |
| Total Mg (kg/Mg dry digestate) | 20.9 ± 0.6 | 10.1 ± 0.3 | 10.5 ± 0.2 | 17.8 ± 0.5 | 17.3 ± 0.7 | 11.6 ± 0.5 | 16.2 ± 0.5 |
| Total Na (kg/Mg dry digestate) | 30.4 ± 1.2 | 21.9 ± 0.4 | 30.2 ± 0.5 | 30.3 ± 1.1 | 30.8 ± 1.1 | 18.3 ± 0.4 | 28.1 ± 0.9 |
| Total Fe (kg/Mg dry digestate) | 8.7 ± 0.2 | 3.0 ± 0.1 | 2.6 ± 0.1 | 5.3 ± 0.1 | 3.5 ± 0.1 | 1.6 ± 0.0 | 2.8 ± 0.1 |

STP – standard temperature pressure at 273 K and 101.325 kPa

For AD plants located near sugar mills and integrated to bioenergy-livestock systems, solid digestates may be used as either bedding for animals or combined with mineral-rich bottom ash from sugar mill cogeneration operations before being applied to the sugarcane fields as organic fertilizer or soil amendment to create a more sustainable biomass to food/bioenergy network [294]. A portion of the residual solids applied as soil amendment will be recalcitrant carbon and will, therefore, likely contribute to long-term carbon storage in the soil. This is an excellent example of bioenergy with carbon capture and storage (BECCS) system [57]. Current sugarcane green harvesting techniques require that approximately 50% of the sugarcane CLM be left on the field to cover the soil in view of increasing nutrient recycling and soil organic matter, whilst minimizing temperature variation and water evaporation from the soil [320].

The potential application of AD digestates with lower organic carbon and higher NPK as soil amendments and organic fertilizers in sugarcane fields may allow for more sugarcane CLM to be removed from the field after harvesting and allocated to bioenergy production, thereby improving bioenergy production yields per hectare of land. Alternatively, the AD digestates can be partial mineral fertilizer replacements, potentially minimizing fertilizer input costs for sugarcane growers [224,226]. However, in- field tests may be necessary to understand the effects of increased CLM removal rates from the sugarcane fields and digestate application as partial mineral fertilizer substitute on the long-term sugarcane crop yields and productivity, soil fertility and environmental impact.

## 6.4 Conclusions

In the present study, the mono-digestion of AFEX™-treated CLM demonstrated the highest methane yield (292 L $CH_4$/kg $VS_{added}$), biogas methane content (59% v/v) and biodegradation rate relative to the mono-digestion of DCM, untreated and StEx-treated SCB and CLM. However, the co-digestion of untreated or pretreated sugarcane residues with DCM significantly enhanced the methane yields and biodegradation rates relative to the corresponding mono-digestion assays, with the methane yields of 290 – 299 L $CH_4$/kg $VS_{added}$ achieved with co-digestion mixtures with C/N ratios

in the range 18 – 35. Further, co-digestion facilitated the production of solid digestates with more concentrated NPK and lower organic matter relative to non-digested controls, suggesting that these digestates could be used to partially replace CLM that is typically left on cane fields and/or reduce mineral fertilizer inputs. Ultimately, the results of this work highlight the potential for incorporating anaerobic co-digestion of DCM and sugarcane residues into the integrated biofuel-livestock nexus. For sugarcane and livestock dense regions, this strategy can create more sustainable food-bioenergy-manure management systems, while possibly avoiding potential indirect land use changes.

# CHAPTER SEVEN:

# CONTRIBUTION 4

**Prepared for submission to:** Energy conversion and Management, ISI 5-year Impact Factor = 6.100

**Title:** Cellulose $III_I$-activation of AFEX™ and steam exploded sugarcane residue pellets for low enzyme loading ethanol production in centralized biorefineries

**Authors:** Thapelo Mokomele, Leonardo da Costa Sousa, Bruce E. Dale, and Johann F. Görgens

**Declaration:** The contents of this chapter have not been published elsewhere at the time of thesis submission. However, this chapter is prepared for submission to Energy Conversion and Management.

## Objective of dissertation and summary of findings in chapter

This chapter addresses the research objectives highlighted in **contribution 4**. This chapter investigates the potential use of StEx and AFEX™ to produce dense and durable biomass pellets that would facilitate easier transportation and storage relative to non-densified controls. As previously identified in **CHAPTER's 4** and **5**, ethanol production from StEx and AFEX™-treated biomass requires enzyme dosages greater than 20 mg per gram glucan to achieve high ethanol yields. To de-bottleneck the high enzyme dosage requirements, this chapter explores the potential use of a room-temperature $CIII_I$-activation step to upgrade the cellulose allomorph of the AFEX™ and StEx pretreated pellets.

Both StEx and AFEX™ vastly improved the bulk density, pellet durability and hydrophobicity of SCB and CLM pellets, achieving pellet physical and mechanical properties akin to corn grains. Coupling the AFEX™-treated SCB and CLM pellets with a $CIII_I$-activation step reduced enzyme loading requirements to lower than 7.5 mg protein per gram glucan (> 60% enzyme dosage reduction), whilst achieving ethanol yields greater than 280 L per Mg raw dry biomass. In contrast, upgrading StEx-treated SCB and CLM pellets could only facilitate ethanol yields of 176 and 201 L per Mg raw dry biomass at an enzyme dosage of 7.5 mg protein/g glucan, respectively. Given uncertainties with the

commercial costs of hydrolytic enzymes, the results of this work demonstrate that $CIII_I$-activation could be a feasible processing strategy for minimizing enzyme-related costs for ethanol production.

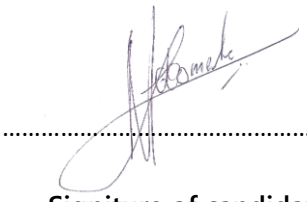
## Candidate declaration

With regards to **Chapter 7**, pages 220 – 275 of this dissertation, the nature and scope of my contributions were as follows:

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental planning | 70 |
| Executing experiments | 100 |
| Intepretation of experiments | 80 |
| Writing of chapter | 100 |

The following co-authors have contributed to **Chapter 7**, pages 220 – 275 of this dissertation:

| Name | e-mail address | Name of contribution | Extent of contribution (%) |
|---|---|---|---|
| Leonardo da Costa Sousa | sousaleo@egr.msu.edu | ✓Experimental planning<br>✓Intepretation of experiments<br>✓Review of chapter | 30<br>20<br>50 |
| Bruce E. Dale | bdale@egr.msu.edu | ✓Review of chapter<br>✓Co-ordination of collaboration | 20<br>50 |
| Johann F. Görgens | jgorgens@sun.ac.za | ✓Review of chapter<br>✓Co-ordination of collaboration | 30<br>50 |



..... October 2018 .....

**Signiture of candidate** **Date**

### Declaration by co-authors

All authors read and approved the final manuscript and hereby confirm that:

I. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to **Chapter 7**, pages 220 – 275 in the dissertation,

II. no other authors contributed to **Chapter 7**, pages 220 – 275 in the dissertation beside those specified above, and

III. potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in **Chapter 7**, page numbers 220 – 275 of this dissertation.



# Cellulose $III_I$-activation of AFEX™ and StEx-treated Sugarcane Residue Pellets for Low Enzyme Loading Ethanol Production in Centralized Biorefineries

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## Abstract

In this study, we studied the potential upgrading of StEx or AFEX™ treated sugarcane residue pellets using a room temperature cellulose $III_I$-activation process to facilitate high ethanol yield production at low enzyme dosages. Both StEx and AFEX™ facilitated the production of sugarcane bagasse (SCB) and cane leaf matter (CLM) pellets that were characterized by significantly higher bulk density, mechanical durability and hydrophobicity relative to their untreated pellet controls. However, ethanol production from these StEx and AFEX™-treated SCB and CLM pellets required enzyme dosages greater than 21 mg/g glucan to achieve enzymatic hydrolysis yields and ethanol titres greater than 75% and 40 $g.L^{-1}$, respectively. Coupling the AFEX™-treated SCB or CLM pellets with a room temperature $CIII_I$-activation step lowered enzyme dosage requirements by more than 50%, whilst maintaining ethanol yields greater than 300 L per Mg untreated dry biomass. In contrast, upgrading StEx-treated pellets with $CIII_I$-activation did not result in ethanol yields that were comparable to the AFEX™ treated pellets under enzyme limited conditions (~10 mg/g glucan). A gross energy conversion assessment revealed that low enzyme dosage ethanol and electricity co-production from AFEX™ + $CIII_I$-activated SCB and CLM can recover up to 73% of the energy in the untreated biomass, compared to 54% recovered by StEx + $CIII_I$-activation. The results of this work suggest that StEx or AFEX™ based



pre-processing depots can produce dense and mechanically durable biomass pellets that can be easily upgraded using a room temperature $CIII_I$-activation step to reduce bioconversion enzyme dosage requirements for industrially relevant ethanol production.

**Keywords:** AFEX™, Steam explosion, Uniform Feedstock Supply, Enzyme dosage, Ethanol; Sugarcane residues



## 7.1 Introduction

Meeting future biofuel production targets that are necessary for the industry to substantially contribute to the global energy and sustainability challenges, will require mass mobilization of cellulosic biomass [61]. To supply a regional or national bioeconomy, feedstock supply logistics will need to confront and manage unfavourable biomass characteristics, *i.e.* low bulk density, geographical dispersion, and variable moisture content and chemical composition [228]. It is well documented that biomass transportation and storage costs limit the size of prospective biorefineries, preventing them from achieving the economies of scale necessary to significantly reduce biofuel prices per unit volume produced [7,229,230]. Recent efforts have focused on de-coupling feedstock supply and conversion in view of developing uniform feedstock supply systems that produce commodity-type and infrastructure compatible bulk solid lignocellulosic biomass [7,231].

Sugarcane crop residues (including sugarcane bagasse (SCB) and cane leaf matter (CLM)) are major agricultural residues mostly planted in developing countries (Brazil, India, China, Thailand, Sub-Saharan Africa), with a global annual production estimated at 800 million metric tons per annum [94]. Sugarcane residues typically benefit from sharing “field-to-sugar mill” feedstock supply and handling infrastructure with the existing sugar production process [64]. Establishing decentralized supply chain networks through annexing pre-processing depots to existing sugar mills presents an opportunity to minimise capital and operating costs by leveraging integration benefits [230]. For depots annexed to sugar mills, sugarcane residues would be pretreated and densified to form uniform biomass intermediates prior to being transported to a central biorefinery for upgrading into biofuels or other commodity markets (*e.g.* animal feeds, biochemicals). The densified biomass can, therefore, be blended with other uniform feedstocks and/or be transported long distances to centralized biorefineries with processing capabilities of large feedstock intake, allowing for lower biomass transportation costs, and production cost reductions by economies of scale [229,231]. This creates a system that mimics the existing commodity grain model and facilitates a scenario whereby sugarcane



mill owners would be the suppliers of conversion-ready intermediates that have favourable physical properties for storage and transportation to multiple markets.

Previous work has shown that well-studied technologies StEx and AFEX™ are effective in simultaneously activating biomass binding properties for easier densification and enhancing fungal enzyme accessibility to carbohydrates embedded in the plant cell wall (particularly in herbaceous monocots) [30,321–323]. However, for efficient enzymatic hydrolysis, the requirement of high enzyme dosages (~ 25 mg protein/g glucan) to achieve high carbohydrate-to-sugar conversions (> 75%) from StEx-or AFEX™-treated sugarcane residues presents a significant bottleneck to their value as cellulosic ethanol feedstock for prospective StEx or AFEX™-based uniform-feedstock supply systems [267].

Previous studies have debottlenecked the enzyme dosage requirements from agricultural residues such as corn stover by transforming the native allomorph of cellulose (cellulose I, $CI_\beta$) to the highly digestible allomorph cellulose $III_I$. da Costa Sousa *et al.,* [140] developed a single-step extractive ammonia (EA) process that demonstrated 60% enzyme dosage reduction in high solids loading enzymatic hydrolysis relative to standalone AFEX™. This technology used liquid ammonia in the presence of low amounts of water (~10 %) to combine the benefits of cleaving lignin-carbohydrate crosslinks via ammonolysis, the selective extraction of lignin, and the formation of $CIII_I$. However, drawbacks of the EA technology included the requirement external heating, high pressure operating conditions (~86 bar) and high ammonia-to-biomass loadings (6 g $NH_3$/g DM), which translated to high capital and operating costs for high pressure equipment and downstream ammonia recovery operations. Liquid ammonia is known to facilitate the transformation of native monocot cellulose to $CIII_I$ even room temperature [126,324]. Hence, there lies an opportunity to exploit its potential by evaluating its ability to upgrade the cellulose allomorph of AFEX™ or StEx-treated biomass pellets to $CIII_I$ in view of reducing the enzyme dosages required to achieve high hydrolysis and ethanol yields. Moreover, the use of densified biomass presents a potential solution for reducing ammonia-to-biomass loadings required to completely submerge the biomass in liquid ammonia, hence reducing



pretreatment capital costs and operating costs required to form $CIII_I$ [130]. The process of transforming the crystalline allomorph of native $CI_\beta$ to $CIII_I$ using liquid ammonia at room temperature and low pressure is herein referred to $CIII_I$-activation.

In the present work, we explore a biorefinery concept whereby StEx- or AFEX$^{TM}$ are adopted as technologies in depots annexed to existing sugar/ethanol mills to produce SCB and CLM pellets that are physically and mechanically stable for prospective uniform feedstock biofuel production systems (Figure 7.1). In this approach, we considered a decentralized feedstock supply system whereby pretreated sugarcane residue pellets are transported to large-scale centralized biorefineries and converted to cellulosic ethanol via a $CIII_I$-activation step, with the residual solids from enzymatic hydrolysis used to coproduce energy for the biorefinery.

First, we investigated the effect of AFEX$^{TM}$ or StEx pretreatment on the physical and mechanical properties of SCB and CLM produced using a single-pass pilot-scale pellet mill and compared them with literature reported values for compacted SCB piles, CLM bales, and corn grains. Thereafter, we evaluated the impact of upgrading StEx and AFEX$^{TM}$-treated pellets using a $CIII_I$-activation process in view of reducing the enzyme dosage requirements for efficient high solids loading enzymatic hydrolysis and fermentation of SCB and CLM. Lastly, we performed an energy conversion assessment to evaluate the potential recovery of the inlet feedstock heat of combustion in ethanol and electricity equivalent energy for the low enzyme dosage StEx/AFEX$^{TM}$ coupled with $CIII_I$-activation ethanol and electricity co-production scenario. The results from this work provide insights into the production of fungible sugarcane residue pellets for prospective uniform feedstock biorefineries that are aimed at catalysing feedstock supply chain development, whilst reducing ethanol production sensitivity to variable enzyme on-site production or off-site purchase related costs.

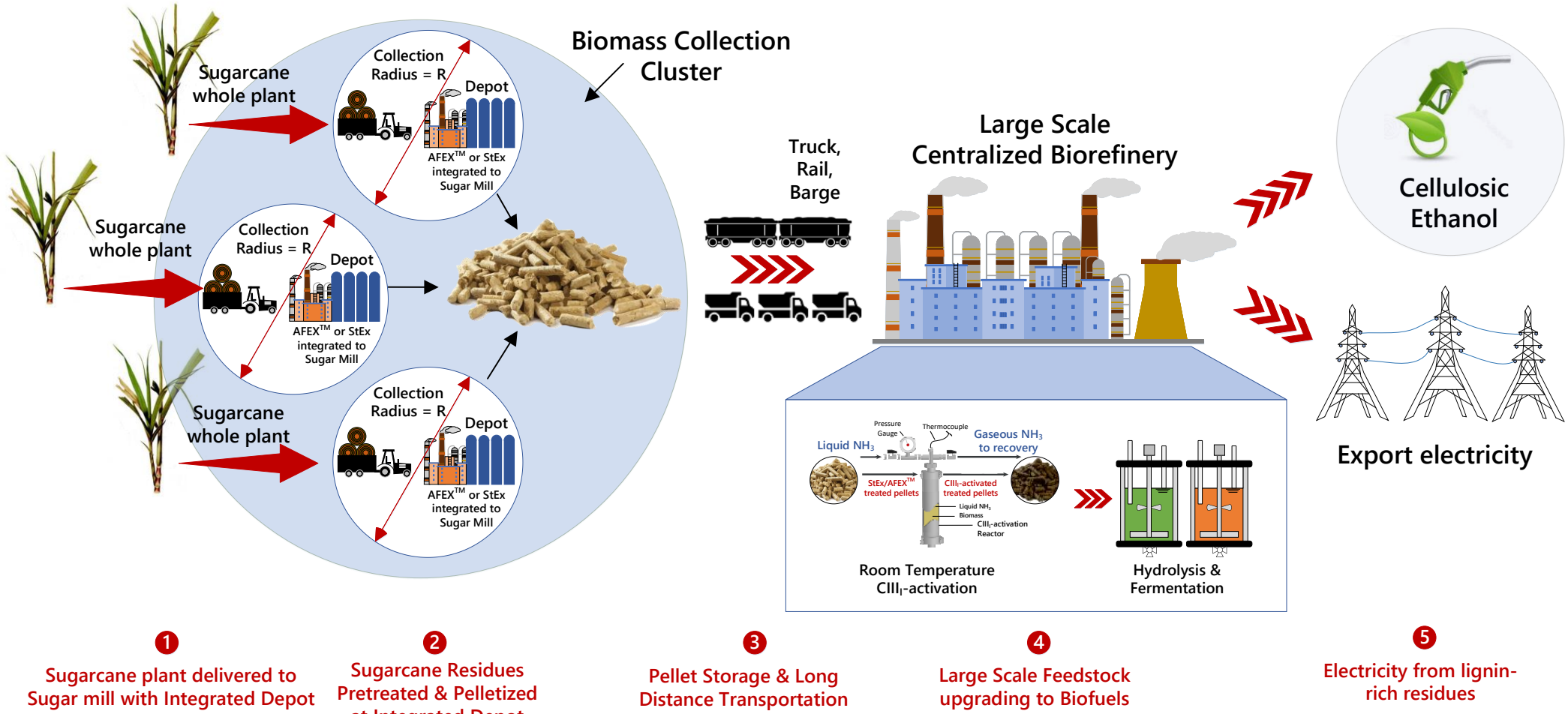



***Figure 7.1:*** Integrating StEx or AFEX$^{TM}$ based decentralized depots to sugar mills for uniform feedstock supply biofuel production systems to service large-scale cellulosic ethanol biorefineries

## 7.2 Materials and Methods

### 7.2.1 *Biomass, Pilot-scale AFEX™- and StEx-pretreatment*

Stockpiled sugarcane bagasse and manually harvested cane leaf matter (green leaves, tops and trash) were collected in the spring season of 2014 from two sugar mills located in Malelane (TSB Sugar, South Africa) and Mount Edgecombe (SASRI, South Africa) and prepared as previously described [267]. The biomass chemical composition was determined according to standard National Renewable Energy Laboratory (NREL, Golden, CO, USA) protocols NREL/TP-510-42618 and NREL/TP-510-42620.

Pilot-scale AFEX™ was performed in a pair of vertical 450 L packed bed reactors (MBI International, Lansing USA) using a protocol previously described in **CHAPTER 5** (section 5.2.2). SCB and CLM were pretreated in separate baskets but the same reactor at the following conditions: 0.7 g NH3/g DM ammonia to biomass ratio, 60% moisture content, 80 – 120 °C, and 60 min reaction time. After AFEX™ pretreatment, residual ammonia was removed from the biomass via low-pressure steam stripping. The pretreated SCB and CLM were transferred to separate burlap sacks and dried to 15% moisture content in a convection oven (Grieve Corporation, IL) to prevent biomass spoilage. Steam explosion was conducted in a 19 L automated batch pilot scale unit (IAP, GmBH, Graz, Austria) equipped with a 100 L blow tank and a steam generator. The StEx pretreatment protocol and pretreatment conditions applied for SCB and CLM were described in **CHAPTER 4** (section 4.2.3). Unwashed StEx SCB and CLM samples were dried to 15% moisture content in a convection oven at 35 °C prior to pelletization.

### 7.2.2 *Biomass pelletization*

Untreated, StEx (non-washed solids) and AFEX™-treated SCB and CLM were pelletized using a Buskirk Engineering PM810 (Ossian, IN) pellet mill equipped with a flat die (aspect ratio 1:6) and two rollers operating at 70 rpm as previously described [273]. Briefly, untreated SCB or CLM samples were recycled through the pellet mill to preheat the pellet die to a minimum temperature of 70 °C. Once the die was preheated, moist biomass (adjusted 20% moisture content) was manually fed into the

pellet mill hopper and the pellets were collected in 20 L buckets before being cooled on a perforated metal tray at room temperature. No external binder was added as pellet adhesive. AFEX™- and StEx-treated samples were passed through the pellet mill once, whereas the untreated samples were recycled at least two times to ensure pellet formation. The cooled pellets were dried at 45 °C in a convection oven to moisture less than 10% and subsequently stored at 4 °C in heat sealed bags until use.

### 7.2.3 *Cellulose $III_I$-activation*

$CIII_I$-activation was conducted in three parallel 820 mL stainless steel tubular reactors equipped with a heating jacket, a PID controller for temperature control and pressure sensors as previously described by da Costa Sousa *et al.,* [140] (Fig S7.1, Supplementary Information). The tubular reactors were loaded with 155 grams (dry basis) of StEx or AFEX™-treated SCB and CLM pellets without adjusting their equilibrium moisture content. Anhydrous liquid ammonia was gravimetrically loaded into the tubular reactors to an ammonia to biomass ratio equivalent to 0.75 g $NH_3$/g DM. Immediately after loading ammonia, the reactors were heated to 25 °C and allowed to soak for 180 min to ensure $CIII_I$-formation. For the duration of the pretreatment, the reactor pressure fluctuated between 9 and 12 bar (absolute). After the pretreatment time had elapsed, the reactor was heated to 40 °C and maintained at that temperature for 10 min before an overhead valve at the top of the reactor was opened to release ammonia gas into an extraction hood. The $CIII_I$-activated biomass was transferred from the reactors to a stainless-steel tray and placed in the hood overnight to remove any residual ammonia. $CIII_I$-activation was performed in duplicate for each biomass. To determine the amount of ammonia chemically bound to the biomass due to $CIII_I$-activation, the nitrogen content of the standalone StEx/AFEX™-treated SCB and CLM pellets and $CIII_I$-activated StEx/AFEX™ SCB and CLM pellets was quantified using the Kjeldahl nitrogen analysis method.

A $CIII_I$ standard was prepared from microcrystalline cellulose I (Avicel PH-101, Sigma Aldrich, St. Louis, MO) using anhydrous liquid ammonia in a high-pressure stirred batch reactor (HEL Inc.,



Borehamwood, UK). $CIII_I$ was formed at an ammonia to biomass loading of 6 g $NH_3$/g DM, 90 °C for 30min residence time [79]. The $CIII_I$-activated Avicel was stored at 4 °C zipped bags prior to use. Evidence of $CIII_I$ formation was confirmed by X-Ray diffraction (described below).

## 7.2.4 *Pellet property characterization*

### 7.2.4.1 *Physical and mechanical properties*

The pellet particle density was determined by measuring the weight of individual pellets to the nearest 0.001 gram and dividing it by its volume, which was measured using digital calliper (Model IP61, Mitutoyo, USA). The pellet unit density was replicated for a representative sample size of 75 pellets to determine the consistency of the pellets produced by the pellet mill under the pseudo-steady state operating conditions. The bulk density was measured by filling a 500 mL beaker with pellets/loose material until it was overflowing. Excess material was removed by striking a straight edge across the top of the beaker. The bulk density was calculated as the weight of the material in the beaker divided by the volume of the beaker. The bulk density measurements were performed in quintuplicate for each sample. The percentage of fines caused by the inefficient pelletization or pellet disintegration at the pellet mill outlet were measured by sieving 500 grams of the pellets collected at the pellet outlet during pseudo-steady state operation through a #7 size wire-cloth mesh and measuring the weight of the retained pellets (ASTM Standard E11-87). The pellet durability index (PDI) was measured according to the ASAE S269.5 standard using a Seedburo pellet durability tester (Seedburo Equipment Company, Des Plaines, IL, USA). Briefly, 500g of fines-free pellets were tumbled in a dust-tight metal box for 10 min at 50 rpm and then sieved through a #3 ½ size wire-cloth mesh to remove the generated fines (ASTM Standard E11-87). The pellet durability index (PDI, %) was calculated as the weight of the pellets retained on the sieve after tumbling divided by their initial weight before tumbling. The water retention value (WRV) was determined to estimate the water holding capacity of pretreated pellets and their non-densified equivalents using the modified SCAN-C 62:00 standard protocol previously described by Bals *et al.,* [321].

#### 7.2.4.2 *Proximate analysis, Ultimate analysis and calorific value*

Proximate analysis was performed by means of thermogravimetric analysis (TGA/DSC 1 Star Systems, Mettler Toledo) to determine the volatile matter content (VM), fixed carbon content (FC) and ash contents of untreated, AFEX$^{TM}$-treated, StEx-treated samples according to ASTM method 1131. Pellet elemental analysis was conducted using a Vario EL Cube elemental analyser (Elementar GmBH, Germany). The biomass higher heating value (HHV) was measured using a bomb calorimeter (Cal2k Eco Calorimeter, RSA), which was previously calibrated with benzoic acid, according to ASTM standard D5865-11a.

#### 7.2.4.3 *X-Ray Diffraction (XRD)*

XRD was carried out in an X-Ray powder diffractometer with its beam parallelized by a global mirror (D8 Advance with Lynxeye detector, Bruker AXS Inc., MI) as previously described by Sousa *et al.,* [140]. Briefly, approximately 0.5 g of biomass samples were mounted in a four circle PMMA goniometer with 25 mm diameter and 8.5 mm height, rotating at 5°/min during analysis. Cu Kα radiation (wavelength = 1.5418 Å) was generated by a rotating Cu anode at 40 kV and 40mA. Samples were scanned using a coupled 2θ/θ scan type with 2θ in the range 8.00°-30.03° at increments of 0.0215°, while θ ranged from 4.00° - 15.014° with increments of 0.0107°.

### 7.2.5 *Ethanol production from AFEX$^{TM}$/StEx pellets*

#### 7.2.7.1 *Low solids loading enzymatic hydrolysis*

Low solids loading enzymatic hydrolysis was conducted to determine the digestibility of StEx- and AFEX$^{TM}$-treated SCB and CLM pellets compared to CIII$_I$-activated StEx/AFEX$^{TM}$ SCB and CLM pellets. Enzymatic hydrolysis was performed in 20 mL screw cap scintillation vials at 1% glucan loading using 15 mg enzyme mixture per gram glucan and incubated at 50 °C, pH 5.0 for 72 h in an orbital shaker (New Brunswick, Scientific, USA). The enzyme mixtures used for StEx and AFEX$^{TM}$-treated SCB and CLM consisted of previously optimized combinations of commercial fungal enzyme preparations Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP [267]. These enzymes were generously donated by



Novozymes (Franklinton, NC, USA). The protein concentration of each enzyme preparation was estimated using the Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Corporative Inc., Ithaca, NY, USA). After 72 h enzymatic hydrolysis, soluble sugars (mainly glucose and xylose) were quantified using an HPLC equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as previously reported [325].

#### 7.2.7.2 *High solids loading separate hydrolysis and fermentation*

High solids loading enzymatic hydrolysis and fermentation were performed to compare the ethanol yields from standalone AFEX™/StEx pellets relative to $CIII_I$-activated AFEX™/StEx pellets. Enzymatic hydrolysis was performed in 250 mL Erlenmeyer flasks with 100 mL working volume, incubated at 50 °C, pH 5.0 on an orbital shaker adjusted to 250 rpm (New Brunswick, Scientific, USA). Chloramphenicol at 50 mg $L^{-1}$ and phosphate buffer at 50 mM were added to the enzymatic hydrolysis mixtures to minimize bacterial contamination and to maintain the hydrolysis pH, respectively. After the enzymatic hydrolysis period, the slurry was centrifuged at 10,000 x *g* for 30 minutes and the supernatant was fermented with xylose-fermenting *S. cerevisiae* 424A (LNH-ST) (kindly provided by Prof. Nancy W.Y. Ho). Fermentations were performed without external nutrient supplementation using our previously reported inoculum preparation and fermentation procedure [171]. The glucose, xylose, arabinose, ethanol, acetate, lactate and glycerol for the enzymatic hydrolysates and fermentation samples were quantified using the above-mentioned HPLC system operated as previously described [267]. All overall mass balances were conducted using a protocol previously published by Gunawan *et al.,* [255].

#### 7.2.7.3 *Experimental design for high solids loading and fermentation*

A statistical approach was undertaken to determine the minimum enzyme dosage requirements for standalone StEx and AFEX™ pellets to reach minimum enzymatic hydrolysis fermentable sugar yields of 75% and ethanol titers of 40 $g.L^{-1}$. A Box Behnken design of experiments (DOE) was used to establish a functional relationship between three process variables: the enzyme dosage, solids loading

and enzymatic hydrolysis residence time; and four response parameters: the glucose yield, xylose yield, ethanol concentration and ethanol yield for StEx and AFEX$^{TM}$-treated SCB and CLM pellets. A total of 15 experimental data points were generated for each pretreated biomass, including triplicates for the center points, and analyzed in Mintab software (Minitab Inc., State College, PA, USA). The experimental design, process variable boundaries, and experimental data are available in Table S7.1 (Supplementary Information). Full quadratic models, including the main, quadratic and interaction effects, were fitted to the experimental data and subsequently refined to include parameters considered significant by ANOVA ($P$ < 0.05) and their influence of the model predictive ability ($R^2_{predicted}$). The fitted regression models were validated and used to predict range of enzyme dosage, solids loading, and residence time combinations that are required to achieve a minimum combined glucose + xylose yield of 75% and a final ethanol concentration of 40 g.L$^{-1}$.

Sugar and ethanol yields from standalone StEx or AFEX$^{TM}$-treated SCB and CLM were compared with those of CIII$_I$-activated AFEX$^{TM}$/StEx pellets performed at enzyme dosages of 15, 10, and 7.5 mg/g glucan, solids loading that corresponded to 10% polymeric glucan + xylan, and enzymatic hydrolysis residence time of 72 hrs.

### 7.2.6 *Estimating cost of enzyme per unit volume ethanol produced*

The enzyme cost contribution to ethanol production remains one of the main obstacles for cost-competitive ethanol production. Literature estimates for the cost contribution of enzymes to ethanol production vary depending on the cited on-site/off-site enzyme production cost, enzyme dosage used during hydrolysis, and the ethanol yield obtained after fermentation. For comparing the effect of CIII$_I$-activation of StEx/AFEX$^{TM}$ pellets on the enzyme cost contribution, the enzyme cost per liter of ethanol produced was estimated by Equation (1) [326,327]:

$$Enzyme\ cost\ per\ liter\ EtOH\ (\frac{\$}{L\ ethanol}) = \frac{D_{enzyme} * P_{enzyme}}{Y_{ethanol}} \quad (1)$$

where $D_{enzyme}$, $P_{enzyme}$ and $Y_{ethanol}$ are the enzyme dosage (kg protein/Mg RDM), the enzyme production price ($/kg protein), and the ethanol yield (L ethanol/Mg RDM).



### 7.2.7 *Gross energy conversion assessment*

The efficiency of converting the energy equivalent to the heat of combustion in untreated SCB and CLM pellets into ethanol and electricity equivalent energy from standalone StEx/AFEX$^{TM}$ and CIII$_I$-activated StEx/AFEX$^{TM}$ pellets was estimated using Equation (2).

$$EC_i(\%) = \frac{m_i \times HHV_i}{m_{RDM} \times HHV_{RDM}} \times 100 \quad (2)$$

In Equation (2) $EC_i$, $m_i$, and $HHV_i$ represent the gross energy conversion factor of product i relative to untreated SCB or CLM pellet, the mass yield of product i, and the gross calorific value of the chosen product i, whereas $m_{RDM}$ and $HHV_{RDM}$ represent the mass and gross calorific value of the untreated and dry SCB or CLM pellet.

### 7.2.8 *Statistical analysis*

Statistical significance of experimental results was determined through a one-way ANOVA in combination with Tukey's HSD *post hoc* test for multiple comparisons (Minitab Inc., State College, PA, USA). A probability value less than 0.05 ($P < 0.05$) was considered statistically significant.

## 7.3 Results and Discussion

### 7.3.1 *Pellet composition, physical and mechanical properties*

The chemical composition, proximate analysis, ultimate analysis and higher heating values (HHV) of untreated, StEx-treated and AFEX$^{TM}$-treated SCB and CLM pellets are presented in Table 7.1. StEx-treated pellets were characterized by increased cellulose and Klason lignin content, primarily due to the solubilization of hemicelluloses during pretreatment [267]. Consequently, StEx-treated pellets were slightly enriched in fixed carbon and HHV relative to the untreated controls. In contrast, AFEX$^{TM}$-treated pellets were characterized by higher nitrogen content and carbohydrate composition similar to the untreated controls. For AFEX$^{TM}$-treated biomass, the additional nitrogen content is generally chemically linked to the biomass due to ammonolysis reactions that cleave lignin-carbohydrate cross-links that are particularly abundant in agricultural residues [120]. Furthermore, the Klason lignin

content was slightly decreased compared to untreated controls ($P < 0.05$), primarily due to the extraction of water and ethanol soluble lignin aromatics and lignin derived decomposition products (*e.g.* phenolic amides, hydrocinnamic acids) during the characterization of the biomass composition [303].

**Table 7.1:** Chemical composition, proximate, ultimate, and gross calorific value analysis for untreated and pretreated sugarcane residue pellets

| | Untreated Pellets | | Pretreated Pellets | | | |
|---|---|---|---|---|---|---|
| | | | StEx | | AFEX™ | |
| **Biomass** | **SCB** | **CLM** | **SCB** | **CLM** | **SCB** | **CLM** |
| **Composition Analysis (%, dry fuel)** | | | | | | |
| Cellulose | 39.5 $^{C}$ | 37.5 $^{D}$ | 59.4 $^{A}$ | 55.3 $^{B}$ | 39.5 $^{C}$ | 37.5 $^{D}$ |
| Hemicellulose | 29.9 $^{A}$ | 29.8 $^{A}$ | 6.1 $^{D}$ | 10.3 $^{C}$ | 25.7 $^{B}$ | 24.2 $^{B}$ |
| Klason Lignin | 19.4 $^{C}$ | 16.2 $^{D}$ | 29.5 $^{A}$ | 27.3 $^{B}$ | 15.9 $^{E}$ | 14.4 $^{F}$ |
| **Proximate Analysis (%, dry fuel)** | | | | | | |
| % Volatile Matter | 80.4 | 76.3 | 78.7 | 75.0 | 81.3 | 76.2 |
| % Fixed Carbon | 15.4 | 15.1 | 17.1 | 16.1 | 15.9 | 16.4 |
| % Ash | 4.2 | 8.6 | 3.4 | 8.5 | 2.8 | 7.4 |
| **Ultimate Analysis** | | | | | | |
| % C | 45.76 | 43.51 | 48.04 | 46.11 | 46.34 | 43.94 |
| % H | 6.55 | 6.34 | 6.23 | 6.23 | 6.64 | 6.37 |
| % N | 0.30 | 0.41 | 0.31 | 0.38 | 1.46 | 1.55 |
| % S | 0.05 | 0.27 | 0.03 | 0.04 | 0.06 | 0.11 |
| **Gross Calorific Value** | | | | | | |
| Higher heating value (GJ/Mg DM) | 18.5 $^{E}$ | 17.7 $^{F}$ | 19.9 $^{A}$ | 18.9 $^{c}$ | 19.4 $^{B}$ | 18.8 $^{D}$ |

Different superscripts within each row indicate significant differences as determined using one-way ANOVA with post-hoc Tukey's HSD test ($P < 0.05$)

An illustration of the untreated, AFEX™-treated and StEx-treated sugarcane residue pellets relative to corn grains is presented in Figure 7.2, with the corresponding physical and mechanical properties presented in Table 7.2. The geometric mean diameter and height of the biomass pellets were 6.8 ± 0.2 mm and 17.4 ± 5.72 mm, respectively. Both StEx and AFEX™ facilitated the production of pellets that were characterized by high particle and bulk density, high durability and low WRV (or higher hydrophobicity) relative to their untreated controls. StEx and AFEX™ produced SCB and CLM pellets with unit particle and bulk densities that ranged from 1094.1 to 1119.5 kg/m$^3$ and 637.3 to

651.7 kg/m$^3$, respectively, with the latter increasing 4 to 14-fold relative to their loose (non-densified) controls ($P < 0.05$).


**Figure 7.2***:* Illustration of the untreated, AFEX™ pretreated, StEx pretreated SCB and CLM pellets and corn grains recovered from a commercial 1G ethanol production mill. (**a**) Untreated SCB, (**b**), AFEX™-SCM, (**c**) StEx-SCB, (**d**) Untreated CLM, (**e**) AFEX™-CLM, (**f**) StEx-CLM, (**g**) corn grains

The bulk densities of StEx and AFEX™-treated SCB and CLM pellets were slightly lower than those reported for corn grains (700-750 kg/m$^3$) and more than 3-fold and 6-fold higher than those for round CLM bales (183 kg/m$^3$) and compacted stockpiles of SCB (100 kg/m$^3$), respectively [328,329]. At quasi-steady state conditions, the pelletization of untreated SCB and CLM resulted in the collection of 12.1% and 7.7% of the total mass as fines, respectively, significantly higher than those achieved by the pretreated pellets ($P < 0.05$). Fines generated from pelletization can be recycled but are generally undesired as they not only reduce the pelletization throughput capacity but also present health and safety hazards for handling, transportation, and storage operations [323,330]. Further, AFEX™ and StEx pretreatment facilitated the production of pellets with durability indexes greater than 98.2%, potentially minimizing dry matter loss as fines and limiting the explosion risks that are typically associated with handling, transporting, and storing low durable biomass pellets [331].

***Table 7.2:*** A summary of the physical and mechanical properties of untreated, AFEX$^{TM}$-treated and StEx-treated sugarcane residue pellets

| Biomass | Pretreatment | Pellet Dimensions (mm) ‡ | | Bulk Density, $\rho_{bulk}$ (kg DM/m$^3$) † | | Unit density (kg DM/m$^3$) ‡ | Durability (%) † | Fines (%) ψ | Water Retention Value (%) ψ |
|---|---|---|---|---|---|---|---|---|---|
| | | Diameter | Height | Pellet | Loose | Pellet | Pellet | Pellet | Pellet |
| SCB | Untreated | 6.97 | 16.96 | 545.1 $^{C}$ | 63.0 $^{C}$ | 1001.7 $^{C}$ | 92.1 $^{D}$ | 12.1 $^{A}$ | 116.6 $^{B}$ |
| SCB | AFEX$^{TM}$ Treated | 6.74 | 19.53 | 637.3 $^{B}$ | 60.4 $^{C}$ | 1107.7 $^{A,B}$ | 99.1 $^{A}$ | 1.4 $^{C}$ | 36.2 $^{D}$ |
| SCB | Steam Explosion | 6.73 | 16.60 | 644.5 $^{A,B}$ | 132.5 $^{B}$ | 1094.1 $^{B}$ | 98.4 $^{A,B}$ | 1.7 $^{C}$ | 6.9 $^{E}$ |
| CLM | Untreated | 6.99 | 17.72 | 518.0 $^{C}$ | 42.3 $^{D}$ | 966.6 $^{D}$ | 94.8 $^{C}$ | 7.7 $^{B}$ | 129.4 $^{A}$ |
| CLM | AFEX $^{TM}$ Treated | 6.69 | 18.70 | 638.9 $^{B}$ | 44.4 $^{D}$ | 1117.7 $^{A,B}$ | 98.4 $^{A,B}$ | 1.3 $^{C}$ | 58.4 $^{C}$ |
| CLM | Steam Explosion | 6.64 | 16.10 | 651.7 $^{A}$ | 149.6 $^{A}$ | 1119.5 $^{A}$ | 98.2 $^{B}$ | 1.1 $^{C}$ | 14.9 $^{E}$ |
| CLM Bale (Round) [1] | N/A | 800 | 1900 | N/A | 183.0 | N/A | N/A | N/A | N/A |
| Compacted SCB pile [2] | Compaction | N/A | N/A | N/A | 100 | N/A | N/A | N/A | N/A |
| Corn Grain (Shelled) [3] | N/A | N/A | N/A | 700 - 750 | N/A | 900-1270 | 97.5-99.7 | N/A | N/A |

†: n = 5; ‡: n = 75; ψ: n = 3

N/A – Not available

[1] Sarto and Hassuani [328] for round CLM bales; [2] Purchase et al., [329]; [3] Boac *et al.,* [332],

Different superscripts within the same column indicate significant differences as determined using one-way ANOVA with post-hoc Tukey's HSD test ($P < 0.05$)



Improvements in pellet durability due to AFEX™ or StEx pretreatment have previously been linked to the activation of lignin as an intrinsic binder through increasing the lignin content of the biomass, the redistribution of lignin towards the outer cell wall during pretreatment, the reduction of the lignin glass transition temperature due, and the presence of a plasticizer (*e.g.* water) during pelletization [321,323]. At last, AFEX™ and StEx pretreatment significantly reduced the water retention capacity of the sugarcane residues, with StEx-treated samples exhibiting the highest hydrophobicity. Stelte and co-workers [323] reported that the simultaneous action of hemicellulose content reduction and increase in lignin content of StEx-treated samples decreases the amount of available hydroxyl groups that can act as hydrogen bonding sites for water, and therefore increases the hydrophobicity of the biomass [333,334]. As a result, these water-resistant pellets would be more desirable for mitigating pellet swelling and self-heating during biomass storage, relative to untreated but densified controls.

Based on these preliminary findings, it is evident that StEx and AFEX™ pretreatment facilitates the production of more dense, durable and hydrophobic SCB and CLM pellets that can potentially reduce the biomass storage footprint and potentially allow for cheaper long distance transportation relative to bulky untreated sugarcane residues [231]. Furthermore, the pellets can be considered aerobically stable provided they are stored at moisture levels below 15% (wet basis), thereby enabling them to be stored and handled under ambient conditions without significant dry matter losses due to microbial activity [2]. Moreover, with consistent physical properties similar to corn grains, these dense pellets have the potential to be integrated into standardized, high efficiency, and high-volume grain handling infrastructure [5]. Given that sugarcane residues are seasonal, producing stable and dense SCB and CLM pellets provides simpler way of reducing the storage footprint of these residues for year-round availability and supply for large-scale 2G biorefineries [335].



### 7.3.2 *Ethanol production from standalone StEx and AFEX™ pellets*

Quadratic regression models were derived to describe the effect of a wide range of enzymatic hydrolysis parameters on four response variables, *i.e.* the glucose yield, xylose yield, final ethanol concentration from fermentation, and the ethanol yield. The inclusion of the main, interaction and quadratic effects in the final refined model was determined by their degree of significance ($P < 0.05$) and their effect on the model predictive ability ($R^2_{predicted}$). The refined regression equations, residual plots, ANOVA, contour plots, and model validations for each model are presented in Figure S7.3 (Supplementary information). According to ANOVA, all the refined models were sufficient to describe the effect of the enzyme dosage, solids loading and enzymatic hydrolysis residence time on the four response variables for each biomass, as evidenced by the insignificant lack of fit and $R^2_{predicted}$ values above 85%. Further, the validity of the models was confirmed by experimental model validation runs, which were all within 5% of the model predicted values.

An overlay of the contour lines representing the range of enzyme dosages and solids loadings that correspond to a minimum combined glucose + xylose yield of 75% and an ethanol titre of 40 $g.L^{-1}$ are presented in Figure 7.3. The contour line intersection region (shaded area) represents the enzyme and solids loading combinations that lead to combined glucose + xylose yields and ethanol concentrations greater than 75% and 40 $g.L^{-1}$, respectively. At these predefined enzymatic hydrolysis and fermentation targets, the minimum enzyme dosage requirements for standalone AFEX™-SCB, AFEX™-CLM, StEx-SCB, and StEx-CLM correspond to 22.5, 21.5, 25, and 25 mg protein/g glucan, respectively. In general, increasing the solids loading had a negative effect in the glucose and xylose yields as high solids loadings require higher enzyme dosages to maintain the same sugar yield. This phenomenon is typically described as the solids effect, where yield reductions have been previously correlated to the reduction in water activity, mass transport phenomena, end-product inhibition, lignin inhibition, and enzyme inhibition by pretreatment decomposition products [153,254]. Conversely, increasing the solids loading had a positive effect on the final ethanol concentration by

facilitating higher sugar concentrations after enzymatic hydrolysis. Nonetheless, even at enzymatic hydrolysis conditions statistically optimized to minimize the solids effect, enzyme dosages greater than 21.5 mg/g glucan are required for standalone AFEX™/StEx pretreated pellets to achieve high ethanol concentrations and hydrolysis efficiencies.

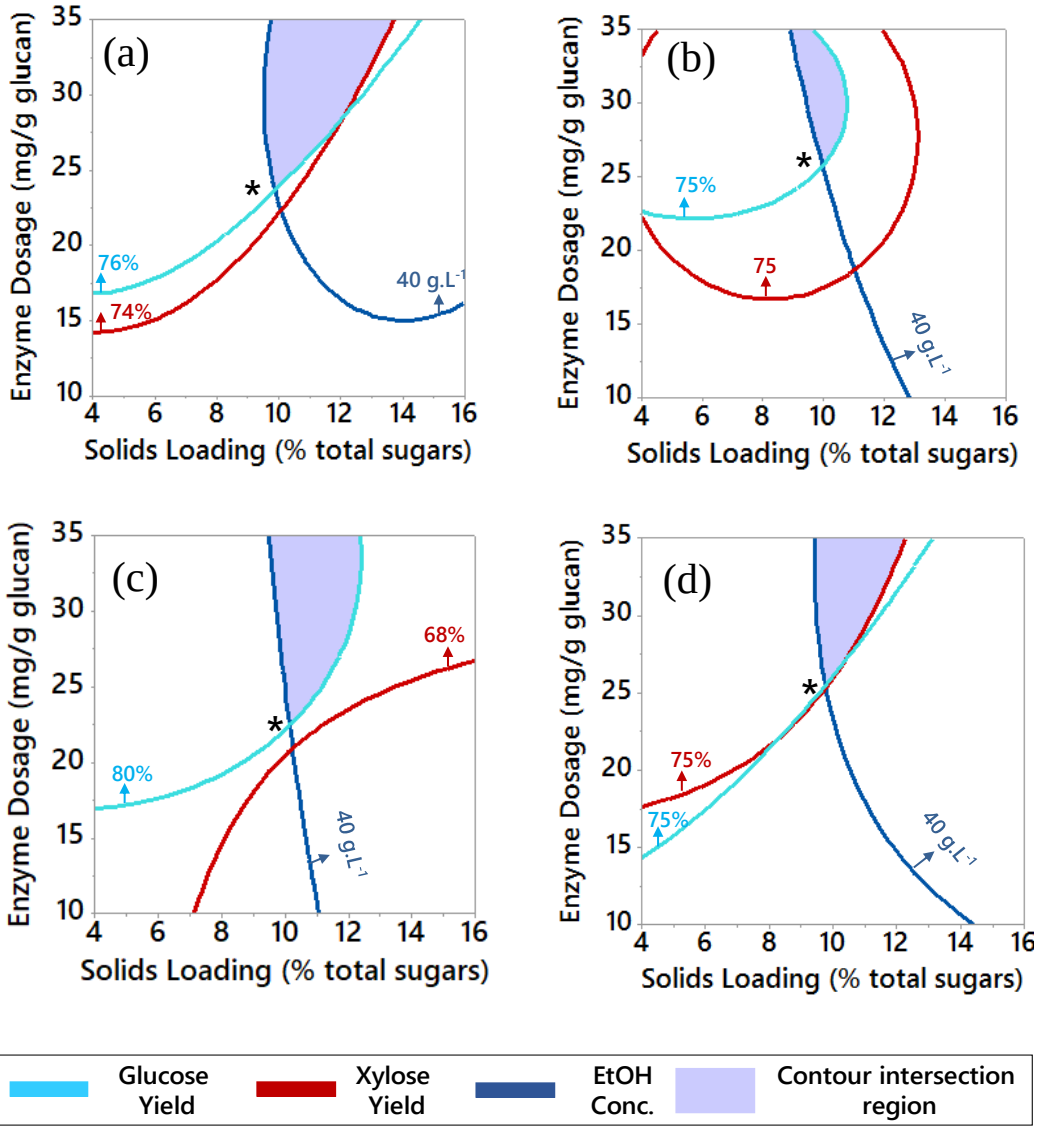



***Figure 7.3:*** Contour plots illustrating the effect of the enzyme dosage and solids loading on the glucose yield, xylose yield and final ethanol concentration. The contour intersection region represents the combinations of enzyme dosage and solids loading that are required to reach minimum combined glucose + xylose yield and ethanol concentrations of 75% and 40 $g.L^{-1}$, respectively. **(a)** – AFEX™-SCB; **(b)** – StEx-SCB; **(c)** – AFEX™-CLM; **(d)** – StEx-CLM.

### 7.3.3 *Upgrading of AFEX$^{TM}$/StEx pellets with CIII$_I$-activation*

Due to high enzyme dosage requirements for standalone StEx or AFEX$^{TM}$ pretreated sugarcane residues, we investigated the potential upgrading of StEx and AFEX$^{TM}$-treated SCB and CLM pellets delivered to a large-scale biorefinery using a low pressure, room temperature, and low ammonia-to-biomass loading CIII$_I$-activation process. The confirmation of the formation of CIII$_I$ from CIII$_I$-activated StEx/AFEX$^{TM}$-treated SCB and CLM pellets was determined qualitatively through the comparison of their XRD spectra to CIII$_I$-controls prepared from microcrystalline cellulose (Avicel PH-101) (Figure 7.4 a – d).

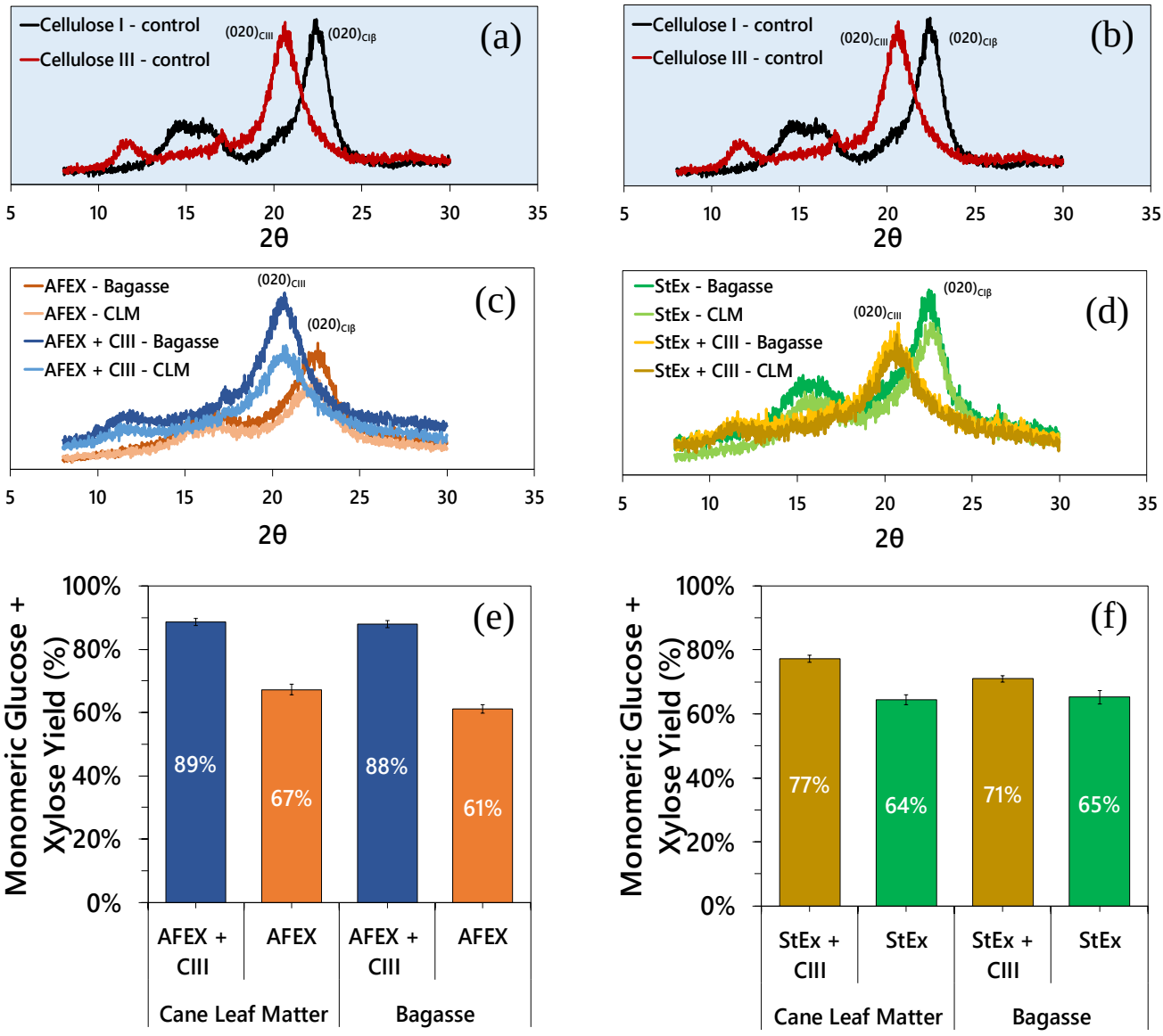



***Figure 7.4:*** XRD spectra confirming CIII$_I$ formation from microcrystalline cellulose (**a,b**), CIII$_I$-activated AFEX$^{TM}$ pellets (**c**), CIII$_I$-activated StEx pellets (**d**). Comparison of low solids loading combined glucose + xylose yields from standalone StEx/AFEX$^{TM}$ and CIII$_I$-activated StEx/AFEX$^{TM}$ pellets (**e,f**).

Consistent with previous literature work, $CIII_I$ was identified by the shift in position of the main cellulose peak (020) from a 2θ value of 22.5° to 20.5° [79,140,324,336]. Conversely, samples treated with standalone StEx or AFEX™ resulted in spectrum similar to microcrystalline Avicel PH-101, indicating that no $CIII_I$ is formed during either of the two standalone pretreatment technologies. The $CIII_I$-activation step submerges cellulose fibers of lignocellulosic biomass in liquid, allowing for ammonia molecules form hydrogen bonds with hydroxyl groups from cellulose, resulting in the formation of an ammonia-cellulose I complex [137,139]. $CIII_I$ is formed once ammonia is removed from the intermediate ammonia-cellulose I complex via vaporization, causing a rewiring of the hydrogen bond network and structural packing of cellulose chains [79,140]. Coupling StEx and AFEX™ with a $CIII_I$-activation step enhanced the low solids loading hydrolysis yields by 33-44% and 9-21%, relative to standalone AFEX™ and StEx pellets, respectively (Figure 7.4 e – f). $CIII_I$ formation using liquid ammonia has been shown to improve the synergistic effects of endocellulases and exocellulases, subsequently enhancing cellulose depolymerization rates by up to 3-fold and enzymatic hydrolysis yields beyond those achieved by cellulose $I_\beta$ and II allomorphs [79]. In agreement with previous work, the low solids loading enzymatic hydrolysis results confirm that even room temperature $CIII_I$-activation facilitates easier cell wall deconstruction by hydrolytic cellulases and hemicellulases [138].

Unlike the EA process which requires high ammonia-to-biomass loadings (6 g $NH_3$/g DM) to completely submerge low bulk density biomass in anhydrous liquid ammonia, $CIII_I$-activation of high bulk density biomass pellets allowed for 8-fold reduction in ammonia-to-biomass loading to facilitate $CIII_I$-formation. As a result, the maximum pressure reached during $CIII_I$-activation was 12 bar (absolute) at room temperature, significantly lower than those required for AFEX™ (21 bar), StEx (18 bar), and EA (86 bar) pretreatment (Figure S7.2, Supplementary Information). Low temperature and pressure systems are generally advantageous for industrial ammonia-based processes where process safety is an important consideration. Moreover, for AFEX™-treated sugarcane residues, $CIII_I$-activation consumed only 1.5 mg/g DM of nitrogen, which would have to be replenished after every cycle, with

the remainder to postulated to be recovered using the same technologies employed for AFEX™ pretreatment (Figure S7.4, Supplementary Information). Hence, minimal nitrogen is chemically linked to the biomass due to ammonolysis, hydrolysis and Maillard reactions during $CIII_I$-activation, potentially ensuring high ammonia recovery for recycling to subsequent pretreatment batches.

### 7.3.4 *Ethanol production from $CIII_I$-activated pellets*

To better understand the potential benefits of enhanced enzymatic hydrolysis efficiency due to $CIII_I$-activation, the potential carbohydrate conversions (monomeric and oligomeric glucose + xylan) and ethanol yields that can be recovered from $CIII_I$-activated pellets were studied under industrially relevant solids loadings. Figure 7.5 demonstrates the comparative combined glucan + xylan conversions and ethanol yields for $CIII_I$-activated AFEX™/StEx sugarcane residue pellets for a range of enzyme dosages (15 mg/g glucan to 7.5 mg/g glucan) relative to standalone AFEX™/StEx pellets at an enzyme dosage of 25 mg/g glucan. As shown in Figure 7.5-a,b, upgrading of AFEX™-treated SCB and CLM pellets with $CIII_I$-activation enabled the reduction of the enzyme dosage from 25mg/g glucan to 7.5 mg/g glucan for maintaining the same combined glucan + xylan conversions relative to standalone AFEX™. At 10 mg/g glucan (~4–3.7 g protein/kg RDM), coupling AFEX™ with $CIII_I$ resulted in ethanol yields greater than 300 L/Mg RDM, statistically similar to standalone AFEX™ using an enzyme dosage of 25 mg/g glucan ($P > 0.05$) (Figure 7.5-e,f). Like the EA technology, augmenting AFEX™ with $CIII_I$-activation combines the benefits of the ammonolysis of cell wall esters during AFEX™ pretreatment and the modification glucan chain packing (or cellulose polymorph) during $CIII_I$-activation to enhance substrate digestibility even under enzyme limited conditions. Further, it has also been postulated that the surface of $CIII_I$ is more hydrophilic than $CI_\beta$, hence $CIII_I$-activation may also contribute to the minimization of the "solids effect" phenomenon [126]. However, more than 10% (62 – 87 kg/Mg RDM) of the glucan and xylan hydrolyzed from $CIII_I$-activated AFEX™ SCB and CLM pellets was recovered in oligomeric form, representing an additional 36-49 L of ethanol per Mg RDM that can be recovered with more efficiently designed enzyme cocktails (data not shown) [155,159].

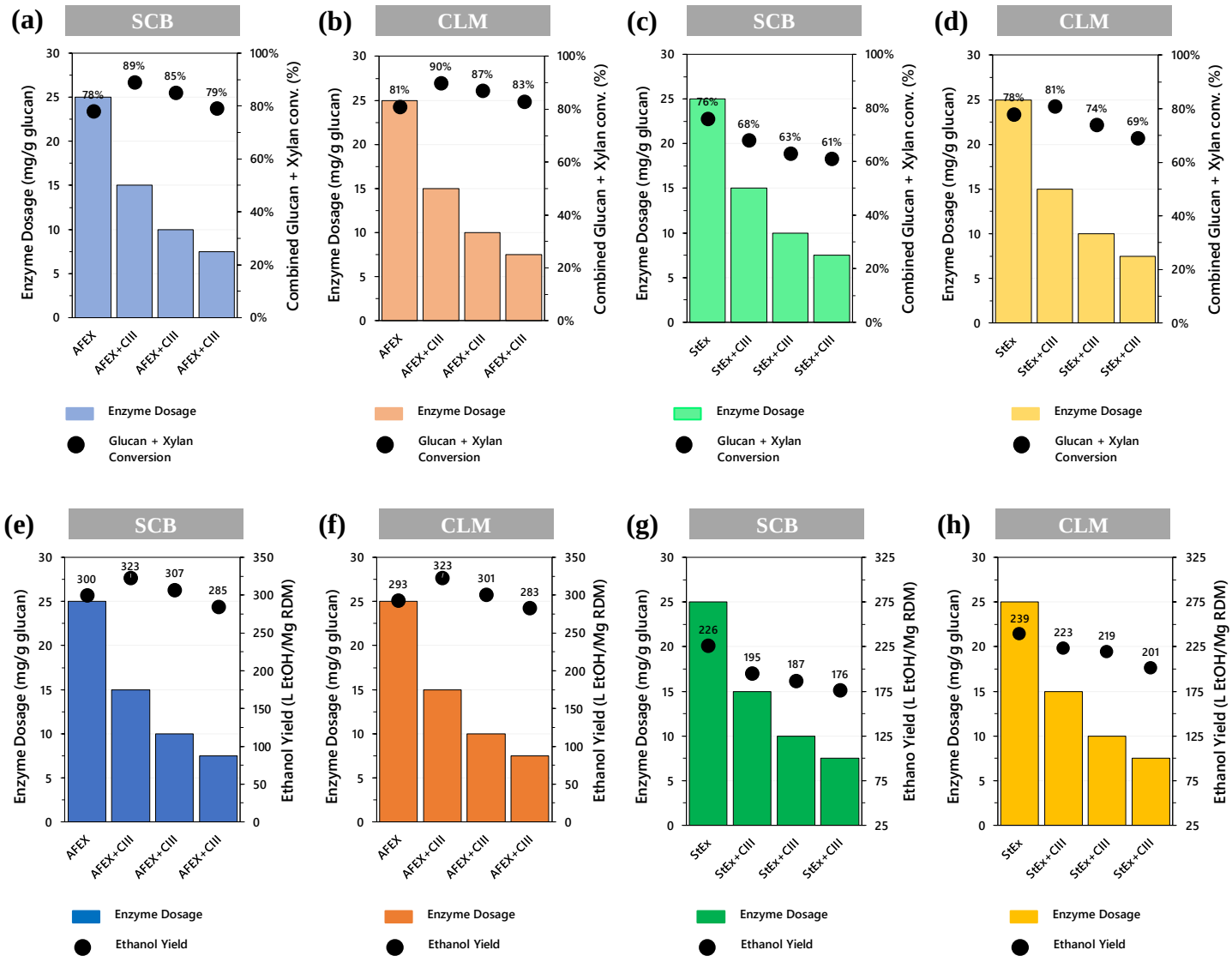



**Figure 7.5:** Comparing the effect of CIII$_I$-activation of AFEX$^{TM}$ and StEx-treated SCB and CLM pellets on **(a-d)** high solids loading enzymatic hydrolysis yields and **(e-f)** the ethanol yield per Mg raw dray feedstock at high and low enzyme dosages.

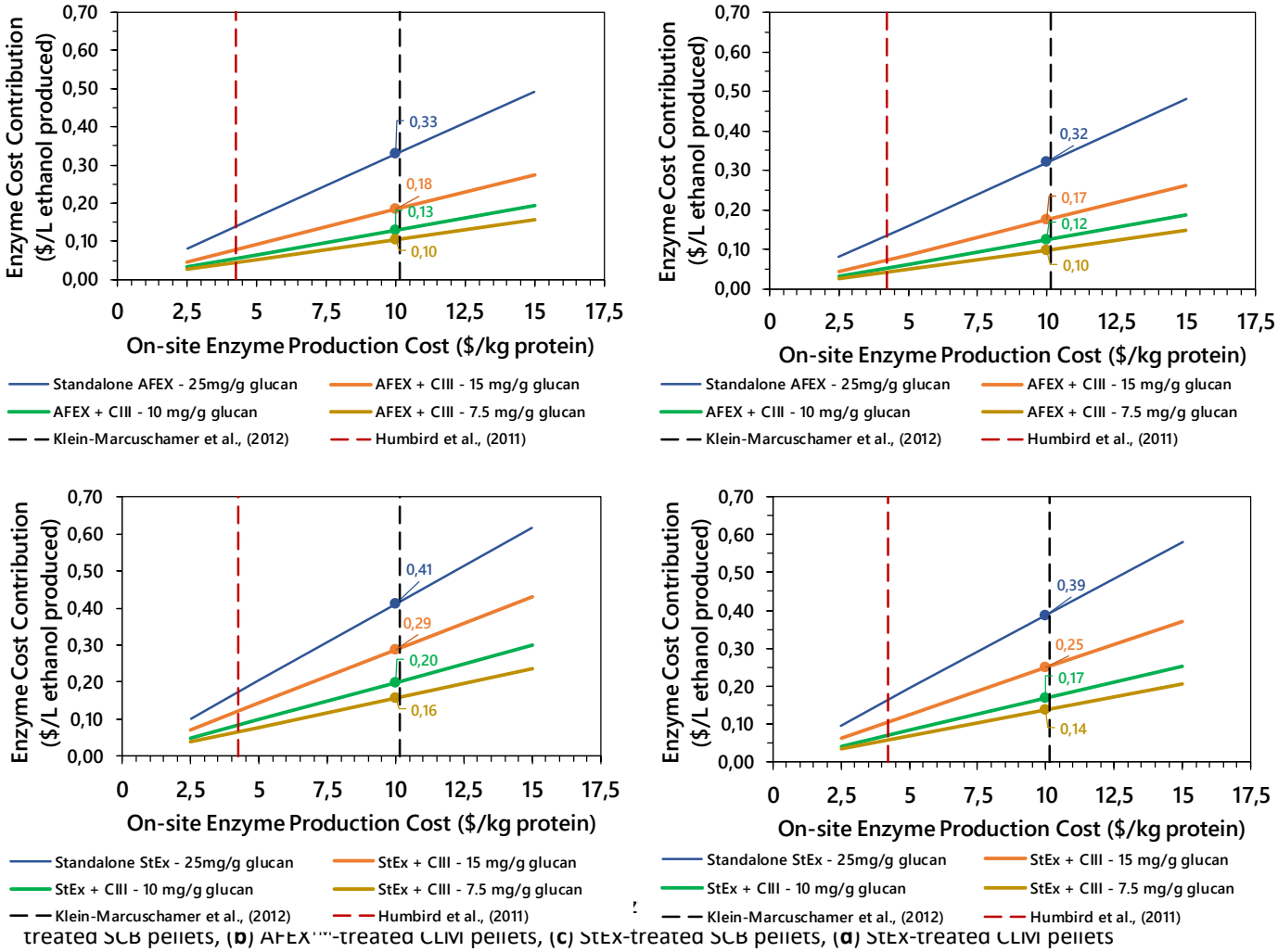
In contrast, augmenting StEx pretreated sugarcane residue pellets with CIII$_I$-activation did not achieve similar carbohydrate conversions and subsequently ethanol yields as observed for AFEX$^{TM}$-treated pellets (Figure 7.5-c,d,g,h). At an enzyme dosage of 10mg/g glucan, CIII$_I$-activated SCB and CLM pellets generated ethanol yields that were 17% and 8.4% lower relative to standalone StEx-treated SCB and CLM pellets at 25mg/g glucan respectively ($P < 0.05$). By removing a significant portion of the hemicellulose fraction of biomass during high temperature pretreatment, StEx not only increased the lignin content of pretreated solids but also facilitated polymerization/condensation reactions that lead to the redeposition of pseudo-lignin compounds on the cell wall surface. In

**CHAPTER 6**, we previously confirmed the presence of pseudo-lignin hydroxyl, carbonyl, and aromatic functional groups in StEx-treated SCB and CLM by ATR-FTIR analysis (see section 6.3.2). These pseudo-lignin type moieties have been demonstrated to limit efficient hydrolytic biomass deconstruction [151].

Recent work has shown that lignin from hydrothermally pretreated corn stover, wheat straw, *Miscanthus* x *giganteus* stalk hindered cellulose hydrolysis by blocking enzyme access to the active cellulose surface binding sites (as a barrier) rather than non-productive binding/adsorption of enzymes [152]. In contrast, Pielhop *et al.,* (2017) found that repolymerized lignin-like structures from autohydrolysis pretreatment of spruce wood significantly intensified enzyme adsorption to lignin and accelerated enzyme deactivation [280,337]. Hence, the lower carbohydrate conversions and ethanol yields recovered in this work can potentially be attributed to substrate blockage by lignin, cellulase deactivation by repolymerized pseudo-lignin at the cell wall surface, and/or enzyme inhibition by soluble products (*i.e.* oligosaccharides, phenolic compounds, furan derivatives, aliphatic acids) generated from pretreatment [267]. A potential solution to this problem could be the redesigning of the StEx pretreatment severity conditions prior to pelletization and the $CIII_I$-activation step. For instance, low severity StEx to facilitate easy pellet formation can be combined with a $CIII_I$-activation step that is augmented with a partial lignin extraction step (akin to the EA process) to minimize the effect of lignin on enzymatic hydrolysis yields at low enzyme dosage conditions [140].

Enzyme cost remains one of the main economic obstacles to cost-competitive cellulosic ethanol production. On-site enzyme production has been predicted to be less expensive than off-site production, even though it amplifies already high capital costs as well as process complexity [54]. With its value often under-estimated, Klein-Marcuschamer and co-workers estimated that on-site enzyme production would cost approximately US$10.4/kg protein, which compares well with the cost of amylase enzymes purchased by the corn ethanol industry (~US$25/kg protein) [326]. Since the enzyme cost contribution (on a $/L EtOH basis) depends on the enzyme dosage (kg protein/Mg RDM),

the ethanol yield (L EtOH/Mg RDM), and the enzyme production or purchase costs (US$/kg protein), the reduction of the enzyme dosage from 25 kg/Mg glucan to 10 kg/Mg glucan facilitated by CIII$_I$-activation of AFEX$^{TM}$-treated SCB and CLM pellets could potentially reduce the enzyme cost contribution to ethanol production from approximately US$0.33/L EtOH to US$0.12/L EtOH (assuming an on-site production cost of US$10.14/kg protein) (Figure 7.6).




treated SCB pellets, (**b**) AFEX$^{TM}$-treated CLM pellets, (**c**) StEx-treated SCB pellets, (**d**) StEx-treated CLM pellets

Similarly, even though enzymatic hydrolysis was less efficient, reducing the enzyme dosage for CIII$_I$-activated StEx SCB and CLM pellets from 25 kg/Mg to 10 kg/Mg could potentially reduce the enzyme cost contribution from US$0.41/L to US$0.17/L. With affordable enzyme dosages and enzyme cost contributions currently estimated at 2 mg protein/g RDM and US$0.066/L, coupling AFEX$^{TM}$ with CIII$_I$-activation process lowers the enzyme dosages to 3.95 – 2.96 mg/g RDM (or 10 – 7.5 mg/g glucan)

and subsequently lowers enzyme cost contribution sensitivity to the cost of on-site enzyme production [100]. This provides a basis for future techno-economic evaluations to determine whether additional capital and operating costs necessary for adding $CIII_I$-activation step at centralized biorefineries would justify the enzyme cost savings achieved through the reduction of enzyme dosage requirements enabled by the modification of the cellulose polymorph.

### 7.3.5 *Energy value of lignin-rich residues for energy cogeneration*

An energy conversion assessment for ethanol production and the equivalent electricity generation from the lignin-rich residual solids is presented in Table 7.3 (next page). Mass balances for each process included in Table 7.3 are available in Figure S7.5 (Supplementary Information). The energy conversion of the heat of combustion energy of the untreated feedstocks to ethanol for standalone AFEX™ and AFEX™ + $CIII_I$-activation was in the range 38-40%, when the enzyme dosage was 25 mg/g glucan and 10 mg/g glucan, respectively. The corresponding HHV values for the lignin-rich solids residues were in the range 20.66–22.91 GJ per dry Mg of lignin residues or 8.35–9.50 GJ per Mg raw dry material. These HHVs are approximately 77–87% of pure lignin (26.8 GJ/MJ), and comparable with sub-bituminous C (19.3–22.1 GJ/Mg) and sub-bituminous B (22.1–24.4 GJ/Mg) grade coal (according to ASTM D 388 coal ranking standard). Assuming 1 GJ of lignin reside HHV generates a theoretical equivalent of 277.8 kWh of electricity [338], a boiler efficiency of 80% and a turbo generator efficiency of 85% [92], the combustion of AFEX™ and AFEX™ + $CIII_I$ lignin residues has the potential to generate an electricity equivalent of 1576– 1795 kWh per Mg raw dry sugarcane residues or 2750 –3304 kWh per Mg of ethanol produced.



**Table 7.3:** Energy conversion assessment for ethanol and electricity co-production from high enzyme dosage standalone StEx/AFEX$^{TM}$ pellets and low enzyme dosage CIII$_I$-activated StEx/AFEX$^{TM}$ SCB and CLM pellets

| Parameter | Standalone | | | | Standalone + CIII$_I$-activation | | | |
|---|---|---|---|---|---|---|---|---|
| | AFEX$^{TM}$ - SCB | AFEX$^{TM}$ - CLM | StEx - SCB | StEx - CLM | AFEX$^{TM}$ - SCB | AFEX$^{TM}$ - CLM | StEx - SCB | StEx - CLM |
| Enzyme Dosage (mg protein/g glucan) | 25 | 25 | 25 | 25 | 10 | 10 | 15 | 15 |
| Residence Time (hours) | 96 | 96 | 96 | 96 | 72 | 72 | 72 | 72 |
| Enzymatic Hydrolysis Monomeric Glucose Yield (%) | 75.7 $^{D}$ | 80.0 $^{B}$ | 74.4 $^{D}$ | 77.0 $^{C}$ | 78.4 $^{B,C}$ | 82.5 $^{A}$ | 62.1 $^{F}$ | 70.1 $^{E}$ |
| Enzymatic Hydrolysis Monomeric Xylose Yield (%) | 73.1 $^{A}$ | 72.4 $^{A,B}$ | 74.2 $^{A}$ | 73.6 $^{A}$ | 64.9 $^{C}$ | 66.6 $^{C}$ | 70.0 $^{B}$ | 71.0 $^{B}$ |
| Final Ethanol Concentration (g/L) | 41.7 $^{C}$ | 42.3 $^{B,C}$ | 41.8 $^{B,C}$ | 44.0 $^{A}$ | 41.4 $^{B,C}$ | 42.9 $^{A,B}$ | 36.8 $^{D}$ | 40.1 $^{B,C}$ |
| EtOH Yield (L EtOH/Mg RDM) | 300.0 $^{A,B}$ | 292.8 $^{B}$ | 226.6 $^{D}$ | 238.7 $^{C}$ | 307.5 $^{A}$ | 300.7 $^{A}$ | 194.9 $^{E}$ | 222.9 $^{D}$ |
| **Ethanol Energy Conversion Factor ‡** | **38%** | **39%** | **29%** | **32%** | **39%** | **40%** | **25%** | **30%** |
| Lignin Residue Yield (Mg dry residues/Mg RDM) | 0.436 $^{A}$ | 0.404 $^{B}$ | 0.295 $^{E}$ | 0.313 $^{D}$ | 0.395 $^{B}$ | 0.405 $^{B}$ | 0.355 $^{C}$ | 0.302 $^{D,E}$ |
| Lignin Residue Yield (Mg dry residues/Mg EtOH) | 1.84 $^{B}$ | 1.75 $^{B}$ | 1.65 $^{C,D}$ | 1.81 $^{B}$ | 1.63 $^{D}$ | 1.69 $^{C,D}$ | 2.31 $^{A}$ | 1.72 $^{C}$ |
| Lignin Residue HHV (GJ/Mg dry residues) | 21.80 $^{C}$ | 20.66 $^{E}$ | 22.35 $^{B}$ | 21.03 $^{D}$ | 22.91 $^{A}$ | 21.27 $^{D}$ | 22.16 $^{B}$ | 20.50 $^{E}$ |
| Potential Energy from Lignin Residues (GJ/Mg RDM) | 9.50 $^{A}$ | 8.35 $^{C}$ | 6.59 $^{E}$ | 6.58 $^{E}$ | 9.05 $^{B}$ | 8.61 $^{C}$ | 7.87 $^{D}$ | 6.19 $^{F}$ |
| Electricity Equivalent (kWh/Mg RDM) † | 1795.5 | 1576.4 | 1245.5 | 1587.4 | 1709.5 | 1627.3 | 1486.1 | 1169.5 |
| Electricity Equivalent (kWh/Mg EtOH) | 3303.6 | 2758.7 | 2055.0 | 2872.6 | 2786.5 | 2750.1 | 3438.8 | 2011.6 |
| **Electricity cogeneration Conversion Factor ‡** | **35%** | **32%** | **24%** | **27%** | **33%** | **33%** | **29%** | **24%** |
| **Combined Ethanol + Electricity Conversion Factor ‡** | **73%** | **71%** | **53%** | **59%** | **72%** | **73%** | **54%** | **54%** |

‡ Energy conversion efficiency as percentage of feedstock higher heating value.

† For electricity production from biomass, a boiler efficiency of 80% and an isentropic turbo generator efficiency of 85% were assumed [92]. 1 GJ of biomass calorific value was assumed to be equivalent to 277.8 kWh of electricity [338]. Calculated as: Electricity Equivalent = Lignin Residue Yield x Lignin Residue HHV x 277.8 x 0.85 x 0.8

Different superscripts within the same row indicate significant differences as determined using one-way ANOVA with *post-hoc* Tukey's HSD test ($P < 0.05$)

Depending on the size of the biorefinery, local regulations, and the price of bio-electricity, the produced electricity would supply the energy demand of the biorefinery with any excess electricity sold to the local or national grid [339]. The combination of ethanol and electricity production from high enzyme dosage standalone AFEX™ or low enzyme dosage AFEX™ + $CIII_I$ recovered approximately 71 – 73% of the heat of combustion of the inlet sugarcane residues.

The ethanol production energy efficiency for standalone StEx and StEx + $CIII_I$ was in the range 25–32%, when the enzyme dosage was 25 mg/g glucan and 15 mg/g glucan, respectively. Like the AFEX™-treated residues, the HHVs of StEx and StEx + $CIII_I$ lignin residues were within high volatile sub-bituminous B grade coal range. With lignin residue yields of 0.295–0.355 Mg dry lignin residues per Mg RDM, an electricity equivalent of 1170–1587 kWh per Mg RDM can potentially be recovered from StEx or StEx + $CIII_I$ lignin residues. The corresponding combined ethanol and electricity production energy conversion efficiencies for standalone StEx and StEx + $CIII_I$ were in the range of 53–59%, significantly lower than those achieved by standalone AFEX™ or AFEX™ + $CIII_I$.

## 7.4 Conclusions

For biofuels to make a meaningful impact on national/global energy and sustainability goals, large scale biomass mobilization and commoditization systems will have to be put in place. In this work, we demonstrated that integrating StEx or AFEX™ based depots to sugar mills can produce dense and conversion-ready sugarcane residue pellets with bulk densities and mechanical durability similar to corn grains, thereby enabling effective storage and long-distance biomass transportation. Coupling AFEX™-treated SCB and CLM pellets with a room temperature $CIII_I$-activation step allowed for the reduction of the enzyme dosages to by more than 50% (reduced to 4 – 3 mg/g RDM), significantly reducing the enzyme cost contribution per unit volume ethanol produced. In contrast, upgrading StEx-treated pellets with $CIII_I$-activation did not achieve similar enzyme dosage reductions as AFEX™-treated pellets, apparently due to the higher lignin content, which may impede effective enzymatic hydrolysis under low enzyme dosage conditions. An energy conversion assessment revealed that low



enzyme dosage AFEX™ + $CIII_I$ can facilitate up to 73% recovery of the heat of combustion energy in untreated SCB or CLM in ethanol and electricity equivalent energy, whereas StEx + $CIII_I$ could only recover up to 59%. For future large-scale sugarcane based biorefineries with uniform feedstock supply systems, the results of this work presented insights into the potential integration of StEx and AFEX™ into sugar/ethanol mills for the preparation of stable, consistent, and conversion ready biomass pellets. Moreover, we outlined the benefits of upgrading StEx- and AFEX™-treated pellets using a low pressure $CIII_I$-activation process in view of minimizing the sensitivity of these prospective biorefineries to variable costs associated with enzyme production.



# 7.5 Supplementary Information – CHAPTER 7

**Table S7.1:** Enzymatic hydrolysis conditions for AFEX™-SCB defined by Box-Behnken DOE and corresponding experimental results used for statistical analysis.

| Box Behnken Design of Experiments - AFEX™-SCB | | | | | | | |
|---|---|---|---|---|---|---|---|
| Run. No | Solids loading (% total sugars) | Enzyme Dosage (mg/g protein) | Residence Time (hrs) | % Glucan Conv. | % Xylan Conv. | EtOH conc. ($g.L^{-1}$) | EtOH Yield (kg/100kg RDM) |
| 1 | 5 | 15 | 72 | 72,2 | 73,7 | 16,3 | 21,6 |
| 6 | 15 | 22,5 | 24 | 54,1 | 50,9 | 42,6 | 14,1 |
| 14 | 10 | 22,5 | 72 | 74,3 | 72,3 | 39,3 | 23,6 |
| 4 | 15 | 30 | 72 | 71,6 | 69,1 | 46,9 | 17,4 |
| 7 | 5 | 22,5 | 120 | 77,0 | 74,8 | 18,9 | 22,6 |
| 10 | 10 | 30 | 24 | 70,8 | 66,4 | 37,3 | 21,5 |
| 11 | 10 | 15 | 120 | 71,8 | 71,4 | 35,9 | 22,1 |
| 2 | 15 | 15 | 72 | 57,8 | 58,5 | 39,4 | 14,6 |
| 3 | 5 | 30 | 72 | 82,4 | 80,9 | 19,1 | 24,0 |
| 5 | 5 | 22,5 | 24 | 68,3 | 67,8 | 17,0 | 20,5 |
| 8 | 15 | 22,5 | 120 | 66,1 | 65,0 | 45,2 | 16,0 |
| 13 | 10 | 22,5 | 72 | 73,6 | 72,1 | 38,7 | 22,5 |
| 9 | 10 | 15 | 24 | 56,0 | 56,4 | 30,5 | 17,2 |
| 15 | 10 | 22,5 | 72 | 74,2 | 72,7 | 39,4 | 23,0 |
| 12 | 10 | 30 | 120 | 76,7 | 75,9 | 42,8 | 24,8 |

**Table S7.1-B:** Enzymatic hydrolysis conditions for AFEX™-CLM defined by Box-Behnken DOE and corresponding experimental results used for statistical analysis.

| Box Behnken Design of Experiments - AFEX™-CLM | | | | | | | |
|---|---|---|---|---|---|---|---|
| Run. Order | Solids loading (% total sugars) | Enzyme Dosage (mg/g protein) | Residence Time (hrs) | % Glucan Conv. | % Xylan Conv. | EtOH conc. ($g.L^{-1}$) | EtOH Yield (kg/100kg RDM) |
| 1 | 5 | 15 | 72 | 77,9 | 68,2 | 18,6 | 21,9 |
| 6 | 15 | 22,5 | 24 | 64,8 | 49,6 | 45,7 | 15,6 |
| 14 | 10 | 22,5 | 72 | 80,5 | 69,2 | 39,8 | 22,7 |
| 4 | 15 | 30 | 72 | 76,4 | 69,9 | 49,2 | 18,3 |
| 7 | 5 | 22,5 | 120 | 83,0 | 72,3 | 20,1 | 23,0 |
| 10 | 10 | 30 | 24 | 79,3 | 63,7 | 38,5 | 21,8 |
| 11 | 10 | 15 | 120 | 76,9 | 67,3 | 39,6 | 22,3 |
| 2 | 15 | 15 | 72 | 66,8 | 55,4 | 44,8 | 15,9 |
| 3 | 5 | 30 | 72 | 84,0 | 72,7 | 19,7 | 22,9 |
| 5 | 5 | 22,5 | 24 | 78,7 | 65,5 | 19,0 | 21,6 |
| 8 | 15 | 22,5 | 120 | 76,0 | 65,5 | 47,8 | 17,2 |
| 13 | 10 | 22,5 | 72 | 78,3 | 67,1 | 38,9 | 21,9 |
| 9 | 10 | 15 | 24 | 67,0 | 54,0 | 33,7 | 18,3 |
| 15 | 10 | 22,5 | 72 | 77,8 | 66,9 | 39,6 | 22,1 |
| 12 | 10 | 30 | 120 | 81,2 | 70,7 | 41,7 | 23,0 |



**Table S7.1-C:** Enzymatic hydrolysis conditions for StEx-SCB defined by Box-Behnken DOE and corresponding experimental results used for statistical analysis.

| Box Behnken Design of Experiments - StEx-SCB | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Run. No** | **Solids loading (% total sugars)** | **Enzyme Dosage (mg/g protein)** | **Residence Time (hrs)** | **% Glucan Conv.** | **% Xylan Conv.** | **EtOH conc. (g.L$^{-1}$)** | **EtOH Yield (kg/100kg RDM)** |
| 1 | 5 | 15 | 72 | 65,2 | 65,8 | 17,7 | 12,9 |
| 6 | 15 | 22,5 | 24 | 39,9 | 52,1 | 43,8 | 8,7 |
| 14 | 10 | 22,5 | 72 | 70,8 | 78,6 | 37,7 | 14,6 |
| 4 | 15 | 30 | 72 | 61,5 | 63,8 | 58,2 | 12,5 |
| 7 | 5 | 22,5 | 120 | 69,5 | 73,5 | 18,7 | 13,6 |
| 10 | 10 | 30 | 24 | 58,1 | 69,5 | 35,4 | 12,3 |
| 11 | 10 | 15 | 120 | 56,7 | 70,3 | 33,7 | 12,0 |
| 2 | 15 | 15 | 72 | 42,3 | 49,7 | 47,6 | 9,1 |
| 3 | 5 | 30 | 72 | 78,7 | 81,2 | 20,3 | 15,9 |
| 5 | 5 | 22,5 | 24 | 61,7 | 69,7 | 17,3 | 12,5 |
| 8 | 15 | 22,5 | 120 | 59,1 | 63,3 | 55,1 | 12,1 |
| 13 | 10 | 22,5 | 72 | 68,9 | 78,6 | 37,7 | 14,7 |
| 9 | 10 | 15 | 24 | 41,8 | 60,8 | 26,5 | 9,3 |
| 15 | 10 | 22,5 | 72 | 72,1 | 79,6 | 39,2 | 14,8 |
| 12 | 10 | 30 | 120 | 74,8 | 79,6 | 41,2 | 15,5 |

**Table S7.1-D:** Enzymatic hydrolysis conditions for StEx-CLM defined by Box-Behnken DOE and corresponding experimental results used for statistical analysis.

| Box Behnken Design of Experiments - StEx-CLM | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Run. Order** | **Solids loading (% total sugars)** | **Enzyme Dosage (mg/g protein)** | **Residence Time (hrs)** | **% Glucan Conv.** | **% Xylan Conv.** | **EtOH conc. (g.L$^{-1}$)** | **EtOH Yield (kg/100kg RDM)** |
| 1 | 5 | 15 | 72 | 73,7 | 72,9 | 18,2 | 15,4 |
| 6 | 15 | 22,5 | 24 | 55,1 | 63,5 | 51,5 | 11,2 |
| 14 | 10 | 22,5 | 72 | 71,4 | 71,3 | 39,3 | 15,1 |
| 4 | 15 | 30 | 72 | 62,6 | 61,7 | 60,3 | 12,4 |
| 7 | 5 | 22,5 | 120 | 83,2 | 82,9 | 20,2 | 16,7 |
| 10 | 10 | 30 | 24 | 71,9 | 73,4 | 39,6 | 14,3 |
| 11 | 10 | 15 | 120 | 64,0 | 62,2 | 34,3 | 12,8 |
| 2 | 15 | 15 | 72 | 52,6 | 63,2 | 47,2 | 11,0 |
| 3 | 5 | 30 | 72 | 85,7 | 83,4 | 20,5 | 16,9 |
| 5 | 5 | 22,5 | 24 | 78,2 | 75,4 | 18,1 | 15,3 |
| 8 | 15 | 22,5 | 120 | 57,6 | 60,6 | 53,6 | 11,6 |
| 13 | 10 | 22,5 | 72 | 71,8 | 73,3 | 39,3 | 14,6 |
| 9 | 10 | 15 | 24 | 65,2 | 73,9 | 31,4 | 13,2 |
| 15 | 10 | 22,5 | 72 | 71,0 | 71,9 | 40,0 | 14,7 |
| 12 | 10 | 30 | 120 | 80,4 | 80,6 | 41,1 | 15,7 |

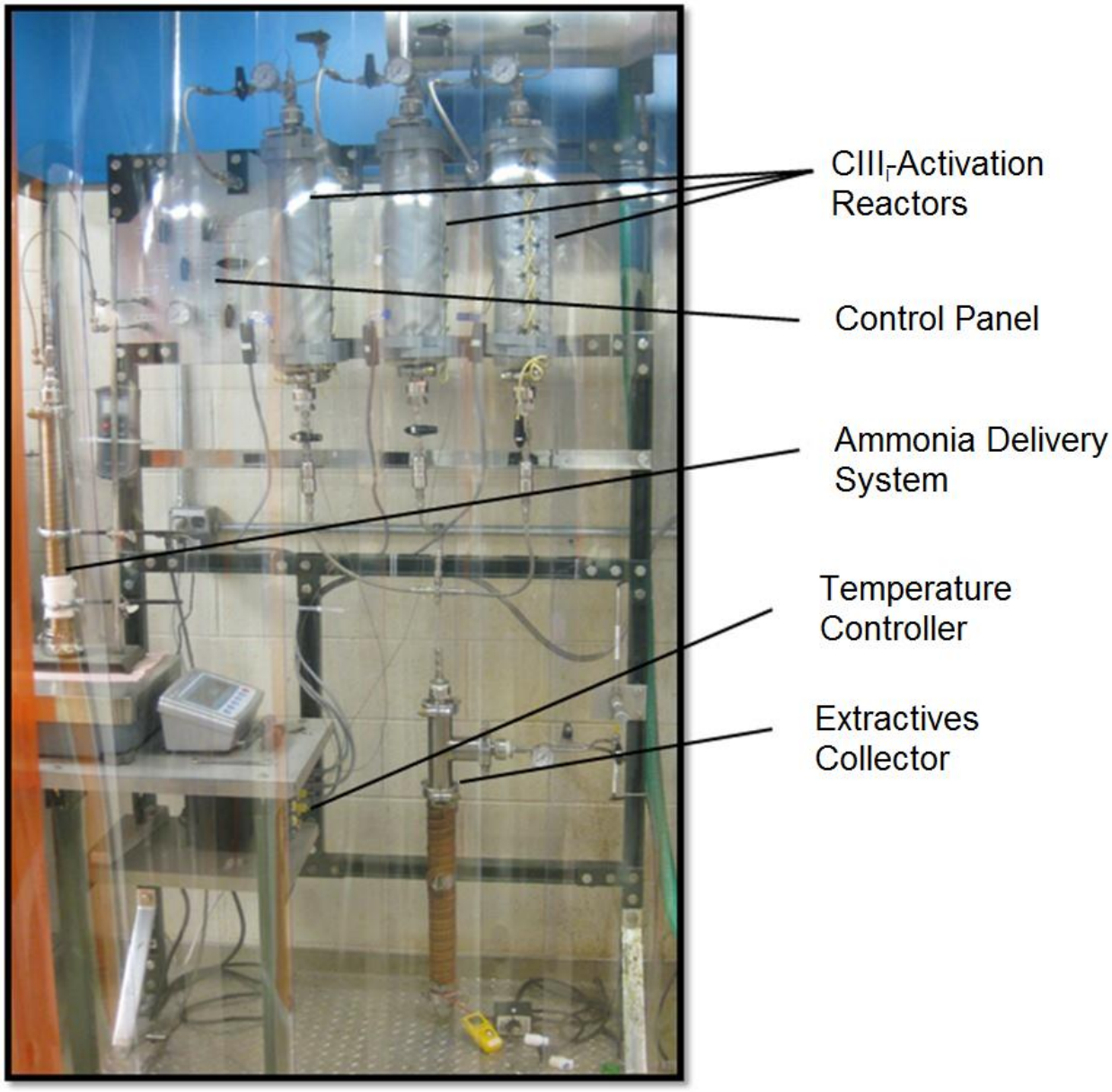



**Figure S7.1:** Picture of experimental set-up of the lab-scale $CIII_I$-activation system composed an ammonia delivery system and three parallel reactors that were equipped with independent heating jackets that were connected to a PID temperature controller (Adapted with permission from da Costa Sousa [17])

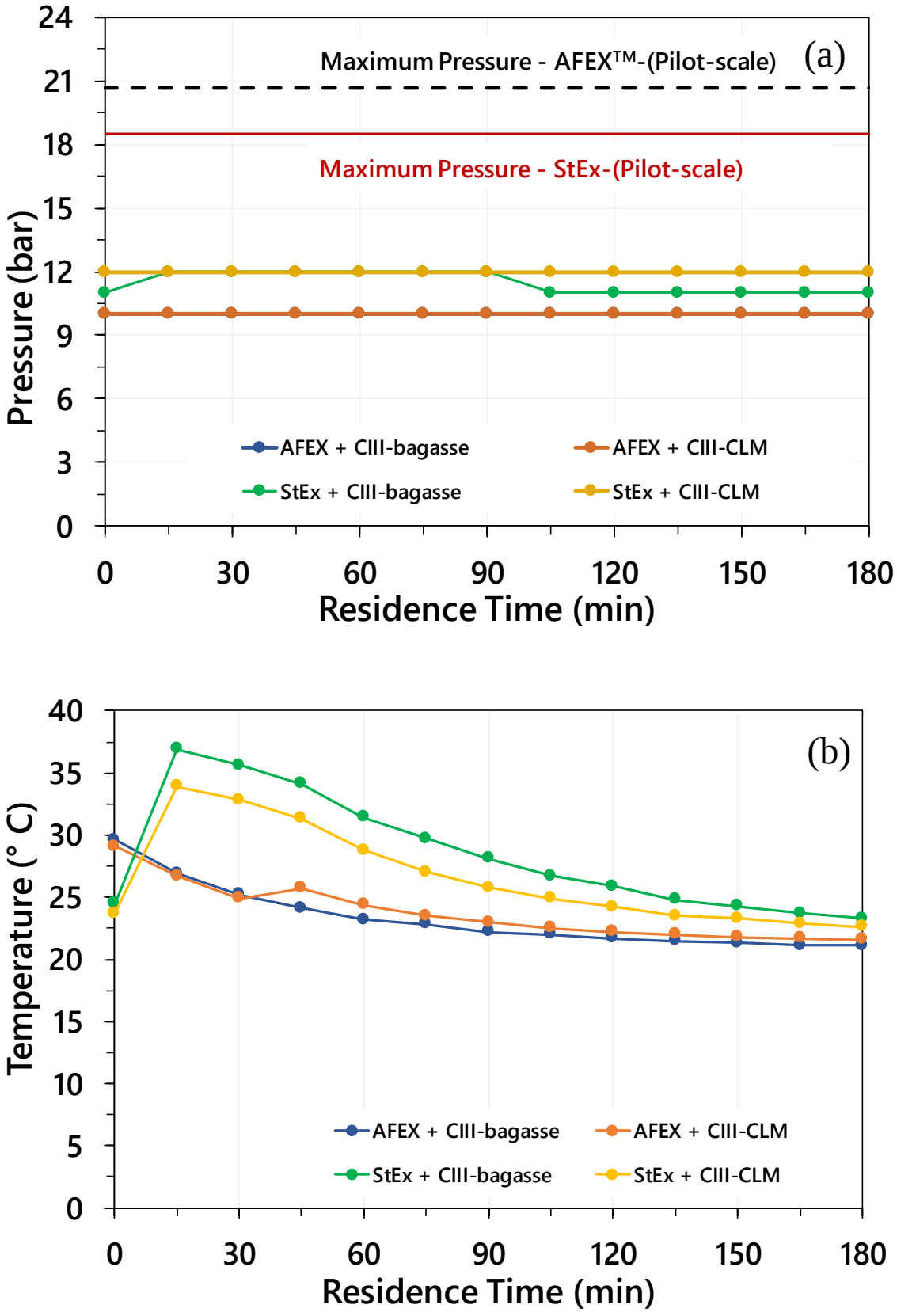



**Figure S7.2:** Pressure **(a)** and temperature **(b)** profiles recorded during the room temperature $CIII_I$-activation of StEx- and AFEX™-treated SCB and CLM pellets.

| Analysis of Variance - AFEX$^{TM}$-SCB (Glucan Conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 8 | 917,205 | 114,651 | 221,92 | 0 |
| **Linear** | 3 | 777,256 | 259,085 | 501,48 | 0 |
| Solids Loading (% total sugars) | 1 | 314,757 | 314,757 | 609,24 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 238,315 | 238,315 | 461,28 | 0 |
| Residence Time (hrs) | 1 | 224,184 | 224,184 | 433,93 | 0 |
| **Square** | 2 | 110,02 | 55,01 | 106,48 | 0 |
| Solids Loading*Solids Loading | 1 | 27,004 | 27,004 | 52,27 | 0 |
| Residence Time*Residence Time | 1 | 89,477 | 89,477 | 173,19 | 0 |
| **2-Way Interaction** | 3 | 29,928 | 9,976 | 19,31 | 0,002 |
| Solids Loading*Enzyme Dosage | 1 | 3,193 | 3,193 | 6,18 | 0,047 |
| Solids Loading*Residence Time | 1 | 2,793 | 2,793 | 5,41 | 0,059 |
| Enzyme Dosage*Residence Time | 1 | 23,942 | 23,942 | 46,34 | 0 |
| **Error** | 6 | 3,1 | 0,517 | | |
| Lack-of-Fit | 4 | 2,828 | 0,707 | 5,19 | 0,168 |
| Pure Error | 2 | 0,272 | 0,136 | | |
| **Total** | 14 | 920,304 | | | |

| Analysis of Variance - AFEX$^{TM}$-SCB (Xylan Conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 894,263 | 127,752 | 128,38 | 0 |
| **Linear** | 3 | 748,612 | 249,537 | 250,77 | 0 |
| Solids Loading (% total sugars) | 1 | 360,437 | 360,437 | 362,21 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 129,296 | 129,296 | 129,93 | 0 |
| Residence Time (hrs) | 1 | 258,879 | 258,879 | 260,16 | 0 |
| **Square** | 2 | 125,372 | 62,686 | 62,99 | 0 |
| Solids Loading*Solids Loading | 1 | 21,904 | 21,904 | 22,01 | 0,002 |
| Residence Time*Residence Time | 1 | 109,835 | 109,835 | 110,38 | 0 |
| **2-Way Interaction** | 2 | 20,279 | 10,139 | 10,19 | 0,008 |
| Solids Loading*Residence Time | 1 | 12,602 | 12,602 | 12,66 | 0,009 |
| Enzyme Dosage*Residence Time | 1 | 7,676 | 7,676 | 7,71 | 0,027 |
| **Error** | 7 | 6,966 | 0,995 | | |
| Lack-of-Fit | 5 | 6,769 | 1,354 | 13,77 | 0,069 |
| Pure Error | 2 | 0,197 | 0,098 | | |
| **Total** | 14 | 901,228 | | | |

| Analysis of Variance - AFEX$^{TM}$-SCB (EtOH conc.) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 1620,1 | 231,44 | 229,34 | 0 |
| **Linear** | 3 | 1419,63 | 473,21 | 468,92 | 0 |
| Solids Loading (% total sugars) | 1 | 1317,48 | 1317,48 | 1305,52 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 72,72 | 72,72 | 72,06 | 0 |
| Residence Time (hrs) | 1 | 29,43 | 29,43 | 29,16 | 0,001 |
| **Square** | 3 | 194,94 | 64,98 | 64,39 | 0 |
| Solids Loading*Solids Loading | 1 | 191,02 | 191,02 | 189,29 | 0 |
| Enzyme Dosage*Enzyme Dosage | 1 | 8,42 | 8,42 | 8,35 | 0,023 |
| Residence Time*Residence Time | 1 | 3,66 | 3,66 | 3,62 | 0,099 |
| **2-Way Interaction** | 1 | 5,52 | 5,52 | 5,47 | 0,052 |
| Solids Loading*Enzyme Dosage | 1 | 5,52 | 5,52 | 5,47 | 0,052 |
| **Error** | 7 | 7,06 | 1,01 | | |
| Lack-of-Fit | 5 | 6,84 | 1,37 | 12,14 | 0,078 |
| Pure Error | 2 | 0,23 | 0,11 | | |
| **Total** | 14 | 1627,16 | | | |

| Analysis of Variance - AFEX$^{TM}$-SCB (EtOH Yield) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 5 | 172,725 | 34,545 | 73,49 | 0 |
| **Linear** | 3 | 126,383 | 42,1277 | 89,62 | 0 |
| Solids Loading (% total sugars) | 1 | 88,908 | 88,9078 | 189,15 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 18,517 | 18,517 | 39,39 | 0 |
| Residence Time (hrs) | 1 | 18,958 | 18,9583 | 40,33 | 0 |
| **Square** | 2 | 46,342 | 23,171 | 49,29 | 0 |
| Solids Loading*Solids Loading | 1 | 41,852 | 41,8523 | 89,04 | 0 |
| Residence Time*Residence Time | 1 | 6,634 | 6,6336 | 14,11 | 0,005 |
| **2-Way Interaction** | | | | | |
| **Error** | 9 | 4,23 | 0,47 | | |
| Lack-of-Fit | 7 | 3,588 | 0,5125 | 1,59 | 0,438 |
| Pure Error | 2 | 0,643 | 0,3214 | | |
| **Total** | 14 | 176,956 | | | |

| Refined Model Summary- AFEX$^{TM}$-SCB (Glucan Conversion) | |
|---|---|
| **Regression Equation** | $Y = 37.12+0.116x_1+0.979x_2+0.532x_3-0.108x_1^2-0.00213x_3^2+0.02383x_1x_2+0.00348x_1x_3-0.0068x_2x_3$ |
| $R^2$ | 99,66% |
| $R^2_{adjusted}$ | 99,21% |
| $R^2_{predicted}$ | 96,81% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-SCB (Xylan Conversion) | |
|---|---|
| **Regression Equation** | $Y = 42.71+0.068x_1+0.813x_2+0.471x_3-0.0971x_1^2-0.00236x_3^2+0.0074x_1x_3-0.00385x_2x_3$ |
| $R^2$ | 99,23% |
| $R^2_{adjusted}$ | 98,45% |
| $R^2_{predicted}$ | 95,45% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-SCB (EtOH conc.) | |
|---|---|
| **Regression Equation** | $Y = -36.01+7.616x_1+1.297x_2+0.102x_3-0.2877x_1^2-0.0269x_2^2-0.00043x_3^2+0.0313x_1x_2$ |
| $R^2$ | 99,57% |
| $R^2_{adjusted}$ | 99,13% |
| $R^2_{predicted}$ | 97,71% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-SCB (EtOH Yield) | |
|---|---|
| **Regression Equation** | $Y = 6.234+2.018x_1+0.203x_2+0.116x_3-0.134x_1^2-0.0005x_3^2$ |
| $R^2$ | 97,61% |
| $R^2_{adjusted}$ | 96,28% |
| $R^2_{predicted}$ | 92,93% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Analysis of Variance- AFEX$^{TM}$-CLM (Glucan Conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 8 | 468,628 | 58,578 | 37,74 | 0 |
| **Linear** | 3 | 419,763 | 139,921 | 90,14 | 0 |
| Solids Loading (% total sugars) | 1 | 195,269 | 195,269 | 125,79 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 130,725 | 130,725 | 84,21 | 0 |
| Residence Time (hrs) | 1 | 93,77 | 93,77 | 60,41 | 0 |
| **Square** | 3 | 20,552 | 6,851 | 4,41 | 0,058 |
| Solids Loading*Solids Loading | 1 | 8,589 | 8,589 | 5,53 | 0,057 |
| Enzyme Dosage *Enzyme Dosage | 1 | 4,09 | 4,09 | 2,64 | 0,156 |
| Residence Time*Residence Time | 1 | 10,821 | 10,821 | 6,97 | 0,039 |
| **2-Way Interaction** | 2 | 28,312 | 14,156 | 9,12 | 0,015 |
| Solids Loading*Residence Time | 1 | 12,17 | 12,17 | 7,84 | 0,031 |
| Enzyme Dosage*Residence Time | 1 | 16,143 | 16,143 | 10,4 | 0,018 |
| **Error** | 6 | 9,314 | 1,552 | | |
| Lack-of-Fit | 4 | 5,302 | 1,325 | 0,66 | 0,676 |
| Pure Error | 2 | 4,012 | 2,006 | | |
| **Total** | 14 | 477,942 | | | |

| Analysis of Variance- AFEX$^{TM}$-CLM (Xylan Conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 6 | 633,368 | 105,561 | 30,17 | 0 |
| **Linear** | 3 | 542,495 | 180,832 | 51,68 | 0 |
| Solids Loading (% total sugars) | 1 | 183,303 | 183,303 | 52,38 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 127,945 | 127,945 | 36,56 | 0 |
| Residence Time (hrs) | 1 | 231,247 | 231,247 | 66,09 | 0 |
| **Square** | 1 | 44,811 | 44,811 | 12,81 | 0,007 |
| Residence Time*Residence Time | 1 | 44,811 | 44,811 | 12,81 | 0,007 |
| **2-Way Interaction** | 2 | 46,062 | 23,031 | 6,58 | 0,02 |
| Solids Loading *Enzyme Dosage | 1 | 24,929 | 24,929 | 7,12 | 0,028 |
| Solids Loading*Residence Time | 1 | 21,133 | 21,133 | 6,04 | 0,039 |
| **Error** | 8 | 27,994 | 3,499 | | |
| Lack-of-Fit | 6 | 24,713 | 4,119 | 2,51 | 0,312 |
| Pure Error | 2 | 3,28 | 1,64 | | |
| **Total** | 14 | 661,362 | | | |

| Analysis of Variance- AFEX$^{TM}$-CLM(EtOH conc.) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 6 | 1678,61 | 279,77 | 308,39 | 0 |
| **Linear** | 3 | 1552,34 | 517,45 | 570,38 | 0 |
| Solids Loading (% total sugars) | 1 | 1514,24 | 1514,24 | 1669,15 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 18,97 | 18,97 | 20,91 | 0,002 |
| Residence Time (hrs) | 1 | 19,12 | 19,12 | 21,08 | 0,002 |
| **Square** | 1 | 121,6 | 121,6 | 134,04 | 0 |
| Solids Loading*Solids Loading | 1 | 121,6 | 121,6 | 134,04 | 0 |
| **2-Way Interaction** | 2 | 4,67 | 2,34 | 2,57 | 0,137 |
| Solids Loading *Enzyme Dosage | 1 | 2,92 | 2,92 | 3,22 | 0,111 |
| Enzyme Dosage*Residence Time | 1 | 1,75 | 1,75 | 1,93 | 0,202 |
| **Error** | 8 | 7,26 | 0,91 | | |
| Lack-of-Fit | 6 | 6,88 | 1,15 | 6,01 | 0,15 |
| Pure Error | 2 | 0,38 | 0,19 | | |
| **Total** | 14 | 1685,87 | | | |

| Analysis of Variance- AFEX$^{TM}$-CLM (EtOH Yield) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 6 | 99,831 | 16,6384 | 67,92 | 0 |
| **Linear** | 3 | 78,955 | 26,3184 | 107,43 | 0 |
| Solids Loading (% total sugars) | 1 | 62,932 | 62,9324 | 256,89 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 7,337 | 7,3367 | 29,95 | 0,001 |
| Residence Time (hrs) | 1 | 8,686 | 8,6859 | 35,46 | 0 |
| **Square** | 2 | 18,981 | 9,4904 | 38,74 | 0 |
| Solids Loading*Solids Loading | 1 | 18,143 | 18,1434 | 74,06 | 0 |
| Residence Time*Residence Time | 1 | 1,481 | 1,4812 | 6,05 | 0,039 |
| **2-Way Interaction** | 1 | 1,895 | 1,8947 | 7,73 | 0,024 |
| Enzyme Dosage*Residence Time | 1 | 1,895 | 1,8947 | 7,73 | 0,024 |
| **Error** | 8 | 1,96 | 0,245 | | |
| Lack-of-Fit | 6 | 1,608 | 0,2681 | 1,53 | 0,447 |
| Pure Error | 2 | 0,351 | 0,1757 | | |
| **Total** | 14 | 101,79 | | | |

| Refined Model Summary- AFEX$^{TM}$-CLM (Glucan Conversion) | |
|---|---|
| **Regression Equation** | $Y = 48.23-0.291x_1+1.783x_2+0.231x_3-0.061x_1^2-0.0187x_2^2-0.00074x_3^2+0.00727x_1x_3-0.00558x_2x_3$ |
| $R^2$ | 98,05% |
| $R^2_{adjusted}$ | 95,45% |
| $R^2_{predicted}$ | 88,53% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-CLM (Xylan Conversion) | |
|---|---|
| **Regression Equation** | $Y = 70,64-3.145x_1+0.133x_2+0.233x_3-0.0015x_3^2+0.0666x_1x_2+0.00958x_1x_3$ |
| $R^2$ | 95,77% |
| $R^2_{adjusted}$ | 92,59% |
| $R^2_{predicted}$ | 82,21% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-CLM (EtOH conc.) | |
|---|---|
| **Regression Equation** | $Y = -16.31+6.805x_1+0.11x_2+0.0736x_3-0.228x_1^2+0.0228x_1x_2-0.00184x_2x_3$ |
| $R^2$ | 99,57% |
| $R^2_{adjusted}$ | 99,25% |
| $R^2_{predicted}$ | 97,99% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- AFEX$^{TM}$-CLM (EtOH Yield) | |
|---|---|
| **Regression Equation** | $Y = 9.898+1.207x_1+0.265x_2+0.104x_3-0.088x_1^2-0.00274x_3^2 - 0.00191x_2x_3$ |
| $R^2$ | 98,07% |
| $R^2_{adjusted}$ | 96,63% |
| $R^2_{predicted}$ | 91,51% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

**Figure S7.3-A:** Analysis of Variance (ANOVA) and refined model summary for % Glucan conversion (monomeric), % Xylan conversion (monomeric), final ethanol concentration (g.L$^{-1}$), ethanol yield (kg/100 kg RDM) for the high solids loading enzymatic hydrolysis and fermentation of AFEX$^{TM}$-treated SCB and CLM pellets



| Analysis of Variance - StEx-SCB (Glucan conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 2041,37 | 291,624 | 81,29 | 0 |
| **Linear** | 3 | 1646,17 | 548,722 | 152,95 | 0 |
| Solids Loading (% total sugars) | 1 | 653,23 | 653,228 | 182,08 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 562,16 | 562,164 | 156,69 | 0 |
| Residence Time (hrs) | 1 | 430,77 | 430,775 | 120,07 | 0 |
| **Square** | 3 | 363,48 | 121,161 | 33,77 | 0 |
| Solids Loading*Solids Loading | 1 | 73,4 | 73,403 | 20,46 | 0,003 |
| Enzyme Dosage *Enzyme Dosage | 1 | 63,68 | 63,682 | 17,75 | 0,004 |
| Residence Time*Residence Time | 1 | 271,09 | 271,093 | 75,56 | 0 |
| **2-Way Interaction** | 1 | 31,72 | 31,717 | 8,84 | 0,021 |
| Solids Loading*Residence Time | 1 | 31,72 | 31,717 | 8,84 | 0,021 |
| **Error** | 7 | 25,11 | 3,588 | | |
| Lack-of-Fit | 5 | 19,81 | 3,963 | 1,5 | 0,447 |
| Pure Error | 2 | 5,3 | 2,65 | | |
| **Total** | 14 | 2066,48 | | | |

| Analysis of Variance - StEx-SCB (Xylan conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 1349 | 192,715 | 54,91 | 0 |
| **Linear** | 3 | 900,33 | 300,111 | 85,5 | 0 |
| Solids Loading (% total sugars) | 1 | 469,72 | 469,717 | 133,83 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 282,21 | 282,205 | 80,4 | 0 |
| Residence Time (hrs) | 1 | 148,41 | 148,411 | 42,28 | 0 |
| **Square** | 3 | 435,06 | 145,019 | 41,32 | 0 |
| Solids Loading*Solids Loading | 1 | 339,91 | 339,91 | 96,84 | 0 |
| Enzyme Dosage *Enzyme Dosage | 1 | 65,26 | 65,26 | 18,59 | 0,004 |
| Residence Time*Residence Time | 1 | 80,33 | 80,334 | 22,89 | 0,002 |
| **2-Way Interaction** | 1 | 13,61 | 13,611 | 3,88 | 0,09 |
| Solids Loading*Residence Time | 1 | 13,61 | 13,611 | 3,88 | 0,09 |
| **Error** | 7 | 24,57 | 3,51 | | |
| Lack-of-Fit | 5 | 23,89 | 4,777 | 14,01 | 0,068 |
| Pure Error | 2 | 0,68 | 0,341 | | |
| **Total** | 14 | 1373,57 | | | |

| Analysis of Variance- StEx-SCB (EtOH conc.) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 2407,76 | 343,97 | 265,97 | 0 |
| **Linear** | 3 | 2328,79 | 776,26 | 600,24 | 0 |
| Solids Loading (% total sugars) | 1 | 2137,36 | 2137,36 | 1652,7 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 109,98 | 109,98 | 85,04 | 0 |
| Residence Time (hrs) | 1 | 81,46 | 81,46 | 62,99 | 0 |
| **Square** | 2 | 38,48 | 19,24 | 14,88 | 0,003 |
| Solids Loading*Solids Loading | 1 | 6,02 | 6,02 | 4,65 | 0,068 |
| Residence Time*Residence Time | 1 | 34,32 | 34,32 | 26,54 | 0,001 |
| **2-Way Interaction** | 2 | 40,49 | 20,24 | 15,65 | 0,003 |
| Solids Loading*Enzyme Dosage | 1 | 15,73 | 15,73 | 12,16 | 0,01 |
| Solids Loading*Residence Time | 1 | 24,76 | 24,76 | 19,14 | 0,003 |
| **Error** | 7 | 9,05 | 1,29 | | |
| Lack-of-Fit | 5 | 7,57 | 1,51 | 2,05 | 0,36 |
| Pure Error | 2 | 1,48 | 0,74 | | |
| **Total** | 14 | 2416,82 | | | |

| Analysis of Variance- StEx-SCB (EtOH Yield) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 71,8745 | 10,2678 | 82,02 | 0 |
| **Linear** | 3 | 54,3548 | 18,1183 | 144,73 | 0 |
| Solids Loading (% total sugars) | 1 | 19,4779 | 19,4779 | 155,6 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 21,3464 | 21,3464 | 170,52 | 0 |
| Residence Time (hrs) | 1 | 13,5304 | 13,5304 | 108,09 | 0 |
| **Square** | 3 | 16,2582 | 5,4194 | 43,29 | 0 |
| Solids Loading*Solids Loading | 1 | 6,311 | 6,311 | 50,41 | 0 |
| Enzyme Dosage*Enzyme Dosage | 1 | 2,2049 | 2,2049 | 17,61 | 0,004 |
| Residence Time*Residence Time | 1 | 9,8925 | 9,8925 | 79,02 | 0 |
| **2-Way Interaction** | 1 | 1,2615 | 1,2615 | 10,08 | 0,016 |
| Solids Loading*Residence Time | 1 | 1,2615 | 1,2615 | 10,08 | 0,016 |
| **Error** | 7 | 0,8763 | 0,1252 | | |
| Lack-of-Fit | 5 | 0,8615 | 0,1723 | 23,29 | 0,042 |
| Pure Error | 2 | 0,0148 | 0,0074 | | |
| **Total** | 14 | 72,7507 | | | |

| Refined Model Summary- StEx-SCB (Glucan conversion) | |
|---|---|
| **Regression Equation** | $Y = -13.6+0.915x_1+4.44x_2+0.571x_3-0.1783x_1^2-0.0738x_2^2$ $0.0037x_3^2+0.01173x_1x_3$ |
| $R^2$ | 98,78% |
| $R^2_{adjusted}$ | 97,57% |
| $R^2_{predicted}$ | 92,56% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-SCB (Xylan conversion) | |
|---|---|
| **Regression Equation** | $Y = -11.2+5.59x_1+4.155x_2+0.304x_3-0.03838x_1^2-$ $0.0747x_2^2-0.00202x_3^2+0.00769x_1x_3$ |
| $R^2$ | 98,21% |
| $R^2_{adjusted}$ | 96,42% |
| $R^2_{predicted}$ | 90,05% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-SCB (EtOH conc.) | |
|---|---|
| **Regression Equation** | $Y = -3.54+2.35x_1-0.034x_2+0.1528x_3-0.051x_1^2-$ $0.00132x_3^2+0.0529x_1x_2-0.01037x_1x_3$ |
| $R^2$ | 99,63% |
| $R^2_{adjusted}$ | 99,25% |
| $R^2_{predicted}$ | 97,49% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-SCB (EtOH Yield) | |
|---|---|
| **Regression Equation** | $Y = -3.24+0.565x_1+0.836x_2+0.106x_3-0.0523x_1^2-$ $0.01374x_2^2+0.00071x_3^2-0.00234x_1x_3$ |
| $R^2$ | 98,80% |
| $R^2_{adjusted}$ | 97,59% |
| $R^2_{predicted}$ | 89,13% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Analysis of Variance - StEx-CLM (Glucan conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 5 | 1401,31 | 280,26 | 342,68 | 0 |
| **Linear** | 3 | 1359,75 | 453,25 | 554,2 | 0 |
| Solids Loading (% total sugars) | 1 | 1078,18 | 1078,18 | 1318,31 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 253,77 | 253,77 | 310,29 | 0 |
| Residence Time (hrs) | 1 | 27,79 | 27,79 | 33,98 | 0 |
| **Square** | 1 | 18,32 | 18,32 | 22,4 | 0,001 |
| Solids Loading*Solids Loading | 1 | 18,32 | 18,32 | 22,4 | 0,001 |
| **2-Way Interaction** | 1 | 23,24 | 23,24 | 28,41 | 0 |
| Enzyme Dosage*Residence Time | 1 | 23,24 | 23,24 | 28,41 | 0 |
| **Error** | 9 | 7,36 | 0,82 | | |
| Lack-of-Fit | 7 | 7,04 | 1,01 | 6,38 | 0,142 |
| Pure Error | 2 | 0,32 | 0,16 | | |
| **Total** | 14 | 1408,67 | | | |

| Analysis of Variance - StEx-CLM (Xylan conversion) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 793,7 | 113,386 | 33,86 | 0 |
| **Linear** | 3 | 628,627 | 209,542 | 62,58 | 0 |
| Solids Loading (% total sugars) | 1 | 537,889 | 537,889 | 160,64 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 90,732 | 90,732 | 27,1 | 0,001 |
| Residence Time (hrs) | 1 | 0,006 | 0,006 | 0 | 0,967 |
| **Square** | 1 | 13,557 | 13,557 | 4,05 | 0,084 |
| Solids Loading*Solids Loading | 1 | 13,557 | 13,557 | 4,05 | 0,084 |
| **2-Way Interaction** | 3 | 151,516 | 50,505 | 15,08 | 0,002 |
| Solids Loading*Enzyme Dosage | 1 | 35,513 | 35,513 | 10,61 | 0,014 |
| Solids Loading*Residence Time | 1 | 26,344 | 26,344 | 7,87 | 0,026 |
| Enzyme Dosage*Residence Time | 1 | 89,659 | 89,659 | 26,78 | 0,001 |
| **Error** | 7 | 23,439 | 3,348 | | |
| Lack-of-Fit | 5 | 21,441 | 4,288 | 4,29 | 0,2 |
| Pure Error | 2 | 1,998 | 0,999 | | |
| **Total** | 14 | 817,139 | | | |

| Analysis of Variance - StEx-CLM (EtOH conc.) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 7 | 2480,5 | 354,36 | 1868,93 | 0 |
| **Linear** | 3 | 2425,31 | 808,44 | 4263,81 | 0 |
| Solids Loading (% total sugars) | 1 | 2301,51 | 2301,51 | 12138,47 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 114,79 | 114,79 | 605,41 | 0 |
| Residence Time (hrs) | 1 | 9,02 | 9,02 | 47,55 | 0 |
| **Square** | 3 | 25,71 | 8,57 | 45,21 | 0 |
| Solids Loading*Solids Loading | 1 | 12,66 | 12,66 | 66,78 | 0 |
| Enzyme Dosage*Enzyme Dosage | 1 | 4,7 | 4,7 | 24,78 | 0,002 |
| Residence Time*Residence Time | 1 | 12,01 | 12,01 | 63,34 | 0 |
| **2-Way Interaction** | 1 | 29,47 | 29,47 | 155,43 | 0 |
| Solids Loading*Enzyme Dosage | 1 | 29,47 | 29,47 | 155,43 | 0 |
| **Error** | 7 | 1,33 | 0,19 | | |
| Lack-of-Fit | 5 | 1,04 | 0,21 | 1,47 | 0,452 |
| Pure Error | 2 | 0,28 | 0,14 | | |
| **Total** | 14 | 2481,82 | | | |

| Analysis of Variance - StEx-CLM (EtOH Yield) | | | | | |
|---|---|---|---|---|---|
| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
| **Model** | 6 | 50,8888 | 8,4815 | 66,71 | 0 |
| **Linear** | 3 | 48,1101 | 16,0367 | 126,14 | 0 |
| Solids Loading (% total sugars) | 1 | 41,2887 | 41,2887 | 324,77 | 0 |
| Enzyme Dosage (mg/g glucan) | 1 | 5,9266 | 5,9266 | 46,62 | 0 |
| Residence Time (hrs) | 1 | 0,8947 | 0,8947 | 7,04 | 0,029 |
| **Square** | 2 | 1,9317 | 0,9658 | 7,6 | 0,014 |
| Solids Loading*Solids Loading | 1 | 1,2698 | 1,2698 | 9,99 | 0,013 |
| Residence Time*Residence Time | 1 | 0,7956 | 0,7956 | 6,26 | 0,037 |
| **2-Way Interaction** | 1 | 0,8471 | 0,8471 | 6,66 | 0,033 |
| Enzyme Dosage*Residence Time | 1 | 0,8471 | 0,8471 | 6,66 | 0,033 |
| **Error** | 8 | 1,0171 | 0,1271 | | |
| Lack-of-Fit | 6 | 0,8789 | 0,1465 | 2,12 | 0,355 |
| Pure Error | 2 | 0,1382 | 0,0691 | | |
| **Total** | 14 | 51,9059 | | | |

| Refined Model Summary- StEx-CLM (Glucan conversion) | |
|---|---|
| **Regression Equation** | $Y = 76.318-0.549x_1+0.269x_2-0.112x_3-0.0883x_1^2-$ $0.006695x_2x_3$ |
| $R^2$ | 99,48% |
| $R^2_{adjusted}$ | 99,19% |
| $R^2_{predicted}$ | 98,46% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-CLM (Xylan conversion) | |
|---|---|
| **Regression Equation** | $Y = 66.72+2.44x_1+0.297x_2-0.188x_3-0.0762x_1^2-$ $0.0795x_1x_2-0.0107x_1x_3+0.01315x_2x_3$ |
| $R^2$ | 97,13% |
| $R^2_{adjusted}$ | 94,26% |
| $R^2_{predicted}$ | 80,20% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-CLM (EtOH conc.) | |
|---|---|
| **Regression Equation** | $Y = 66.72+3,245x_1+0.684x_2+1,35x_3-0.0741x_1^2-$ $0,0201x_2^2-0,000783x_3^2+0.0724x_1x_2$ |
| $R^2$ | 99,95% |
| $R^2_{adjusted}$ | 99,89% |
| $R^2_{predicted}$ | 99,67% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

| Refined Model Summary- StEx-CLM (EtOH Yield) | |
|---|---|
| **Regression Equation** | $Y = 14,78+0.013x_1+0.0227x_2+0,0071x_3-0.0234x_1^2-$ $0,000201x_3^2+0.00128x_2x_3$ |
| $R^2$ | 98,04% |
| $R^2_{adjusted}$ | 96,57% |
| $R^2_{predicted}$ | 91,57% |

$x_1$ - solids loading, $x_2$ - enzyme dosage, $x_3$ - residence time

**Figure S7.3-B:** Analysis of Variance (ANOVA) and refined model summary for % Glucan conversion (monomeric), % Xylan conversion (monomeric), final ethanol concentration (g.L$^{-1}$), and ethanol yield (kg/100 kg RDM) for the high solids loading enzymatic hydrolysis and fermentation of StEx-treated SCB and CLM pellets

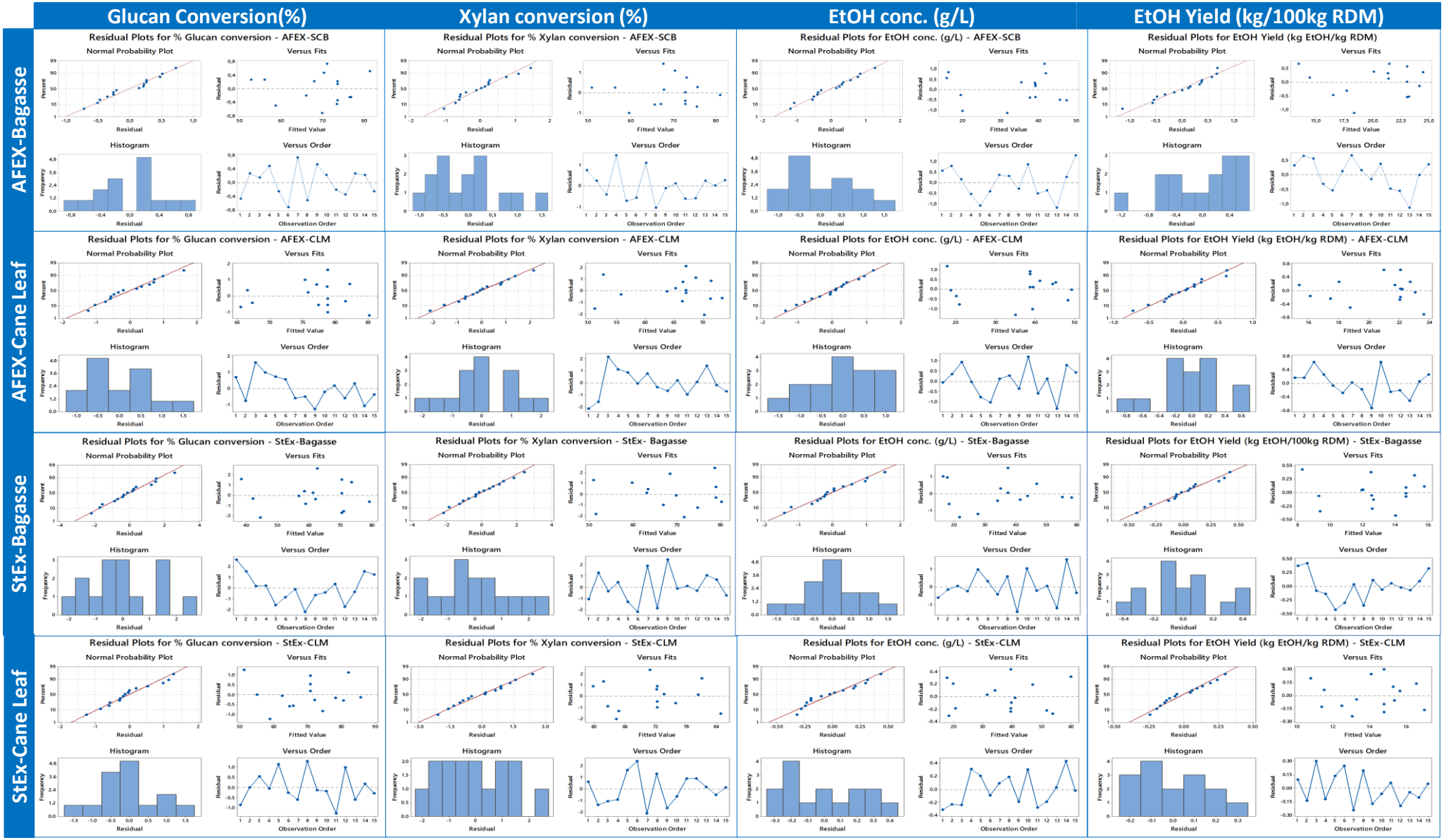



**Figure S7.3-C:** Normal probability and residual plots % Glucan conversion (monomeric), % Xylan conversion (monomeric), final ethanol concentration (g.L$^{-1}$), and ethanol yield (kg/100 kg RDM) for the high solids loading enzymatic hydrolysis and fermentation of StEx-treated SCB and CLM pellets

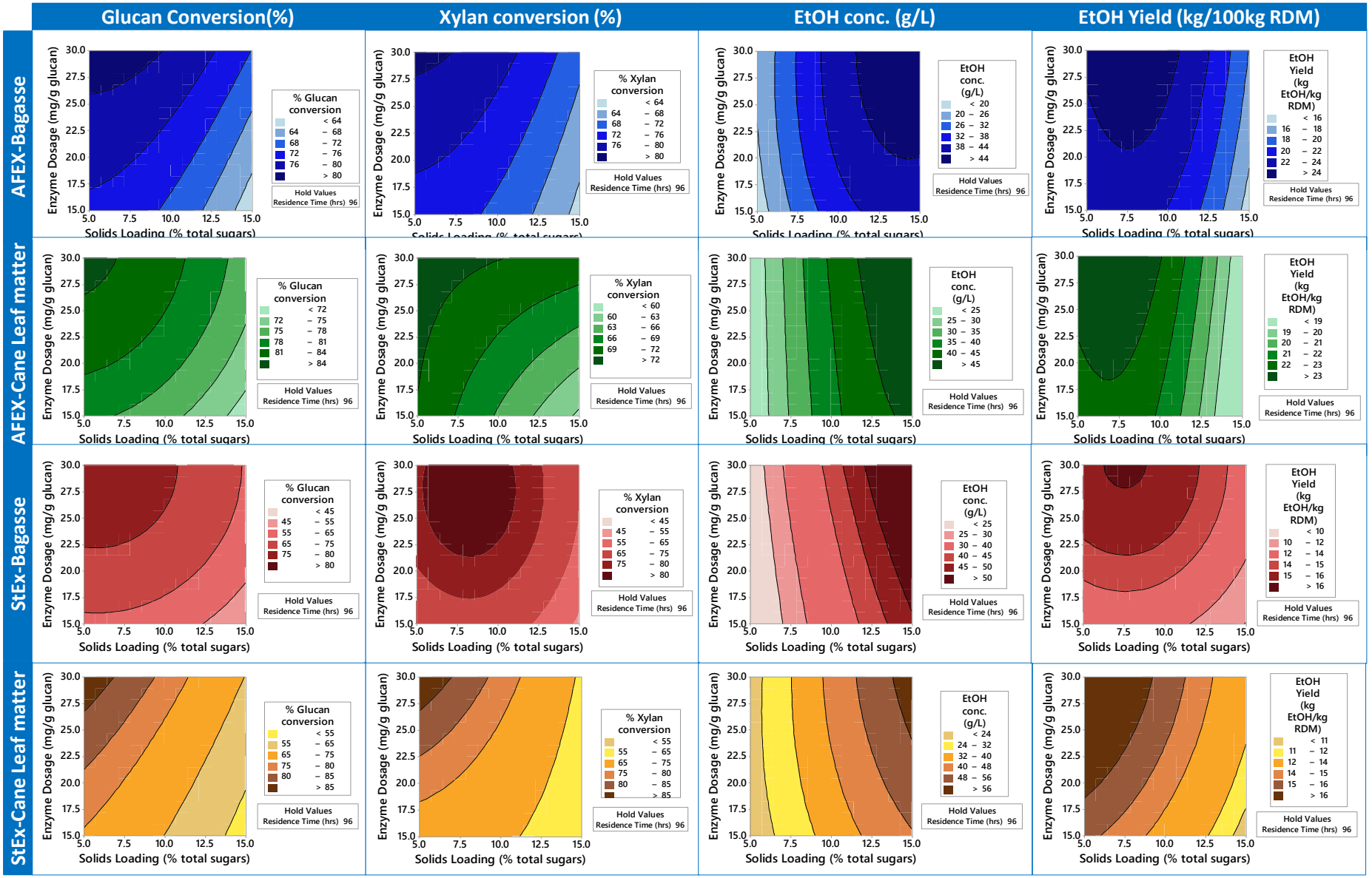
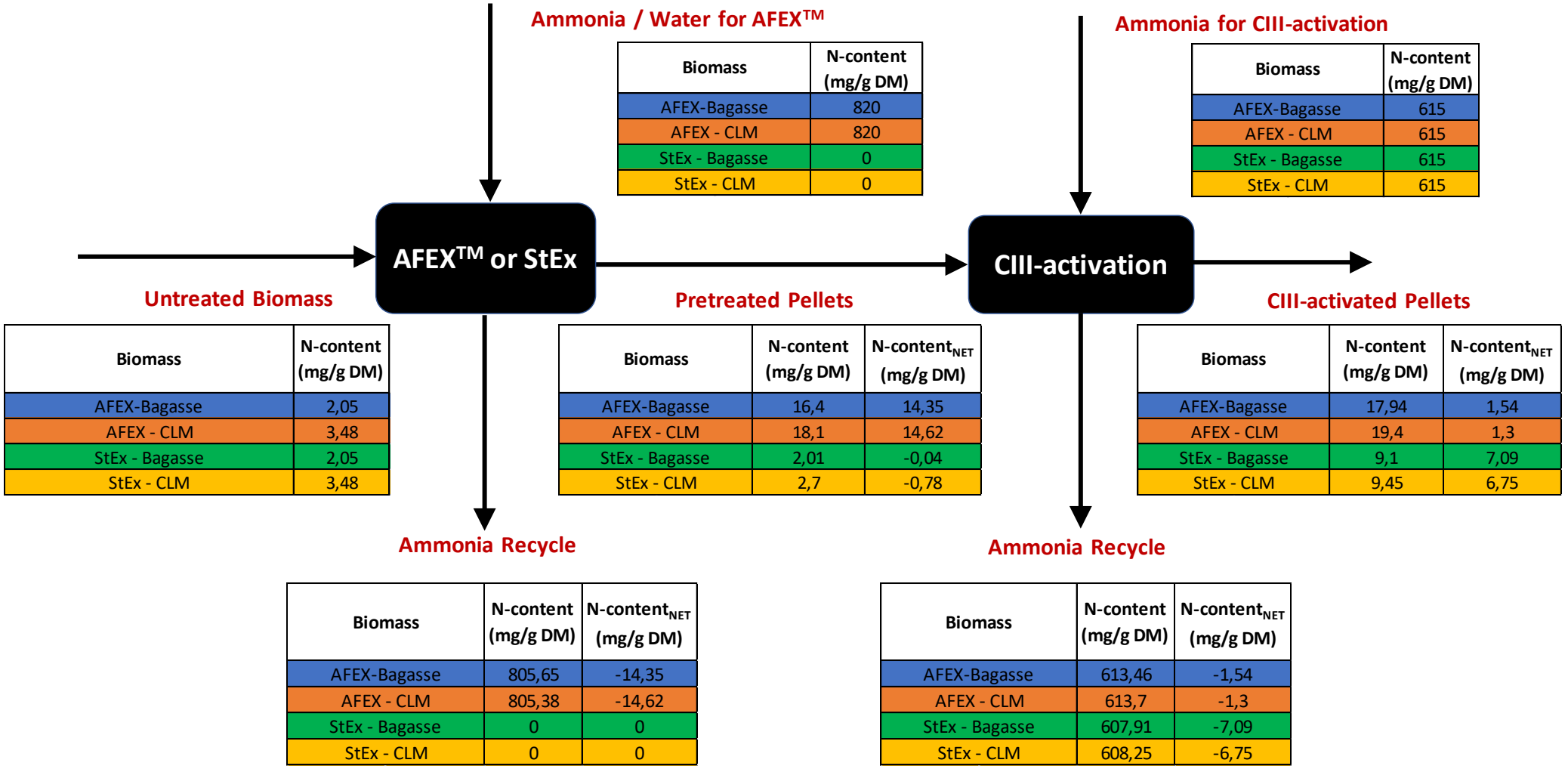



**Figure S7.3-D:** Full contour plots for % Glucan conversion (monomeric), % Xylan conversion (monomeric), final ethanol concentration (g.L$^{-1}$), and ethanol yield (kg/100 kg RDM) for the high solids loading enzymatic hydrolysis and fermentation of StEx-treated SCB and CLM pellets



| Model Validation - Glucan conversion | | |
|---|---|---|
| **Biomass** | **Glucan conv. (Model)** | **Gluc conv. (Experimental)** |
| AFEX$^{TM}$-Bagasse | 76,11% | 75,78% |
| AFEX$^{TM}$-CLM | 80,60% | 80,02% |
| StEx-Bagasse | 73,62% | 74,42% |
| StEx-CLM | 75,41% | 77,03% |
| **Model Validation - Xylan conversion** | | |
| **Biomass** | **Xylan conv. (Model)** | **Xylan conv. (Experimental)** |
| AFEX$^{TM}$-Bagasse | 74,72% | 73,10% |
| AFEX$^{TM}$-CLM | 70,21% | 72,37% |
| StEx-Bagasse | 80,64% | 74,22% |
| StEx-CLM | 73,21% | 73,52% |
| **Model Validation - EtOH conc. (g/L)** | | |
| **Biomass** | **Conc. (Model)** | **Conc. (Experimental)** |
| AFEX$^{TM}$-Bagasse | 40,68 | 41,77 |
| AFEX$^{TM}$-CLM | 41,1 | 42,28 |
| StEx-Bagasse | 39,71 | 41,84 |
| StEx-CLM | 41,73 | 44,04 |
| **Model Validation - EtOH Yield (kg EtOH/100 kg RDM)** | | |
| **Biomass** | **Yield (Model)** | **Yield (Exp)** |
| AFEX$^{TM}$-Bagasse | 23,88 | 23,67 |
| AFEX$^{TM}$-CLM | 22,65 | 23,1 |
| StEx-Bagasse | 15,37 | 16,14 |
| StEx-CLM | 15,04 | 15,08 |

**Figure S7.3-E:** Comparison of the % glucan conversion, % xylan conversion, final ethanol concentration and ethanol yield as predicted by the model to the experimental data. Model validation was performed at an enzyme dosage of 25mg/g glucan, solids loading corresponding to 10% total sugars, and an enzymatic hydrolysis residence time of 96 hrs. The selected validation points were not included in the original Box-Behnken DOE.




| Biomass | N-content (mg/g DM) |
|---|---|
| AFEX-Bagasse | 820 |
| AFEX - CLM | 820 |
| StEx - Bagasse | 0 |
| StEx - CLM | 0 |

| Biomass | N-content (mg/g DM) |
|---|---|
| AFEX-Bagasse | 615 |
| AFEX - CLM | 615 |
| StEx - Bagasse | 615 |
| StEx - CLM | 615 |

| Biomass | N-content (mg/g DM) |
|---|---|
| AFEX-Bagasse | 2,05 |
| AFEX - CLM | 3,48 |
| StEx - Bagasse | 2,05 |
| StEx - CLM | 3,48 |

| Biomass | N-content (mg/g DM) | N-content$_{NET}$ (mg/g DM) |
|---|---|---|
| AFEX-Bagasse | 16,4 | 14,35 |
| AFEX - CLM | 18,1 | 14,62 |
| StEx - Bagasse | 2,01 | -0,04 |
| StEx - CLM | 2,7 | -0,78 |

| Biomass | N-content (mg/g DM) | N-content$_{NET}$ (mg/g DM) |
|---|---|---|
| AFEX-Bagasse | 17,94 | 1,54 |
| AFEX - CLM | 19,4 | 1,3 |
| StEx - Bagasse | 9,1 | 7,09 |
| StEx - CLM | 9,45 | 6,75 |

| Biomass | N-content (mg/g DM) | N-content$_{NET}$ (mg/g DM) |
|---|---|---|
| AFEX-Bagasse | 805,65 | -14,35 |
| AFEX - CLM | 805,38 | -14,62 |
| StEx - Bagasse | 0 | 0 |
| StEx - CLM | 0 | 0 |

| Biomass | N-content (mg/g DM) | N-content$_{NET}$ (mg/g DM) |
|---|---|---|
| AFEX-Bagasse | 613,46 | -1,54 |
| AFEX - CLM | 613,7 | -1,3 |
| StEx - Bagasse | 607,91 | -7,09 |
| StEx - CLM | 608,25 | -6,75 |

**Figure S7.4:** Quantification of the biomass nitrogen consumption/depletion after StEx/AFEX$^{TM}$-pretreatment and CIII$_I$-activation.

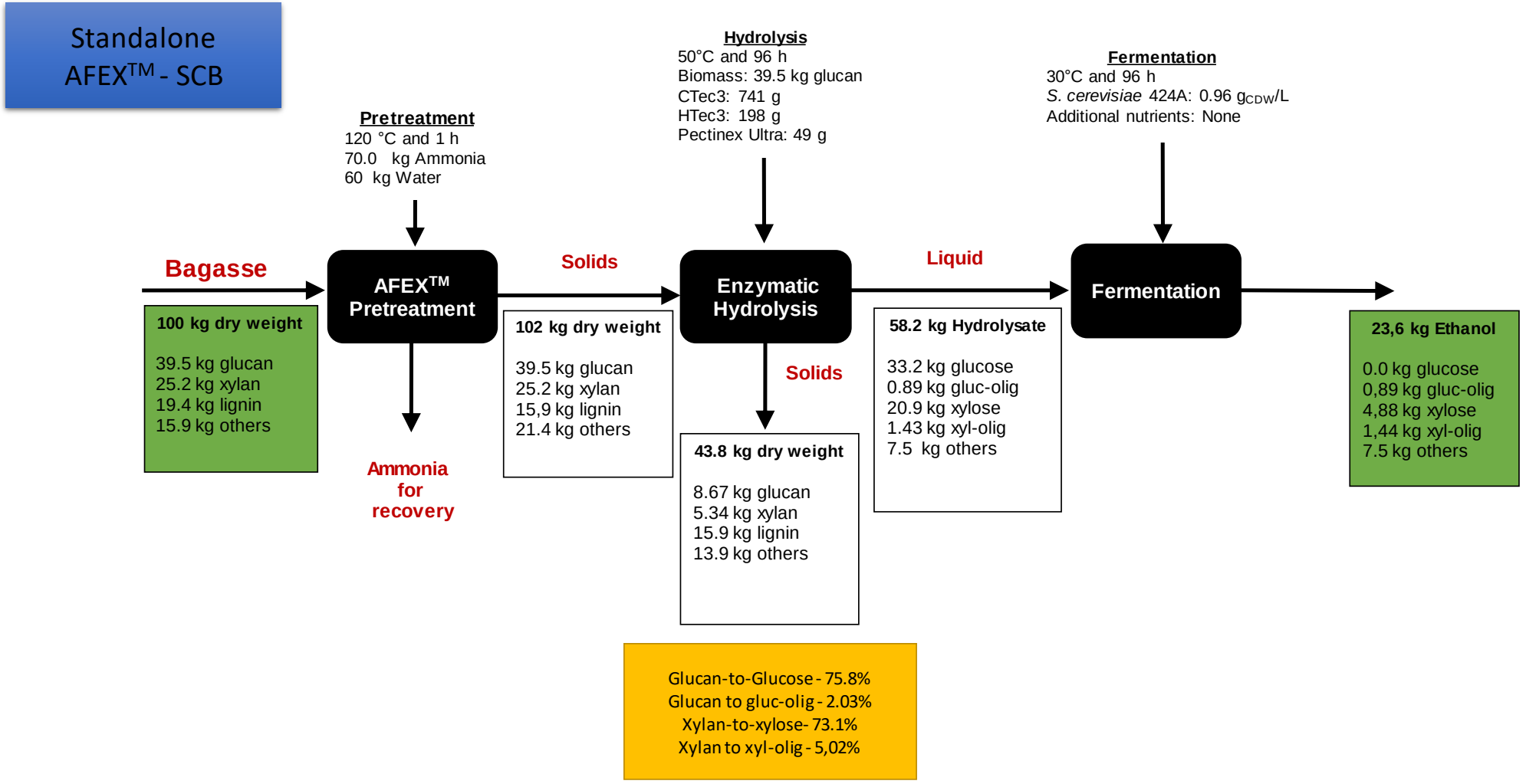



**Figure S7.5-A:** Mass balances for standalone AFEX$^{TM}$-treated SCB conversion to ethanol via high solids loading enzymatic hydrolysis and fermentation.
Enzymatic hydrolysis conditions: Enzyme dosage – 25mg/g glucan; Solids loading: 10% total sugar (~15.2% w/w); Enzymatic hydrolysis residence time: 96 hrs; Temperature: 50 °C.
Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

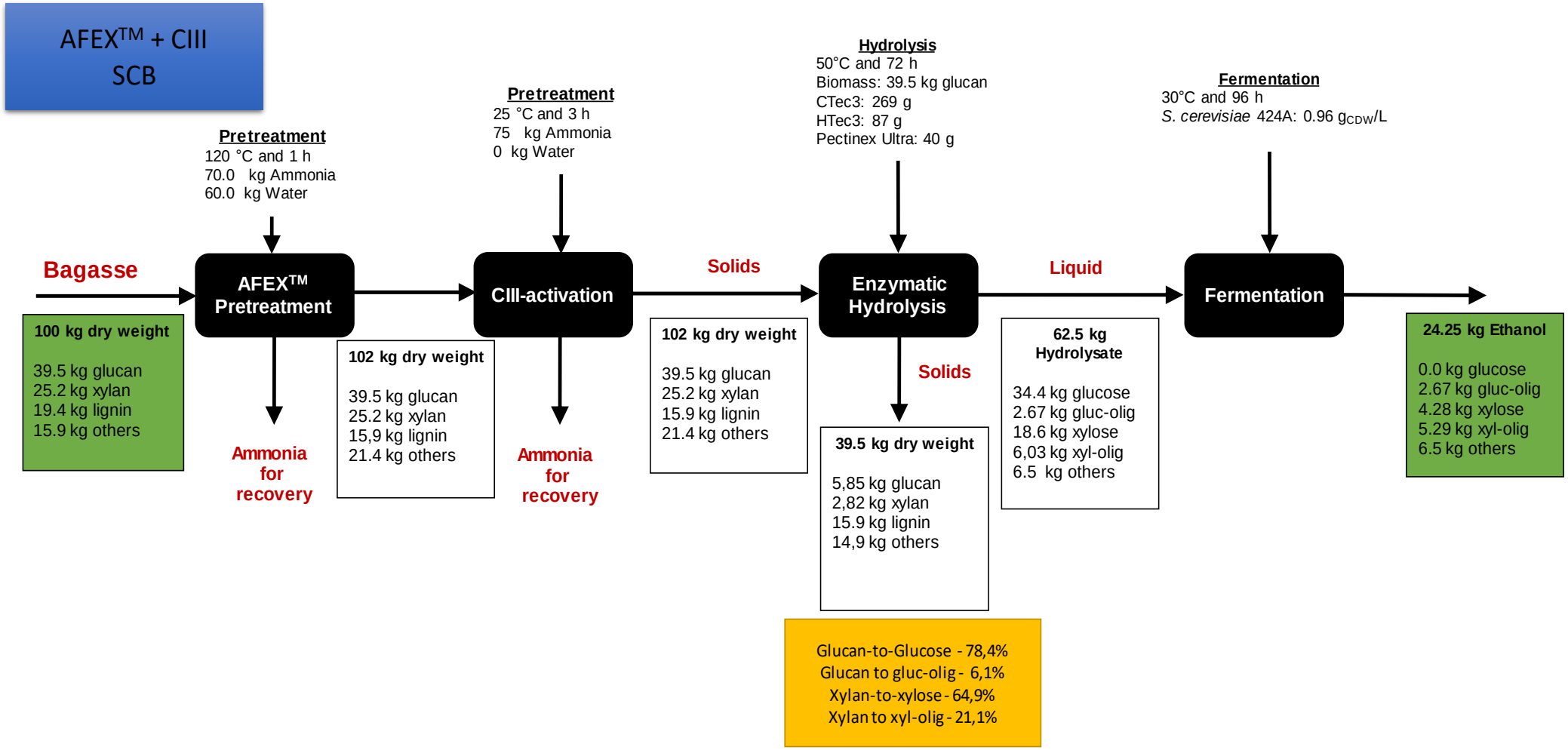



**Figure S7.5-B:** Mass balances for standalone AFEX$^{TM}$ + CIII treated SCB conversion to ethanol via high solids loading enzymatic hydrolysis and fermentation.
Enzymatic hydrolysis conditions: Enzyme dosage – 10 mg/g glucan; Solids loading: 10% total sugar (~15.2% w/w); Enzymatic hydrolysis residence time: 72 hrs; Temperature: 50 °C.
Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

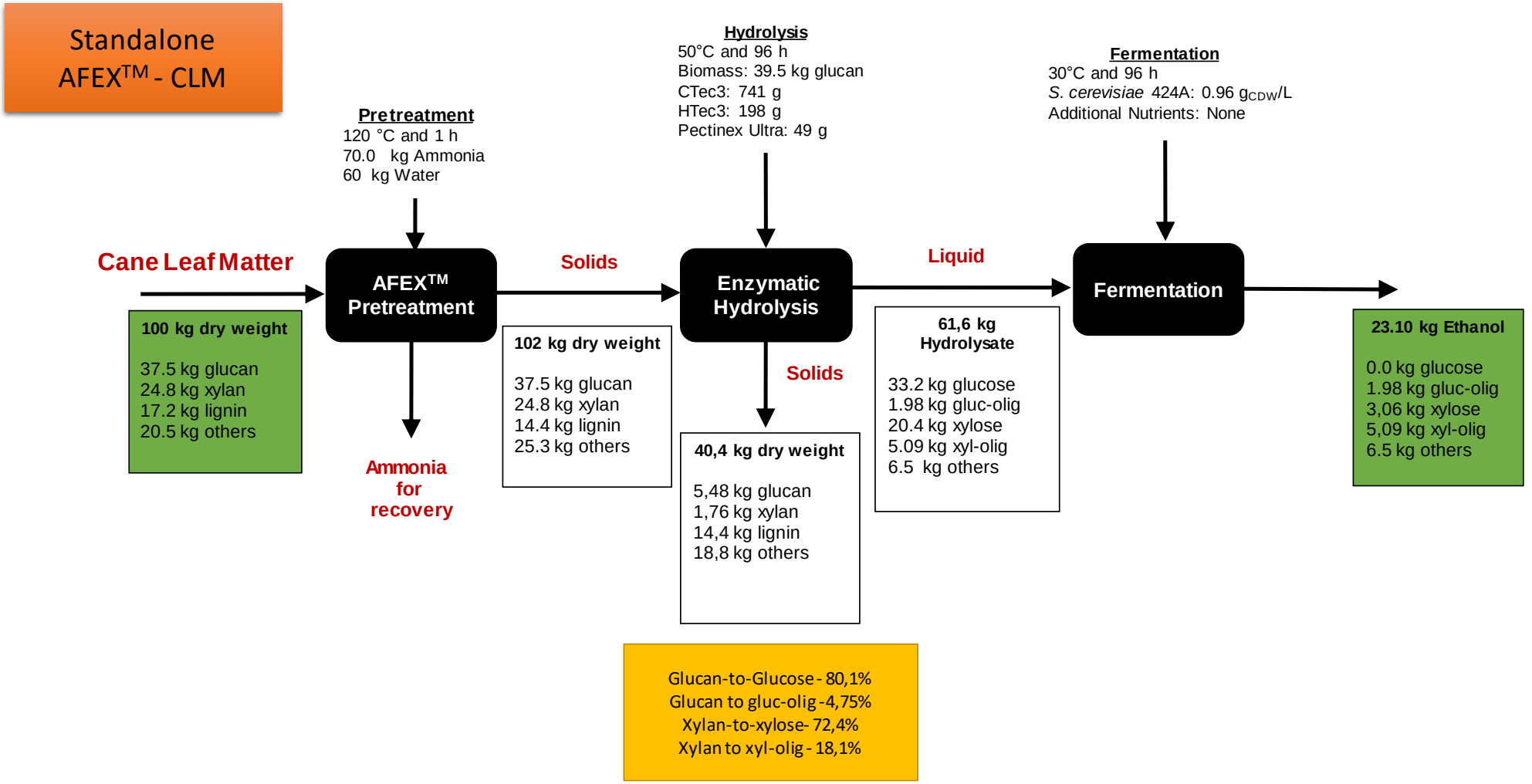



**Figure S7.5-C:** Mass balances for standalone AFEX$^{TM}$ treated CLM conversion to ethanol via high solids loading enzymatic hydrolysis and fermentation.
Enzymatic hydrolysis conditions: Enzyme dosage – 25 mg/g glucan; Solids loading: 10% total sugar (~16% w/w); Enzymatic hydrolysis residence time: 96 hrs; Temperature: 50 °C.
Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

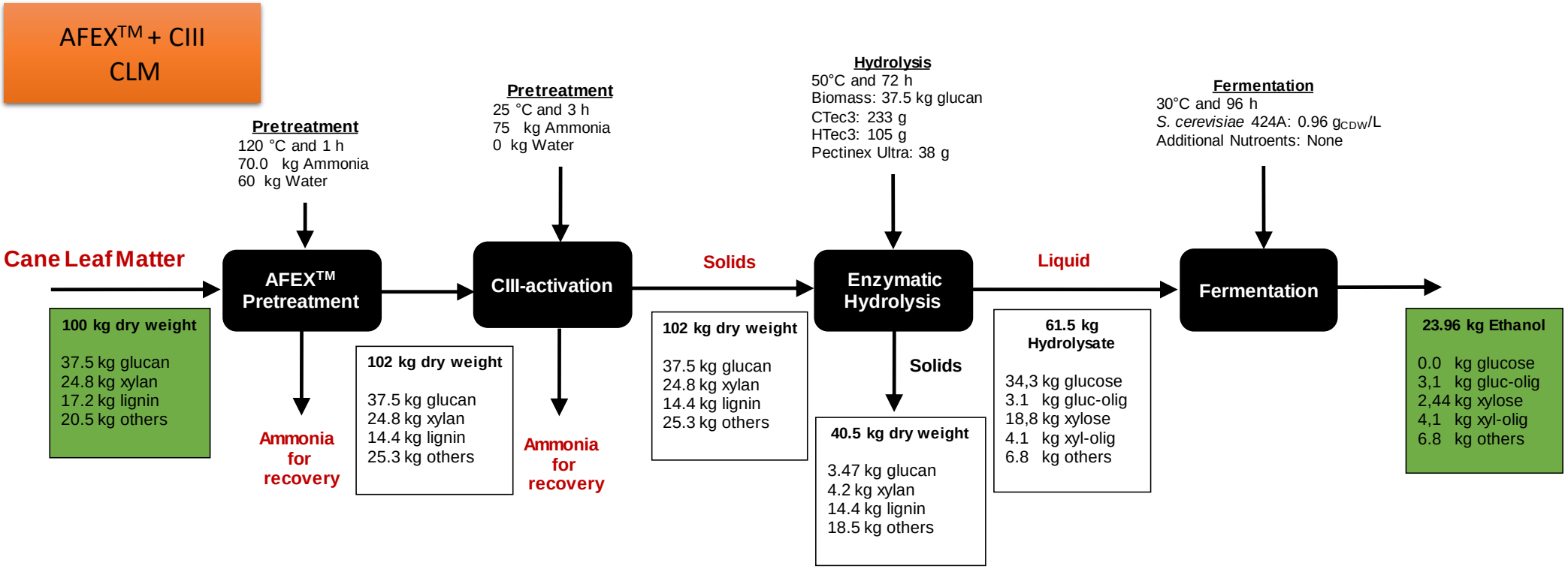



**Figure S7.5-D:** Mass balances for standalone AFEX$^{TM}$ + CIII treated CLM conversion to ethanol via high solids loading enzymatic hydrolysis and fermentation.
Enzymatic hydrolysis conditions: Enzyme dosage – 10 mg/g glucan; Solids loading: 10% total sugar (~16% w/w); Enzymatic hydrolysis residence time: 72 hrs; Temperature: 50 °C.
Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

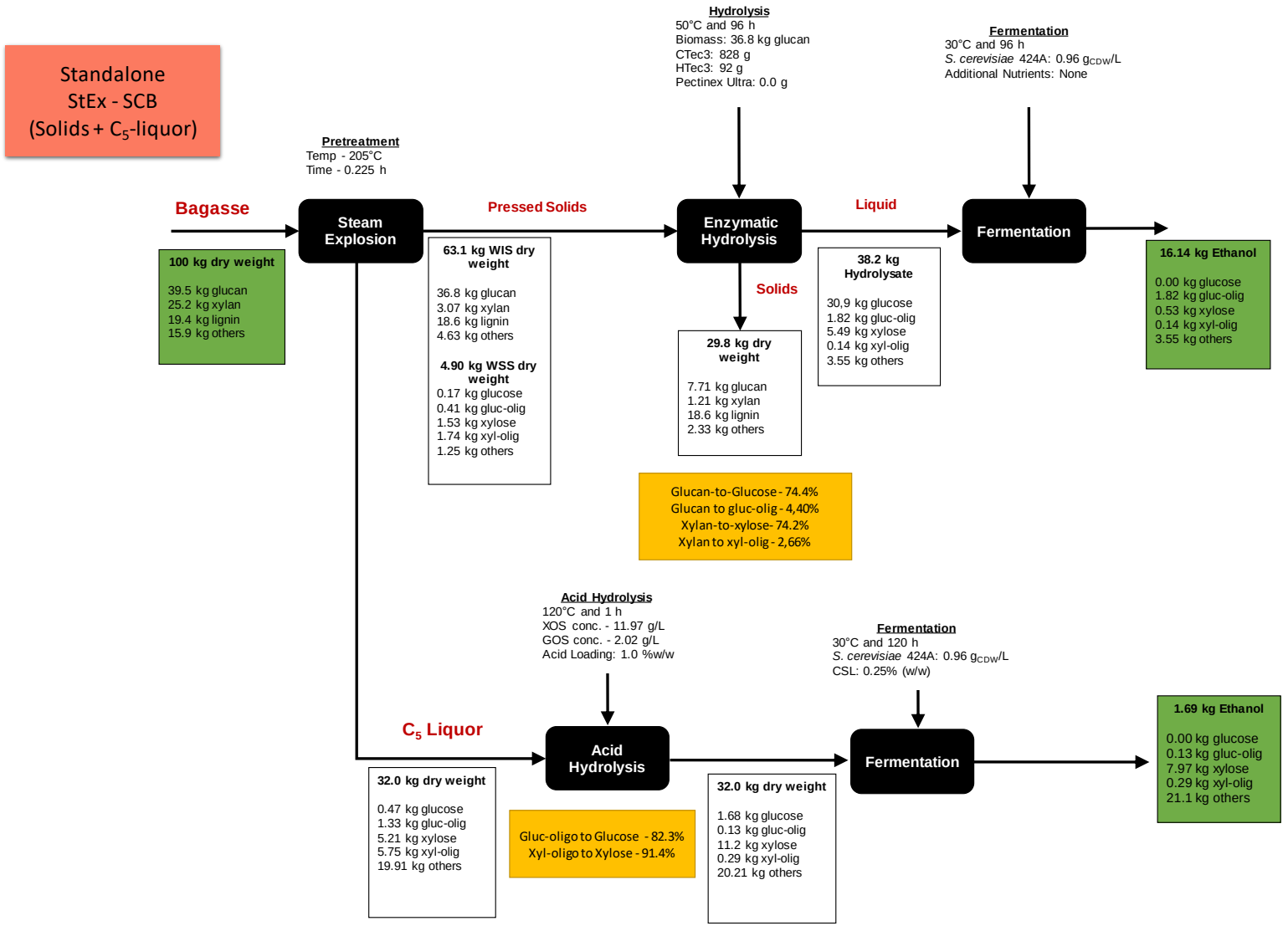



**Figure S7.5-E:** Mass balances for standalone StEx treated SCB conversion to ethanol via high solids loading enzymatic hydrolysis, hydrolysate fermentation and $C_5$-liquor fermentation. Enzymatic hydrolysis conditions: Enzyme dosage – 25 mg/g glucan; Solids loading: 10% total sugar (~15.6% w/w); Enzymatic hydrolysis residence time: 96 hrs; Temperature: 50 °C. Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

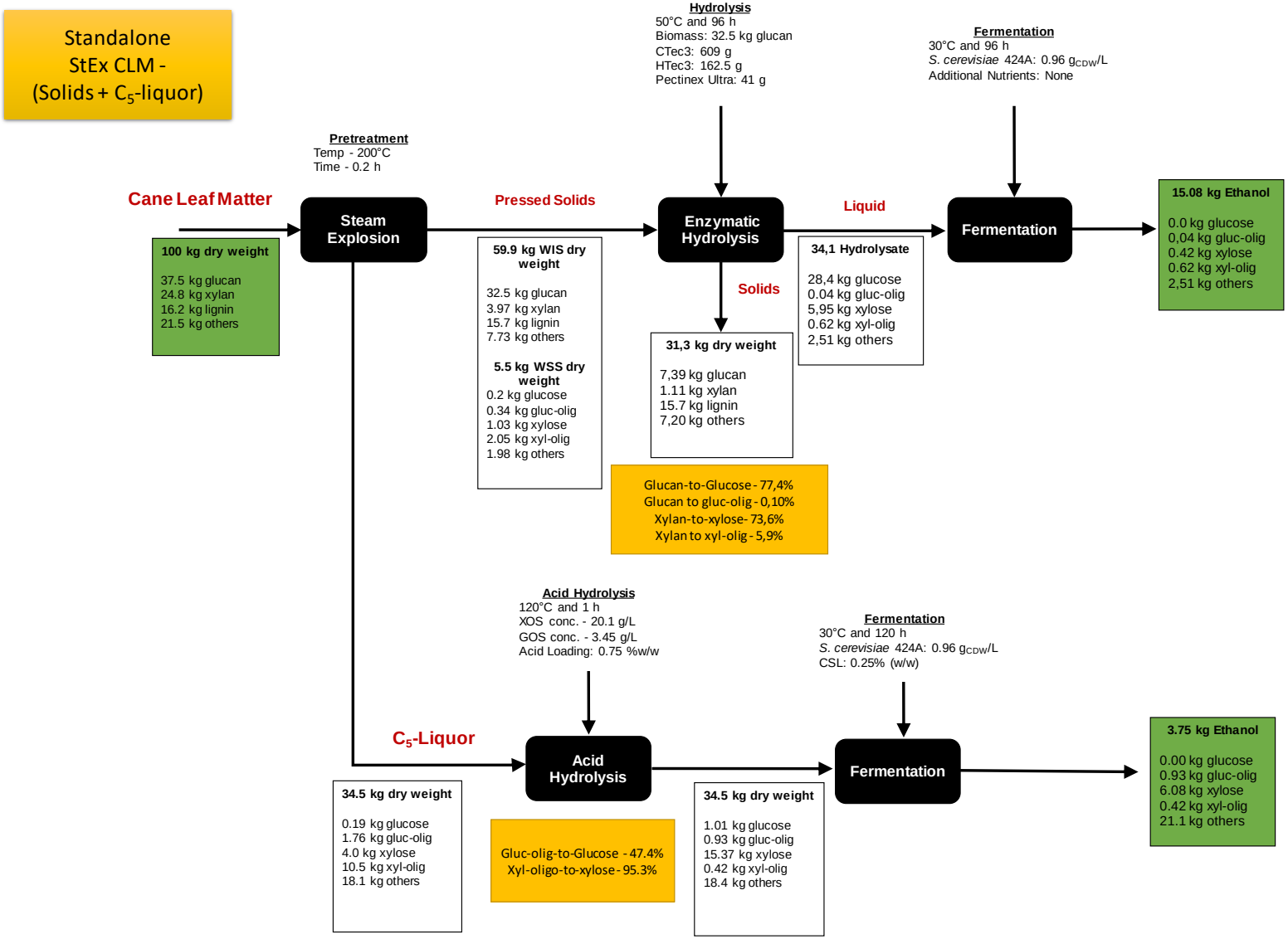



**Figure S7.F:** Mass balances for standalone StEx + CIII treated SCB conversion to ethanol via high solids loading enzymatic hydrolysis, hydrolysate fermentation and $C_5$-liquor fermentation. Enzymatic hydrolysis conditions: Enzyme dosage – 15 mg/g glucan; Solids loading: 10% total sugar (~15.6% w/w); Enzymatic hydrolysis residence time: 72 hrs; Temperature: 50 °C. Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

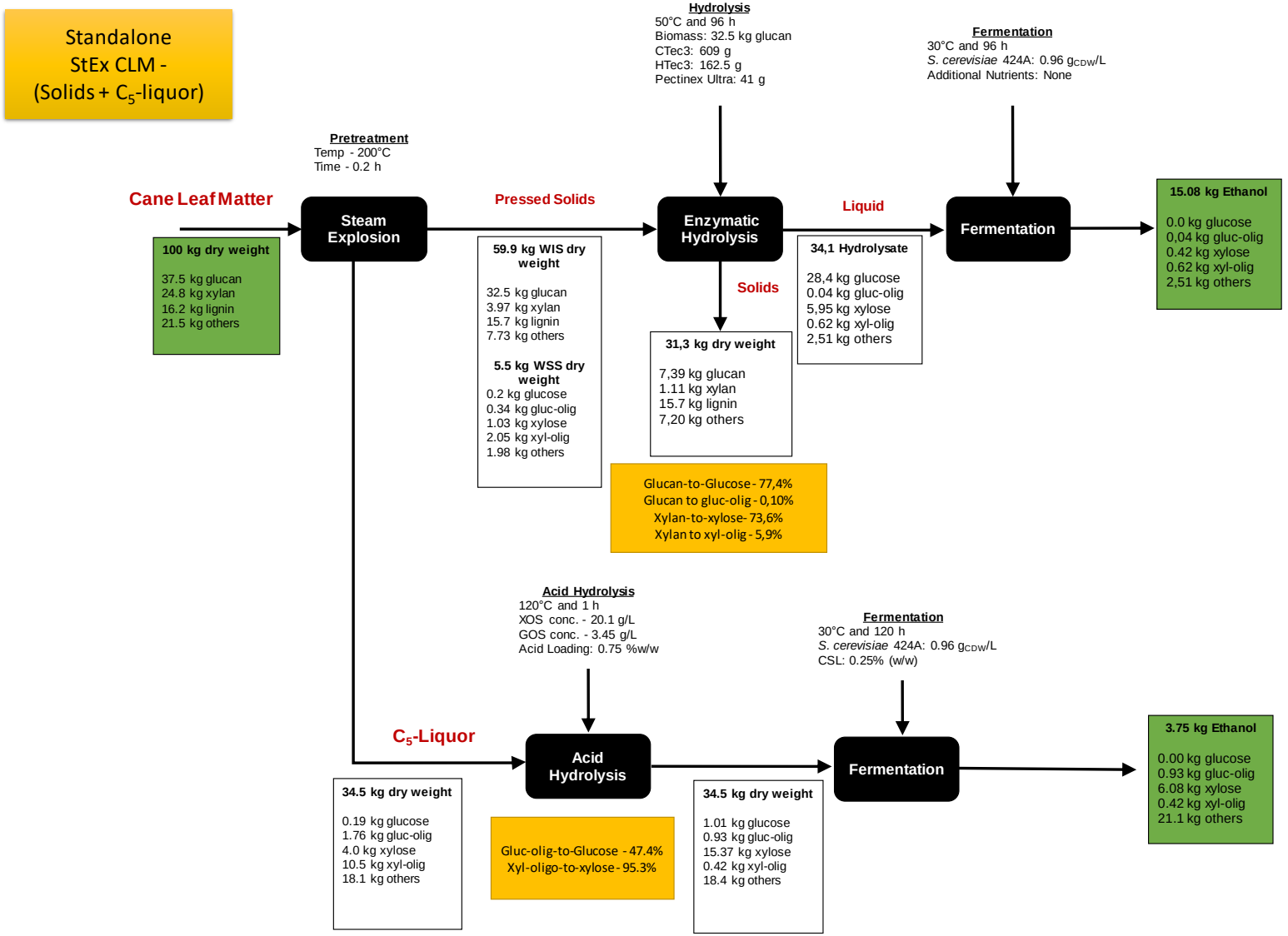



**Figure S7.5-G:** Mass balances for standalone StEx treated CLM conversion to ethanol via high solids loading enzymatic hydrolysis, hydrolysate fermentation and $C_5$-liquor fermentation. Enzymatic hydrolysis conditions: Enzyme dosage – 25 mg/g glucan; Solids loading: 10% total sugar (~16.4% w/w); Enzymatic hydrolysis residence time: 96 hrs; Temperature: 50 °C. Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

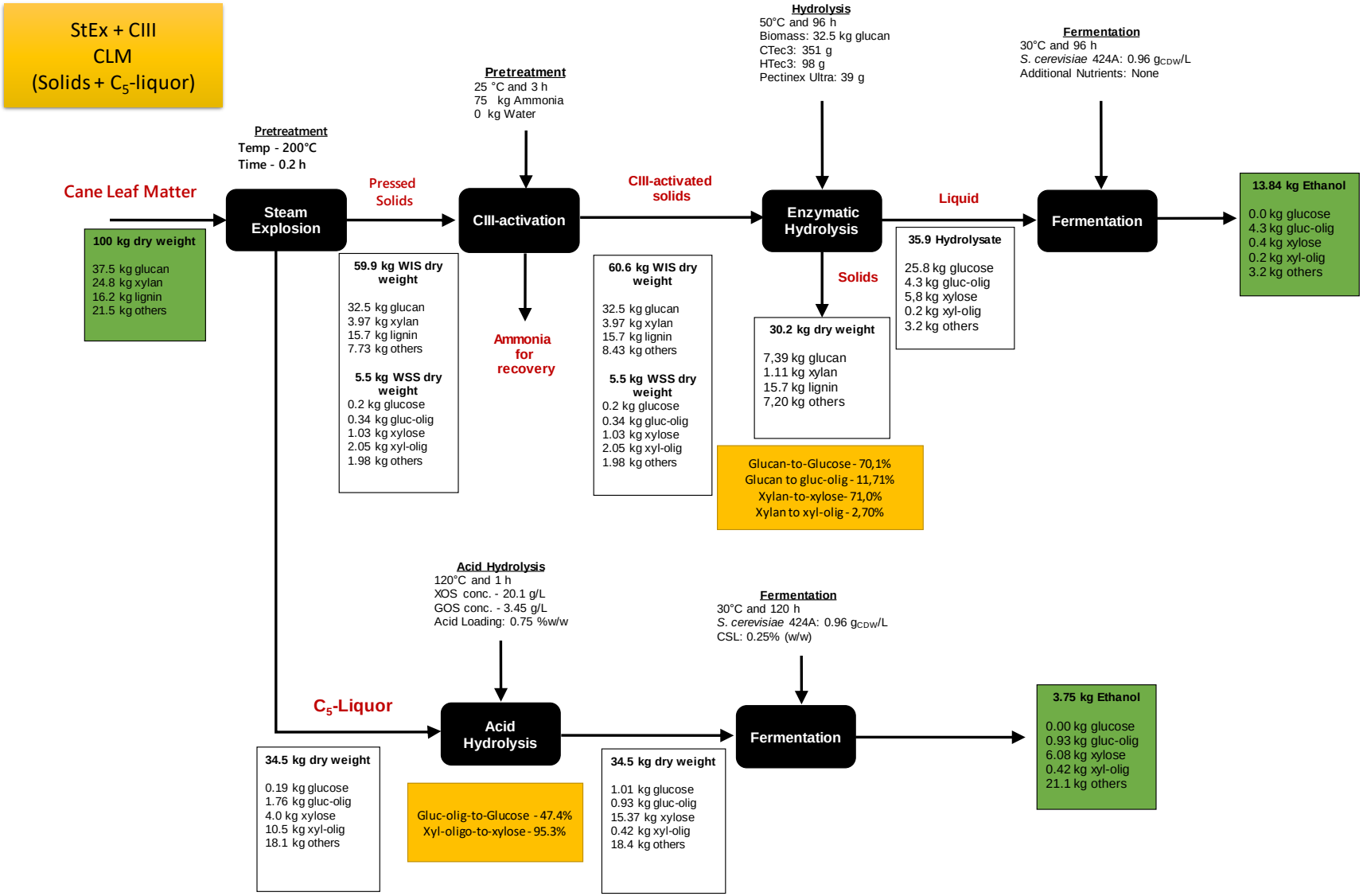



**Figure S7.5-H:** Mass balances for standalone StEx + CIII treated CLM conversion to ethanol via high solids loading enzymatic hydrolysis, hydrolysate fermentation and $C_5$-liquor fermentation. Enzymatic hydrolysis conditions: Enzyme dosage – 15 mg/g glucan; Solids loading: 10% total sugar (~16.4% w/w); Enzymatic hydrolysis residence time: 72 hrs; Temperature: 50 °C. Fermentation conditions: Nutrient supplementation – None; Yeast inoculum: 0.96 $g_{CDW}$/L; Temperature: 30 °C, Residence Time: 96 hrs.

**Figure S7.6:** Lab-scale Seedburo® pellet durability tester with four dust-tight compartments

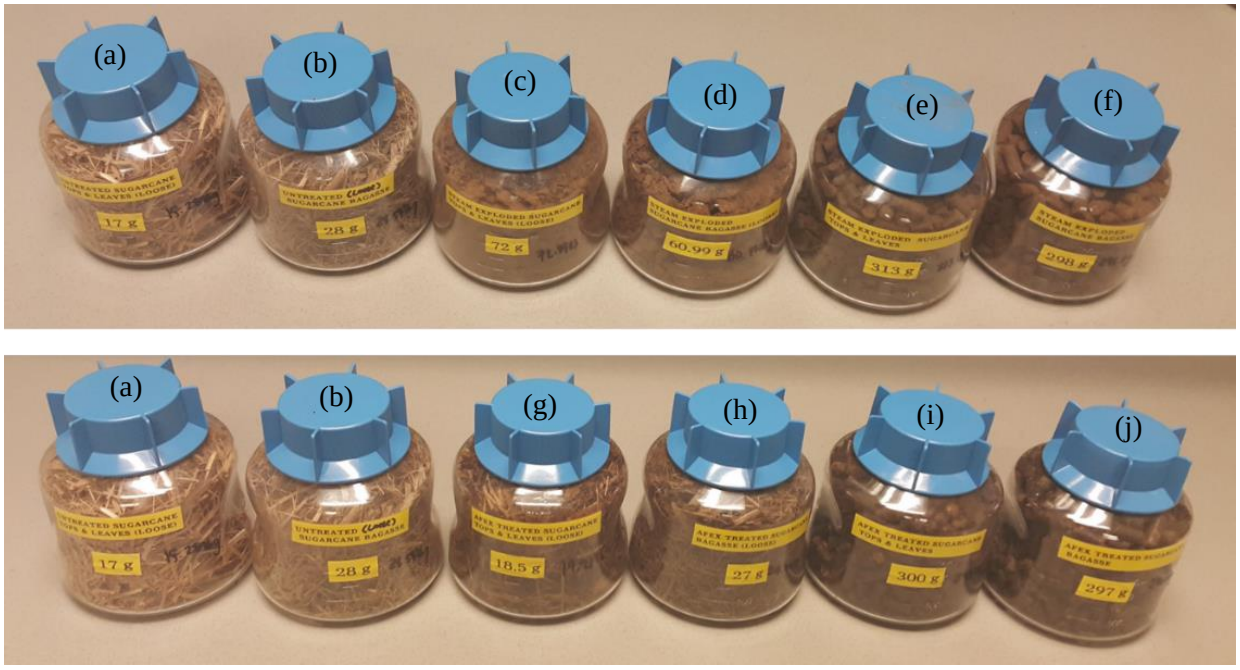



**Figure S7.7:** Demonstration of the differences in packing densities for (**a**) untreated CLM, (**b**) untreated SCB, (**c**) StEx-CLM (loose), (**d**) StEx-SCB (loose), (**e**) StEx-CLM (pellet), (**f**) StEx-SCB (pellet), (**g**) AFEX$^{TM}$-CLM (loose),(**h**) AFEX$^{TM}$-SCB (loose), (**i**) AFEX$^{TM}$-CLM (pellet), (**j**) AFEX$^{TM}$-SCB (pellet)



# CHAPTER EIGHT: CONTRIBUTION 5

**Chapter Title:** Evaluating the fermentability of steam exploded and non-detoxified sugarcane bagasse whole slurry using industrial xylose-fermenting Saccharomyces cerevisiae strains

**Authors of manuscript: Mokomele, T.**, Brandt, B.A, Görgens, J.F

**Disclosure:** This chapter contains proprietary information that should remain confidential and should not be disclosed to the public domain.

## Objective of dissertation and summary of findings in chapter

This chapter addresses the research objectives highlighted in **contribution 5**. In **CHAPTER 4**, the low fermentability of xylose in inhibitor-laden whole slurry hydrolysates generated was identified as one of the major bottlenecks for maximising ethanol yields from steam exploded sugarcane residues. This chapter evaluates the fermentability of steam exploded and non-detoxified SCB whole slurries using four industrial xylose-fermenting *Saccharomyces cerevisiae* strains, *viz.* TP-1, TP-50, CelluX$^{TM}$ E50, and CelluX$^{TM}$ 4. First, the four yeast strains were evaluated and compared for the ability to efficiently ferment both glucose and xylose from whole slurry hydrolysates derived from high solids loading enzymatic hydrolysis with a high enzyme dosage. The two best performing strains from whole slurry hydrolysate fermentation were subsequently used to compare ethanol yields that can be recovered from the pre-hydrolysis simultaneous saccharification and co-fermentation (PSSCF) of non-detoxified StEx whole slurry's and AFEX$^{TM}$-treated sugarcane bagasse.

*S. cerevisiae* TP-1 and CelluXT$^{M}$ 4 demonstrated high acetate resistance and enhanced furan and phenolic aldehyde detoxification phenotypes, resulting in near complete combined glucose and xylose conversion (> 96%) and high ethanol concentrations (> 50 g.L$^{-1}$) from the fermentation of StEx-treated and non-detoxified SCB whole slurry hydrolysates. The fermentation performance of these



two strains was among the more promising industrial xylose-fermenting yeast strains reported in literature. Under industrially relevant PSSCF solids loadings (15% w,w) and enzyme dosages (8 mg/g RDM), *S. cerevisiae* TP-1 and CelluX™ 4 facilitated ethanol yields of 208 and 224 L per Mg raw dry SCB from inhibitor-laden StEx-SCB whole slurry biomass, respectively, with near complete glucose and xylose consumption. In comparison, the PSSCF of AFEX™ pretreated SCB achieved ethanol yields of 234 and 251 L per Mg raw dry SCB using TP-1 and CelluX™ 4, respectively. The results suggest that the limitation of ethanol yields as a consequence of microbial inhibition from StEx-whole slurry fermentations (at the same pretreatment severity used in this work) can be alleviated by using *S. cerevisiae* TP-1 and CelluX™ 4. However, the lower yields from the PSSCF of StEx-SCB whole slurries relative to AFEX™-treated SCB suggest that sugar loss during pretreatment and potential enzyme inhibition could still be limiting factors for maximising ethanol yields. Nevertheless, whilst the results from **Chapter 4** indicated a significantly larger gap in the ethanol yields from StEx whole slurry's relative to AFEX™-treated biomass, this chapter emphatically shows that this difference can be minimized through the use of a suitably robust and inhibitor tolerant ethanologen.



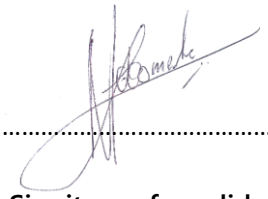
# Candidate declaration

With regards to **Chapter 8**, pages 276 – 311 of this dissertation, the nature and scope of my contributions were as follows:

| Name of contribution | Extent of contribution (%) |
|---|---|
| Experimental planning | 90 |
| Executing experiments | 100 |
| Intepretation of experiments | 80 |
| Writing of chapter | 100 |

The following co-authors have contributed to **Chapter 8**, pages 276 – 311 of this dissertation:

| Name | e-mail address | Name of contribution | Extent of contribution (%) |
|---|---|---|---|
| Bianca B.Brandt | Bbrandt@sun.ac.za | ✓Experimental planning<br>✓Intepretation of experiments | 10<br>20 |
| Johann F. Görgens | jgorgens@sun.ac.za | ✓Review of chapter<br>✓Conceived study | 100<br>100 |



..... October 2018

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**Signiture of candidate** **Date**

**Declaration by co-authors**

All authors read and approved the final manuscript and hereby confirm that:

IV. The declaration above accurately reflects the nature and extent of the contributions of the candidates and co-authors to **Chapter 8**, pages 276 – 311 in the dissertation,

V. no other authors contributed to **Chapter 8**, pages 276 – 311 in the dissertation beside those specified above, and

VI. potential conflicts of interest have been revealed to all interested parties and that are necessary arrangements have been made to use the material in **Chapter 8**, pages 276 – 311 of the dissertation.



# Evaluating the fermentability of steam exploded and non-detoxified sugarcane bagasse whole slurry using industrial xylose-fermenting *Saccharomyces cerevisiae* strains

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## Abstract

The efficient co-fermentation of hexose and pentose sugars from lignocellulosic hydrolysates in the presence of microbial stresses such as pretreatment derived inhibitors remains one of the major bottlenecks for steam explosion (StEx) based processes. In this study, we evaluated and compared the glucose and xylose fermentation capacity of four industrial xylose-fermenting *S. cerevisiae* strains: TP-1, TP-50, CelluX™ E50, and CelluX™ 4; in non-detoxified StEx sugarcane bagasse (SCB) whole slurry hydrolysates. Out of the four strains evaluated, fermentation of non-detoxified whole slurry hydrolysates with the strains TP-1 and CelluX™ 4 yielded near complete xylose consumption (≥ 88%), thereby achieving the highest ethanol concentrations (≥ 50 $g.L^{-1}$) and ethanol metabolic yields (≥ 89% relative to theoretical maximum), even in the presence of approximately 8 $gL^{-1}$ of acetic acid. Under industrially relevant pre-hydrolysis simultaneous saccharification and co-fermentation (PSSCF) conditions of high solids loading (15%, w/w) and low enzyme dosage (8 mg protein per gram untreated biomass), the fermentation of StEx-treated SCB whole slurry achieved ethanol yields of 208 and 224 L per Mg raw dry SCB using *S. cerevisiae* TP-1 and CelluX™ 4, respectively. Under the same solids loading

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and enzyme dosages, the PSSCF of ammonia fiber expansion (AFEX™) pretreated SCB achieved ethanol yields of 234 and 251 L per Mg raw dry SCB using TP-1 and CelluX™ 4, respectively. Ultimately, the results of this work provide insights into the potential use of inhibitor tolerant *S. cerevisiae* strains TP-1 and CelluX™ 4 as ethanologens for the fermentation of steam exploded and non-detoxified SCB whole slurries.

**Keywords:** Sugarcane bagasse, whole slurry, fermentation, *Saccharomyces cerevisiae*, CelluX™ 4, TP-1



## 8.1 Introduction

Feedstock or raw material costs represent the largest contribution for 2G biorefineries, hence maximising ethanol yields through the efficient conversion of all the available polymeric carbohydrate substrates in the feedstock is essential for improving the economic viability of prospective cellulosic biorefineries [4,340]. Current technologies for the biologically-mediated conversion of lignocelluloses to ethanol commence with a thermochemical pretreatment step to render the polymeric fractions embedded in the plant cell wall more accessible for enzymatic degradation [64]. However, a majority of these pretreatment technologies result in the generation of intrinsic biomass- and pretreatment-derived degradation products, which have inhibitory effects in subsequent enzymatic hydrolysis and fermentation processes [1]. Hence, for maximising ethanol yields, the fermentation of biomass derived hexoses (*i.e.* glucose, mannose, and galactose) and pentoses (*i.e.* xylose, arabinose) in the presence of pretreatment derived inhibitors is one of the fundamental bottlenecks for large scale and economical cellulosic ethanol production [341].

*Saccharomyces cerevisiae*, the most widely used microorganism in industrial sucrose and corn starch ethanol production, remains one of the leading candidate ethanologens for cellulosic ethanol production due to its general robustness and relatively high tolerance to microbial stresses such as ethanol and fermentation metabolites [342]. Through rational metabolic engineering interventions, pentose (particularly xylose) fermenting capacity in *S. cerevisiae* has been pursued through the cloning of fungal xylose reductase and xylitol dehydrogenase (XR-XDH) or the cloning of bacterial or fungal xylose isomerase (XI) pathways into *S. cerevisiae* strains [343]. However, genetically engineered xylose-fermenting yeast strains generally display higher sensitivity phenotypes to stressful conditions (*e.g.* in the presence of high weak acid, chemical inhibitors, and ethanol concentrations), resulting in lower overall fermentation yields [344].

Steam explosion (StEx) is a mature thermochemical pretreatment that uses water as the solvent/catalyst to overcome the recalcitrance of a wide array of feedstocks. However, at industrially



relevant pretreatment conditions, StEx generates weak acids (particularly acetic acid from the deacetylation of *O*-acetyl groups in hemicellulose), furan aldehydes (derived from the dehydration of glucose and xylose) and phenolic compounds (from the cleavage of acid- and alkali-labile lignin-carbohydrate complexes) that are found in the resultant pretreated slurry [163]. Among the pretreated slurry (herein referred to as whole slurry) processing options available, the use of the whole slurry in downstream enzymatic hydrolysis and co-fermentation is one of the strategies considered for reducing the biorefinery capital expenditure (CAPEX) and operating expenditures (OPEX) by avoiding costs associated with solid/liquid separation, washing, reclamation of excess process water, detoxification, and salt disposal [146,149]. However, the fermentation of inhibitor-laden StEx whole slurry hydrolysates (without detoxification) by laboratory recombinant strains such as *S. cerevisiae* 424A (LNH-ST) was significantly limited by the synergistic action of microbial stresses such as pretreatment derived-inhibitors, ethanol and fermentation metabolites [341]. In comparison, the same recombinant strain achieved near complete xylose consumption (96%) and high ethanol yields (0.46 g ethanol/g sugars) in hydrolysates derived from pretreatment technologies, such as ammonia fiber expansion (AFEX$^{TM}$), that known to generate limited amounts of microbial inhibitors

Recently, several works have reported on the successful fermentation of non-detoxified whole slurry hydrolysates derived from autohydrolysis-type pretreatments by recombinant *S. cerevisiae* strains, demonstrating high xylose consumption (> 80%), ethanol concentrations (> 38 g.L$^{-1}$), metabolic yields (> 78%) and overall ethanol productivities (0.57 g.L$^{-1}$.h$^{-1}$) [261,345–347]. Furthermore, Brandt *et al.,* (manuscript in preparation) overexpressed six genes in industrial recombinant strain *S. cerevisiae* CelluX$^{TM}$ 1 (Leaf Technologies, France) to confer enhanced strain resistance to weak acid, furan aldehyde and phenolic compound stresses [348]. The resultant transformant strain, *S. cerevisiae* TP-1, demonstrated higher inhibitor resistance, detoxification and ethanol production phenotypes relative to the parent strain in inhibitor laden and non-detoxified spent sulphite liquor. These studies demonstrate the impressive advances in the development of sufficiently hardened yeast strains to support the efficient fermentation of non-detoxified whole slurry hydrolysates without detoxification.



In this study, we evaluated the potential ethanol production from steam exploded sugarcane bagasse (SCB) using four industrial recombinant *S. cerevisiae* strains under industrially relevant conditions (*i.e.* high solids loadings to achieve high ethanol concentrations). First, we evaluated and compared the fermentability of StEx-pretreated SCB whole slurry hydrolysates using recombinant *S. cerevisiae* strains: TP-1, TP-50, CelluX™ E50, and CelluX™ 4. Subsequently, the two best performing yeast strains were selected and used as ethanologens to compare the fermentability of StEx-SCB whole slurry to AFEX™-pertreated SCB in a pre-hydrolysis simultaneous saccharification and co-fermentation (PSSCF) configuration operating under industrially relevant solids loadings and enzyme dosages. Finally, mass balances from the PSSCF experiments for both StEx- and AFEX™-treated SCB were developed to estimate the overall ethanol yields per tonne untreated SCB, and subsequently compared with literature reported yields for SCB. Ultimately, the results of this work provide insights into the improvements in the overall ethanol yields from steam exploded SCB facilitated by the use of efficient xylose-fermenting and inhibitor tolerant ethanologens.



## 8.2 Materials and methods

### 8.2.1 *Biomass, StEx and AFEX™ pretreatment*

Fresh sugarcane bagasse (SCB) was collected from two local sugar mills in Malelane (Mpumalanga, South Africa) and Mount Edgecombe (Kwazulu Natal, South Africa) and prepared as previously described [341]. Untreated SCB was composed of 39.5 ± 0.4% glucan, 25.2 ± 0.1% xylan, 19.4 ± 0.1% lignin, and 2.9 ± 0.7% ash content. StEx pretreatment of SCB was conducted in an automated batch pilot-scale unit (IAP GmBH, Graz, Austria) equipped with a 19 L StEx reaction vessel, a 100 L discharge vessel and a 40 bar steam generator as previously described by [247]. SCB was pretreated at 200 °C and 10 min, with three 100 gram samples of the pretreated slurry collected and characterized in terms of the total solids (TS), water soluble solids (WSS), water insoluble solids (WIS) and degradation product content in the WSS. The remaining slurry was vacuum packed and stored at -20°C and used within seven days. Pilot-scale AFEX™ pretreatment of SCB was performed in a pair of 450 L vertical packed-bed reactors at Michigan Biotechnology Institute (Lansing, MI, USA) as previously described in [35]. Pretreatment conditions included: ammonia to biomass loading of 0.7 g $NH_3$/g dry biomass, water loading of 0.6 g $H_2O$/g dry biomass, temperature range 80 – 120 °C, and residence time of 60 min.

### 8.2.2 *Enzymes*

Commercial fungal enzyme preparations Cellic® CTec2 (138 mg protein.$mL^{-1}$), Cellic® HTec2 (138 mg protein.$mL^{-1}$) and Pectinex Ultra-SP (31 mg protein.$mL^{-1}$) were used in enzymatic hydrolysis and ethanol production experiments. These enzymes were generously donated by Novozymes (Copenhagen, Denmark). The protein content for each cocktail was estimated using Kjeldahl nitrogen analysis (AOAC Method 2001.11). Combinations of CTec2, HTec2, and Pectinex ultra-SP previously optimized by Mokomele *et al.,* [341] for StEx-treated SCB and AFEX™ treated SCB were used during enzymatic hydrolysis and fermentation.



### 8.2.3 *Microbial strains*

Four industrial genetically engineered xylose-fermenting yeast strains were used to ferment StEx pretreated SCB whole slurry hydrolysates. Recombinant strains *S. cerevisiae* TP-1, *S. cerevisiae* TP-50 and *S. cerevisiae* CelluX™ E-50, and *S. cerevisiae* CelluX™ 4 were kindly provided by Brandt *et al.,* (manuscript prepared for submission) with permission from Lesaffre (Leaf Technologies, France). *S. cerevisiae* TP-1 was derived from recombinant parent strain *S. cerevisiae* CelluX™ 1 and was rationally engineered to overexpress six genes to confer increased resistance to selected weak acids, furan aldehydes, and phenolic compounds. *S. cerevisiae* TP-50 and *S. cerevisiae* CelluX™ E-50 are evolutionary engineered variants of *S. cerevisiae* TP-1 and CelluX™ 1, respectively, that have been evolved for a period of 50 generations in repeated batch cultures using a synthetic inhibitor-rich cocktail. *S. cerevisiae* CelluX™ 4 is the fourth generation of the CelluX™ commercial strain collection and has been reported to efficiently consume xylose derived from lignocellulosic hydrolysates within 40 h [349,350]. Stock culture aliquots of each strain were contained in 20% (v/v) glycerol and stored at -80 °C.

### 8.2.4 *Inoculum preparation*

Seed or inoculum for non-detoxified whole slurry hydrolysate or PSSCF fermentations was performed using a two-step protocol to prime the yeast cells for improved fermentation performance upon exposure to inhibitor stressed conditions [351]. Pre-seed cultures of the four industrial strains used in this work were initially cultivated from the glycerol stock cultures in test tubes containing 10mL YPDX media (20 $g.L^{-1}$ glucose, 4 $g.L^{-1}$ xylose, 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ tryptone) and incubated at 30 °C and 150 rpm in a rotary shaker for 24 h. The seed culture was prepared by inoculating 1.5 g CDW/L of the pre-seed cultures into 250 mL Erlenmeyer flasks containing pre-conditioning media that was composed of 75 mL YPDX media and 25 mL of StEx pretreatment liquor. StEx pretreatment liquor was derived by filtering the solids from the StEx pretreatment whole slurry. After inoculating the pre-conditioning media, the seed-cultures were incubated at 30 °C and 150 rpm in an orbital shaker for 24

h. The pre-conditioned seed culture was thereafter harvested by centrifuging at 1,500 x *g* for 10 min and the yeast pellets were used as inoculum for whole slurry hydrolysate fermentation and PSSCF experiments.

### 8.2.5 *Fermentation of StEx SCB whole slurry*

The fermentation capability of the four selected industrial xylose-fermenting yeast strains was evaluated in two sets of experiments as shown in Figure 8.1. In the first set, the fermentation capability of the four selected recombinant strains was evaluated on non-detoxified StEx-SCB whole slurry hydrolysate and the performance of the strains was compared in terms of the ethanol yield, final ethanol concentration and specific ethanol productivity. In the second set of experiments, two of the best performing strains from the first set were subsequently used to evaluate their suitability as ethanologens for the PSSCF of AFEX™ pretreated SCB and non-detoxified StEx-treated SCB whole slurry.

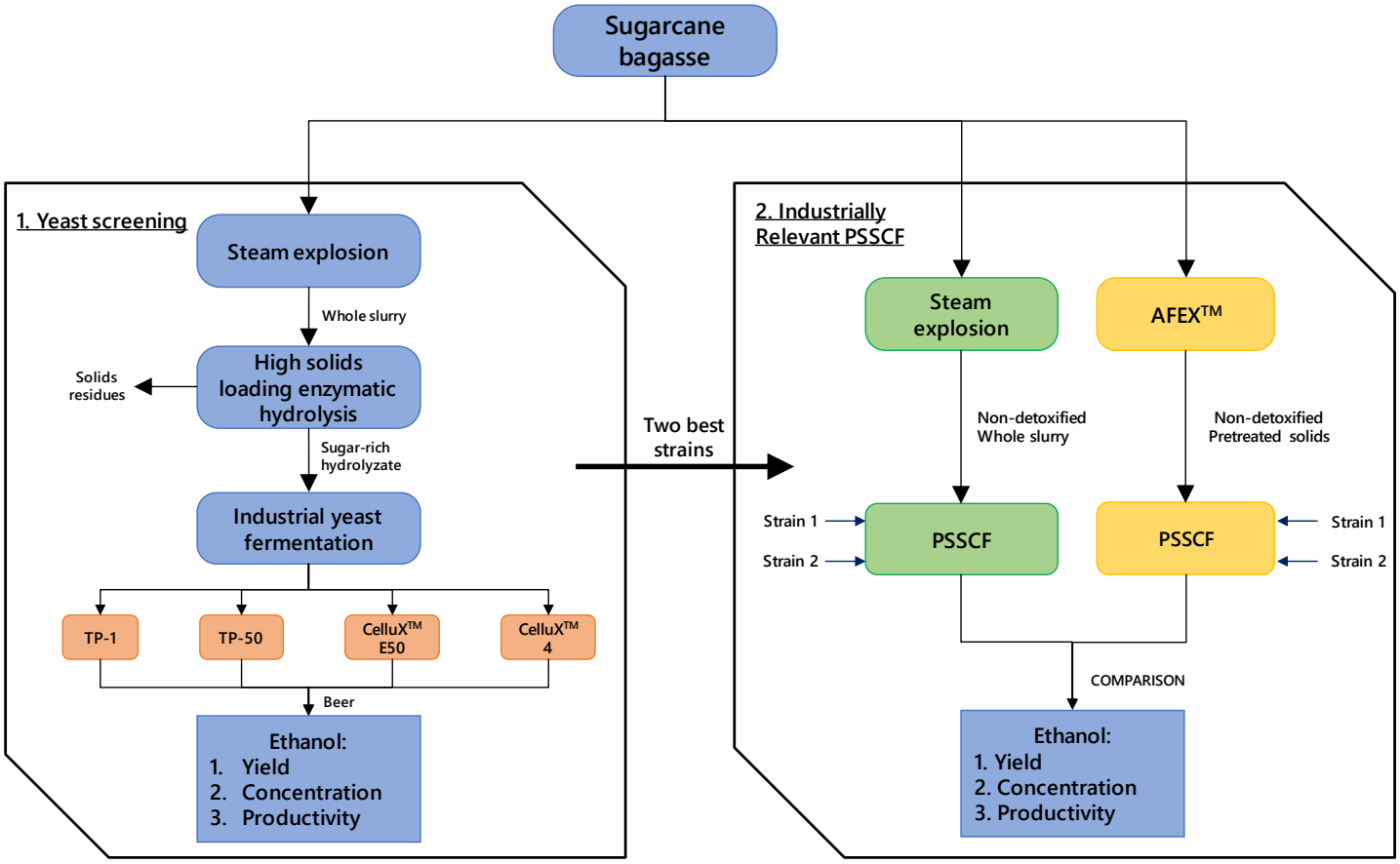


**Figure 8.1:** Experimental approach for evaluating the fermentability of StEx-treated SCB whole slurry and AFEX™ treated biomass in pre-hydrolysis simultaneous saccharification and co-fermentation process configuration.



#### 8.2.5.1 *Yeast screening: Preparation and fermentation of StEx-SCB whole slurry hydrolysate*

The StEx-treated SCB whole slurry was enzymatically hydrolysed in 1000 mL baffled Erlenmeyer flasks with a working volume of 400 grams at a solids loading of 15% (w/w) and an enzyme dosage of 40 mg protein per gram glucan. A relatively high enzyme dosage was used to overcome the impact of the inhibitors and end-product inhibition on the activity of the hydrolytic enzymes, and therefore enable the production of non-detoxified enzymatic hydrolysates with relatively high sugar concentrations that are anticipated for commercial biorefineries [352]. To avoid mixing issues associated with high solids loading enzymatic hydrolysis, the whole slurry was added using a fed-batch strategy, with half the slurry added at $t$ = 0 h and the remainder at $t$ = 3 h. The enzymatic hydrolysis mixtures were supplemented with 50 mM potassium phosphate buffer and 50 mg.L$^{-1}$ chloramphenicol to regulate the hydrolysis/fermentation pH and prevent bacterial contamination, respectively, and subsequently incubated at 50 °C and 180 rpm in an orbital shaker. After 72 h of hydrolysis, the hydrolysis slurry was centrifuged at 8,000 x $g$ for 30 min. The sugar-rich supernatant was supplemented with 0.5% (w/w) corn steep liquor (CSL), pH adjusted to 5.5 using 10M KOH, before being filter sterilized through a 0.22 µm polyethersulfone filter into a sterile bottle (Millipore Stericup). The sterile whole slurry hydrolysate was refrigerated at 4 °C until use (used within one day).

The fermentation of the sterile StEx-SCB whole slurry hydrolysates was performed in 100 mL serum bottles with a working volume of 40 mL and incubated at 30 °C and 150 rpm in an orbital shaker. Each serum bottle was inoculated with the pre-conditioned seed culture at $OD_{600}$ of 3 (~1.8 g CDW/L). After inoculating the hydrolysate containing serum bottles, they were capped with sterile butyl rubber stoppers, sealed with an aluminium crimp, and pierced with two hypodermic needles to facilitate $CO_2$ release, sampling, and semi-anaerobic fermentation conditions. Samples were withdrawn once every 24 h and the sugar, ethanol and furan aldehydes content of the cell-free supernatants was quantified HPLC analysis (described below). Each hydrolysate fermentation assay was performed in triplicate.



#### 8.2.5.2 *Simultaneous saccharification and co-fermentation with pre-saccharification (PSSCF)*

Fed-batch PSSCF of both the StEx-treated SCB whole slurry and the AFEX™-treated SCB at 15% (w/w) solids loading were carried out in baffled 250 mL Erlenmeyer flasks using an enzyme dosage of 20 mg protein/g glucan and a total mixture weight of 100 grams. Like the yeast screening hydrolysates, the biomass was added in two steps (half at $t$ = 0 h and the remainder at $t$ = 3 h) and the PSSCF flasks were supplemented with 50mM phosphate buffer, 0.5% (w/w) CSL, 50 $g.L^{-1}$ chloramphenicol (antibiotic) and the pH was adjusted using 10M KOH or 8M HCl after adding the biomass. PSSCF was conducted with an initial 48 h pre-saccharification step at 50 °C, pH 5 and 200 rpm to liquefy the pretreated biomass and to produce high sugar concentrations prior to yeast inoculation [105]. After pre-saccharification, seed cultures of the recombinant strains were inoculated at to an initial $OD_{600}$ of 3, the incubation temperature and shaking speed were lowered to 35 °C and 180 rpm, respectively, and the PSSCF proceeded for an additional 96 h (144 h total pre-saccharification + SSCF time). For the duration of the PSSCF, samples were withdrawn once every 24 h and quantified by HPLC analysis. Each PSSF assay was performed in triplicate.

### 8.2.6 *Analytical techniques*

Monomeric sugars (glucose, xylose, arabinose), aliphatic acids (acetic acid, formic acid), and furan aldehydes (furfural, 5-HMF) fermentation products (lactate, xylitol, glycerol and ethanol) were determined by Thermo Separation Product HPLC system on an Aminex HPX-87H ion exchange column equipped with a Bio-Rad H cartridge guard column (Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 65 °C, with 5mM $H_2SO_4$ as the mobile phase at a flowrate of 0.6 mL. $min^{-1}$. Peak detection for sugars, fermentation products and aliphatic acids performed using a refractive index detector (Shodex, RI-101), whereas the furan aldehydes were detected using a RS Variable Wavelength UV detector set at 280 nm. Phenolic compounds were analysed on Dionex UltiMate™ 3000 HPLC System equipped with an Aminex HPX-87H (7.8x250 mm) and a RS Variable



Wavelength UV detector. The mobile phases used for elution was water (A) and acetonitrile (B) at a flow rate of 0.7 mL $min^{-1}$.

### 8.2.7 *Calculations*

For hydrolysate fermentation experiments, the ethanol yield ($Y_{p/s}$) was determined from the amount of ethanol generated relative to the sum of monomeric glucose and xylose at $t$ = 0 h. The ethanol metabolic yield was calculated from the ethanol generated relative to the stoichiometric maximum ethanol from the consumed glucose and xylose. The specific glucose or xylose uptake ($q_{glucose}$ or $q_{xylose}$) and specific ethanol production rates ($q_{ethanol}$) were determined from the amount of substrate consumed or ethanol produced per cell mass. The maximum growth rate ($\mu_{max}$) was estimated during the exponential growth phase by plotting the natural logarithm of the cell $OD_{600}$ as a function of time. The ethanol yields from PSSCF were estimated based on the weight ethanol produced relative to the weight of initial polymeric glucan and xylan content input to the flasks. The overall PSSCF productivity was calculated amount of ethanol generated relative to the overall processing time, *i.e.* 144 h.

### 8.2.8 *Statistical analysis*

The experimental data is presented as means ± standard deviation of triplicate experimental runs. The statistical significance of the experimental results was determined through a one-way analysis of variance (ANOVA) in combination with Tukey's *post hoc* HSD test for multiple comparisons (Minitab Inc., State College, PA, USA). The null hypothesis was accepted or rejected at 95% confidence interval ($P < 0.05$).



## 8.3 Results and discussion

### 8.3.1 *Chemical composition of StEx and AFEX™ pretreated SCB and whole slurry enzymatic hydrolysate*

The chemical composition of the StEx-SCB whole slurry, StEx-SCB whole slurry hydrolysate and AFEX™-treated SCB are presented in Table 8.1. The water insoluble solids of the StEx-SCB whole slurry were enriched in glucan and Klason lignin content, whereas the pretreatment liquor was composed of predominantly hemicellulose derived total sugars (34 $g.L^{-1}$), acetate (5.87 $g.L^{-1}$), formate (0.57 $g.L^{-1}$), and furan aldehydes (0.958 $g.L^{-1}$). Vanillic acid, ferulic acid, p-coumaric acid, 3,4-dihydrobenzoic acid, syringic acid, vanillin, syringaldehyde, and coniferyl aldehyde were quantified as the predominant phenolic compounds in the pretreatment liquor, with their cumulative concentration being 231.8$mg.L^{-1}$.

High solids loading enzymatic hydrolysis of the non-detoxified whole slurry generated a hydrolysate with increased glucose (80.8 $g.L^{-1}$), xylose (33.7 $g.L^{-1}$) and acetate (8.11 $g.L^{-1}$) concentrations due to the enzyme-mediated degradation of the structural carbohydrates and soluble oligosaccharides that were present in the pretreated slurry ($P < 0.05$). However, the formate (0.81$g.L^{-1}$), furan aldehyde (0.717 $g.L^{-1}$) and phenolic compound concentrations (157.7 $mg.L^{-1}$) were slightly reduced compared to the pretreatment liquor, primarily due to the addition of water and enzymes during the hydrolysis ($P < 0.05$). Based on the total fermentable sugar (monomeric glucose + xylose) concentration in the whole slurry hydrolysate, a maximum ethanol concentration of 58.4 $g.L^{-1}$ (based on a theoretical yield of 0.51 g ethanol/g sugar) could be generated from the hydrolysate. This would be significantly higher than the minimum recommended ethanol concentration (40 $g.L^{-1}$) from fermentation to minimise energy costs for downstream ethanol recovery. The structural composition of AFEX™-treated SCB was adopted from **CHAPTER 6**.



**Table 8.1:** Chemical composition of AFEX™-treated SCB, StEx-treated SCB whole slurry, and StEx-treated SCB whole slurry hydrolysate

| Polymeric structural components in solids | | | |
|---|---|---|---|
| **Components** | **AFEX™-SCB** | **StEx-SCB pretreatment whole slurry** | **StEx-SCB pretreatment whole slurry hydrolysate** |
| Glucan (g/100g DM)† | 39.15 ± 0.88 | 56.53 ± 2.60 | - |
| Xylan (g/100g DM)† | 24.53 ± 0.44 | 6.74 ± 0.81 | - |
| Arabinan (g/100g DM)† | 1.34 ± 0.48 | 0.28 ± 0.06 | - |
| Acetyl (g/100g DM)† | 0.62 ± 0.11 | 1.44 ± 0.22 | - |
| Klason Lignin (g/100g DM)† | 16.92 ± 0.51 | 27.14 ± 0.45 | - |
| Ash (g/100g DM)† | 2.88 ± 0.09 | 3.44 ± 0.65 | - |
| **Water soluble structural components in pretreatment liquor or hydrolysate** | | | |
| Monomeric glucose (g/L) | - | 1.05 ± 0.18 | 80.82 ± 2.21 |
| G-OS (g/L) | - | 2.99 ± 0.54 | 3.10 ± 1.01 |
| Monomeric xylose (g/L) | - | 8.62 ± 0.22 | 33.71 ± 0.96 |
| X-OS (g/L) | - | 19.26 ± 3.62 | 6.02 ± 1.61 |
| Monomeric Arabinose (g/L) | - | 1.37 ± 0.37 | 1.15 ± 0.05 |
| A-OS (g/L) | - | 0.76 ± 0.34 | BDL |
| Acetic Acid (g/L) | - | 5.87 ± 0.72 | 8.11 ± 0.43 |
| Formic Acid (g/L) | - | 0.57 ± 0.24 | 0.81 ± 0.07 |
| Lactic Acid (g/L) | - | BDL | BDL |
| Furfural (mg/L) | - | 863.92 ± 85.56 | 651.33 ± 16.03 |
| 5-HMF (mg/L) | - | 93.78 ± 22.16 | 66.00 ± 1.89 |
| Vanillic Acid (mg/L) | - | 12.20 ± 3.70 | 10.35 ± 0.73 |
| Ferulic Acid (mg/L) | - | 29.78 ± 3.34 | 17.02 ± 1.22 |
| *p*-Coumaric Acid (mg/L) | - | 75.61 ± 8.11 | 60.23 ± 3.34 |
| 3,4-Dihydrobenzoic Acid (mg/L) | - | 7.01 ± 1.26 | 2.48 ± 0.61 |
| Syringic Acid (mg/L) | - | 26.75 ± 3.45 | 9.61 ± 3.51 |
| Vanillin | - | 51.65 ± 0.48 | 36.82 ± 1.21 |
| Syringaldehyde (mg/L) | - | 20.31 ± 1.78 | 14.38 ± 1.13 |
| Coniferyl Aldehyde (mg/L) | - | 8.44 ± 0.31 | 6.81 ± 0.81 |

† - dry basis

Abbreviations: G-OS – Glucooligosaccharides; X-OS – Xylooligosaccharides; A-OS – Arabinoologosaccharides; BDL – Below Detection Limit; SCB – sugarcane bagasse

### 8.3.2 *Screening of industrial xylose-fermenting yeast strains for tolerance of inhibitors in SCB whole slurry hydrolysate*

The fermentation profiles for converting StEx-treated SCB whole slurry hydrolysate to ethanol are presented in Figure 8.2 and the corresponding kinetic parameters are presented in Table 8.2. The non-detoxified hydrolysates were generated from high solids loading enzymatic hydrolysis to attain high initial sugar and inhibitor concentrations to simulate the synergistic action of multiple stress



conditions (including inhibitors and osmotic stress) at the beginning of the fermentation and high ethanol stress on xylose-utilization towards the end of the fermentation [346,353].

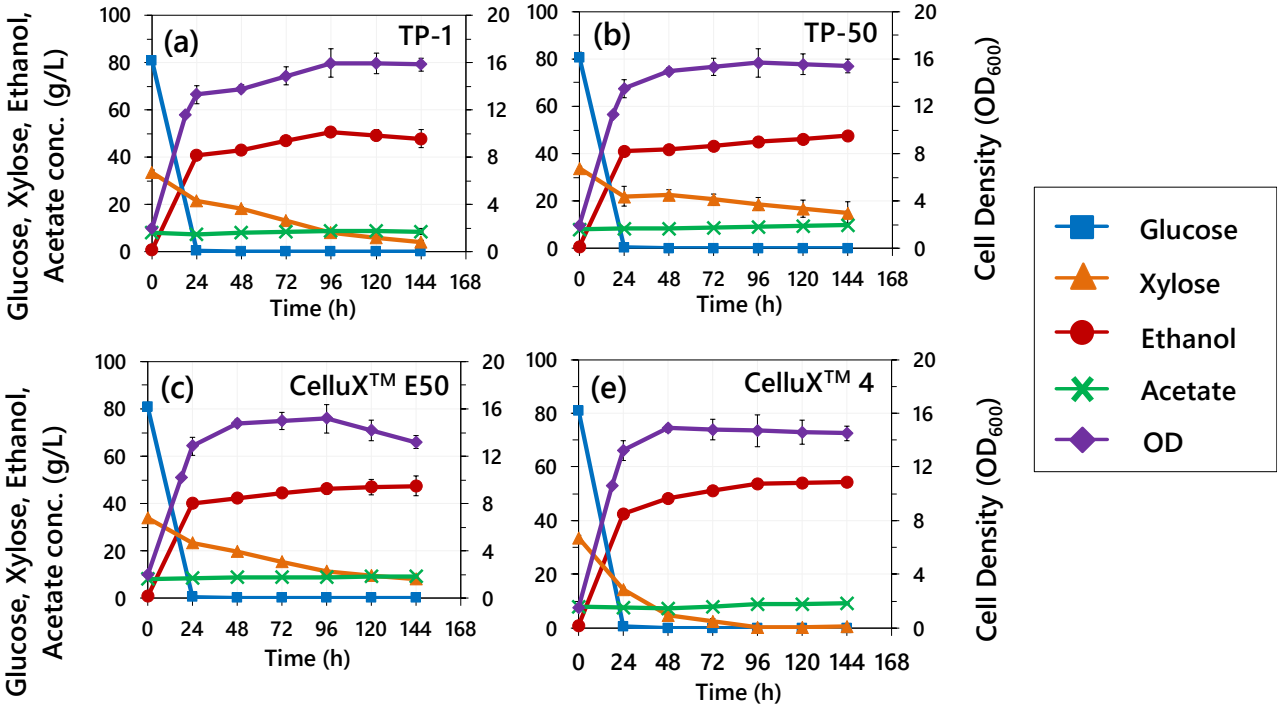


**Figure 8.2:** Time profiles for the fermentation of steam exploded and undetoxified sugarcane bagasse whole slurry hydrolysate using (**a**) *S. cerevisiae* TP-1, (**b**) *S. cerevisiae* TP-50, (**c**) *S. cerevisiae* CelluX™ E50, (**d**) *S. cerevisiae* CelluX™ 4. Fermentations were performed with an initial inoculum of 1.5 g CDW.$L^{-1}$ at pH 5.5, 30 C and a shaking speed of 150 rpm for 120 h. All hydrolysates were supplemented with 0.5% (w/w) corn steep liquor.

For all four yeast strains, fermentation proceeded without a noticeable lag phase with glucose rapidly consumed to completion within 24 h, resulting in ethanol concentrations greater than 38 g.$L^{-1}$ even in the presence of acetic acid concentrations of approximately 8.3 g.$L^{-1}$. The fast-initial glucose consumption by these strains demonstrates their higher affinity for glucose and their robustness for the rapid utilization of glucose in the inhibitor-laden whole slurry hydrolysate. Furthermore, all four strains did not show diauxic xylose fermentation traits, even though xylose was consumed at a significantly slower rate relative to glucose. In **CHAPTER 4**, *S. cerevisiae* 424A (LNH-ST) demonstrated a similar trend, with glucose consumed to completion within 24 h and slow xylose fermentation phenotype potentially due to the lack of high-affinity xylose transporters in the presence of glucose.



**Table 8.2:** Comparing the literature reported yeast fermentation of non-detoxified whole slurry hydrolysates to *S. cerevisiae* TP-1, TP-50, CelluX™ E50, and CelluX™ 4.

| Yeast Strain | Hydrolysate | Initial sugar conc. ($g.L^{-1}$)ψ | Glucose Cons (%) | Xylose cons (%) | EtOH conc. ($g.L^{-1}$) | $Y_{P/S}$ ($g.g^{-1}$) * | EtOH prod. ($g.L^{-1}.h^{-1}$) ϕ | Metabolic Yield (%) ** | $q_{EtOH}$ ($g.g^{-1}.h^{-1}$) | $q_{Glucose}$ ($g.g^{-1}.h^{-1}$) | $q_{Xylose}$ ($g.g^{-1}.h^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. cerevisiae* TP-1 | StEx-SCB whole slurry hydrolysate | G: 80<br>X: 34 | 100% | 88% | 50.0 | 0.44 | 0.347 | 89% | 0.063 | 0.406 | 0.034 | This study |
| *S. cerevisiae* TP-50 | StEx-SCB whole slurry hydrolysate | G: 80<br>X: 34 | 100% | 55% | 46.7 | 0.41 | 0.325 | 92% | 0.040 | 0.418 | 0.029 | This study |
| *S. cerevisiae* CelluX™ E50 | StEx-SCB whole slurry hydrolysate | G: 80<br>X: 34 | 100% | 77% | 46.7 | 0.41 | 0.324 | 86% | 0.052 | 0.418 | 0.029 | This study |
| *S. cerevisiae* CelluX™ 4 | StEx-SCB whole slurry hydrolysate | G: 80<br>X: 34 | 100% | 98% | 53.8 | 0.47 | 0.747 | 92% | 0.096 | 1.105 | 0.154 | This study |
| *S. cerevisiae* 424A (LNH-ST) | AFEX™-SCB hydrolysate | G: 59<br>X: 37 | 100% | 96% | 44.2 | 0.46 | 0.368 | 92% | 0.080 | 1.067 | 0.067 | [341] |
| *S. cerevisiae* 424A (LNH-ST) | AFEX™-CLM hydrolysate | G: 58<br>X: 35 | 100% | 95% | 41.7 | 0.44 | 0.350 | 89% | 0.073 | 1.033 | 0.061 | [341] |
| *S. cerevisiae* 424A (LNH-ST) | StEx-SCB whole slurry hydrolysate | G: 70<br>X: 26 | 98% | 37% | 34.6 | 0.36 | 0.288 | 87% | 0.056 | 0.752 | 0.015 | [341] |
| *S. cerevisiae* 424A (LNH-ST) | StEx-CLM whole slurry hydrolysate | G: 68<br>X: 29 | 99% | 41% | 35.1 | 0.36 | 0.293 | 87% | 0.059 | 0.762 | 0.019 | [341] |
| *S. cerevisiae* GLBRC Y128 | AFEX™ corn stover hydrolysate | G: 60<br>X: 30 | 100% | 58% | 39.0 | 0.43 | 0.325 | 98% | 0.083 | 0.854 | 0.037 | [156] |
| *S. cerevisiae* MEC1122 | LHW corn cobs pretreatment liquor | G: 2<br>X: 26 | 100% | 99% | 7.7 | 0.28 | 0.107 | 58% | N/R | N/R | N/R | [260] |
| *S. cerevisiae* RWB 218 | StEx wheat straw hydrolysate | G: 50<br>X: 20 | 99% | 88% | 38.1 | 0.47 | 0.693 | 99% | N/R | N/R | N/R | [354] |
| *S. cerevisiae* TMB 3400 | Dilute Acid spruce hydrolysate † | G: 16<br>X: 7 | 100% | 100% | 16 | 0.43 | 0.16 | 80% | 0.005 | 0.021 | 0.005 | [355] |
| *S. cerevisiae* GS1.11-26 | $SO_2$-StEx spruce pretreatment liquor ‡ | G: 62<br>X: 18 | 100% | 86% | 39.0 | 0.41 | 0.361 | 84% | N/R | N/R | N/R | [346] |
| *S. cerevisiae* XH7 | StEx corn stover hydrolysate | G: 78<br>X: 39 | 98% | 80% | 41.5 | 0.39 | 0.569 | 78% | 0.146 | 1.67 | 0.124 | [261] |
| *S. cerevisiae* LF1 | StEx corn stover hydrolysate | G: 80<br>X: 40 | 99% | 89% | 49.0 | 0.41 | 1.021 | 84% | 0.254 | 1.62 | 0.181 | [261] |

**Superscripts**: **Ψ** – **G**: glucose, **X**: Xylose; **ϕ** - volumetric ethanol productivity; **†** - Hydrolysate also composed of fermentable 4 $g.L^{-1}$ galactose and 10 $g.L^{-1}$ mannose; **‡** - Hydrolysate also composed of 15 $g.L^{-1}$ fermentable mannose and supplemented with 50 $g.L^{-1}$ synthetic glucose. * - Ethanol Yield: ethanol produced relative to the available sugars in the hydrolysate; ** - Metabolic yield: ethanol produced relative to the theoretical maximum ethanol based on consumed sugars in the hydrolysate.

**Abbreviations: CLM** – Cane leaf matter; **LHW** – Liquid hot water; **AFEX™** – ammonia fiber expansion; **StEx** – steam explosion;



Overall, the highest xylose consumption, ethanol concentration, ethanol metabolic yield and overall ethanol productivity were achieved by CelluX™ 4, reaching 98%, 53.8 $g.L^{-1}$, 92% (based on consumed sugars), 0.747 $g.L^{-1}.h^{-1}$ after 72 h of fermentation, respectively. Fermentation with the TP-1 transformant was also characterised by high xylose consumption (88%) and metabolic yield (89%), with the final ethanol concentration reaching 50 $g.L^{-1}$. However, TP-1 required 120 h of fermentation to achieve high xylose consumption and demonstrated 4.5-fold lower specific xylose fermentation rate ($q_{xylose}$) compared to CelluX™ 4, suggesting that the TP-1 transformant may have lower xylose affinity or lower ethanol and/or inhibitor stress tolerance phenotypes relative to CelluX™ 4. The evolved strains (TP-50 and CelluX™ E50) demonstrated lower overall xylose consumption and specific xylose consumption rates compared to CelluX™ 4 and TP-1, suggesting that these evolved strains might have lost some part of their inhibitor and/or ethanol stress tolerance phenotypes during the course of xylose evolution [345,356]. Furthermore, no xylitol formation was detected from the fermentation runs of all four yeast strains.

Figure 8.3 illustrates the time-based profiles of the furan aldehydes (furfural and 5-HMF) during the fermentation of the non-detoxified hydrolysate and the final concentrations of the phenolic compounds quantified at the end of the 120 h of fermentation period. As shown in Figures 8.3 (a – e), all four strains demonstrated near complete furfural and 5-HMF detoxification phenotypes within 24h of fermentation, indicating that these strains were effective in assimilating both furan aldehydes [163]. Traditionally, microbial stresses caused by both furfural and 5-HMF inhibit yeast glycolysis, deplete intracellular NAD(P)H and ATP pools, and damage intracellular proteins [163]; hence, their assimilation during the glucose consumption phase mitigates their effect on the xylose fermentation capacity of these four industrial strains. When present at sub-lethal concentrations, Almeida *et al.,* [357] suggested that both furfural and 5-HMF can be reduced by some inhibitor tolerant yeast strains to form their furan alcohol equivalents (furfuryl alcohol and 2,5-*bis*-hydroxymethylfuryl alcohol, respectively).



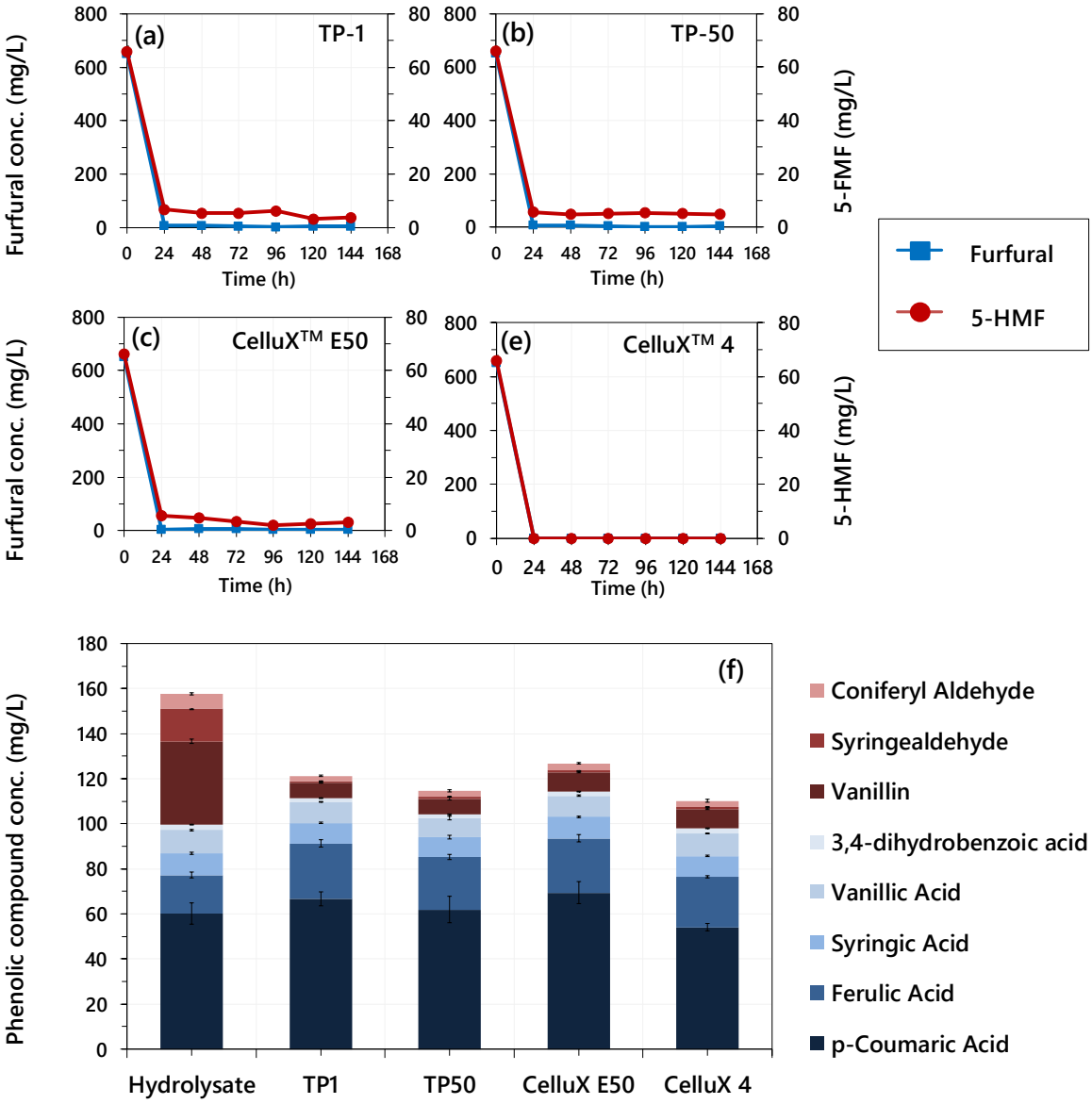


**Figure 8.3**: (**a-e**) An illustration of the time-based detoxification of furan derivatives (furfural and 5-HMF) during the fermentation of steam exploded and undetoxified sugarcane bagasse whole slurry hydrolysate using yeast strains TP-1, TP-50, CelluX™ E50, and CelluX™ 4. (**f**) A comparison of the phenolic acid and aldehydes before and after fermentation of the steam exploded and undetoxified sugarcane bagasse whole slurry hydrolysate.

Similarly, lignin-derived phenolic aromatic aldehydes, *viz.* vanillin, syringaldehyde, and coniferyl aldehyde, were also quantified at significantly lower concentrations after the fermentation period for all four yeast strains relative to the initial hydrolysate ($P < 0.05$). Although initially present at significantly lower concentrations relative to furan aldehydes and aliphatic acids, phenolic



aldehydes such as coniferyl aldehyde and vanillin have previously demonstrated significantly higher *S. cerevisiae* toxicity even at low concentrations [112,119]. Hence, these phenolic aldehyde detoxification traits suggest that microbial stress due to phenolic aldehydes contribute to the more efficient xylose fermentation capacity of these industrial strains.

However, apart from CelluX$^{TM}$ 4, there were minor increases in the total phenolic aromatic acid (*p*-coumaric acid, ferulic acid, syringic acid, vanillic acid, and 3-4-dihydrobenzoic acid) concentration after fermentation relative to the hydrolysate ($P < 0.05$). The minor increments in *p*-coumaric acid and ferulic acid were the primary factors for the higher total phenolic aromatic compound concentration. Like the phenolic aldehydes, Larsson *et al.,* [358] found that the inhibitory effect of phenolic acids is significantly stronger than that of aliphatic acids such as acetate and formate. As a result, phenolic acids in the hydrolysate are potential contributors to microbial inhibition to the xylose-fermentation capacity of TP-1, TP-50, and CelluX$^{TM}$ E50 in non-detoxified StEx-SCB whole slurry hydrolysate

Recent research has suggested that hydroquinones (*e.g. p*-benzoquinone) and small aliphatic aldehydes (*e.g.* formaldehyde), which both can be found in high severity steam exploded hydrolysates, can have an even more pronounced inhibitory effect on *S. cerevisiae* relative to some phenolic acids and aliphatic acids such as acetate [161,359]. Hence, future quantification of the phenolic aldehyde, phenolic acid, hydroquinone and small aliphatic aldehyde concentrations accumulated in the cells or in the reaction medium as a function of fermentation time could provide valuable insights of the fate of these products in the presence of high ethanol and acetic acid concentrations in the fermentation of inhibitor-laden hydrolysates [360].

### 8.3.3 *Comparative fermentation of hydrolysates with literature yeast strains*

Direct comparison of the fermentation of non-detoxified hydrolysates of various strains presented in literature is not trivial due to the differences in the source of raw biomass, type of pretreatment, pretreatment conditions, the microbial stress tolerance and background of the selected



strains, the application of detoxification methods and ultimately the levels multiple stress factors (*e.g.* ethanol, sugars, salt content, inhibitors) present in the hydrolysates. Nonetheless, Table 8.2 compares the fermentation capacity of the four industrial yeast strains used in this work to some of the most promising xylose-fermenting yeast strains (fermenting non-detoxified hydrolysates) reported in literature.

In **CHAPTER 4**, we previously compared the fermentability of inhibitor-laden StEx pretreated SCB whole slurry hydrolysate to the fermentability of inhibitor-deficient AFEX™ pretreated SCB hydrolysate using xylose-fermenting *S. cerevisiae* 424A (LNH-ST) [341]. Whereas near complete xylose consumption (96%), high ethanol concentration (44.2 $g.L^{-1}$) and high metabolic yield (92%) were achieved from the AFEX™ hydrolysates, only 37% xylose consumption was achieved in StEx-SCB whole slurry hydrolysates at the same fermentation conditions. *S. cerevisiae* 424A (LNH-ST) demonstrated diauxic xylose fermentation and the low xylose uptake from the StEx-derived hydrolysates was attributed to the potential cumulative inhibitory effect of high ethanol, acetate, and phenolic compound concentrations that were present in the hydrolysate during the xylose fermentation phase.

Among the most efficient xylose fermenting yeasts reported in literature, recombinant *S. cerevisiae* strains RWB218, GS1.11-26, XH7, and LF1 have demonstrated high xylose consumption (> 80%), ethanol concentrations (> 38 $g.L^{-1}$), metabolic yields (> 78%) and overall ethanol productivities (0.57 $g.L^{-1}.h^{-1}$) in non-detoxified StEx generated whole slurry hydrolysates derived from various lignocellulosic residues. Despite its low specific xylose uptake rate and therefore low overall volumetric productivity, transformant TP-1 achieved xylose consumption (88%) akin to RWB218, XH7, and LF1 with higher metabolic yields and ethanol concentrations. However, the results of this work have demonstrated that CelluX™ 4 can produce volumetric ethanol productivities that were 2-fold higher than TP-1, while generating xylose consumption, metabolic yield ethanol concentrations higher than those demonstrated by RWB218, GS1.11-26, XH7, and LF1. Even with a volumetric ethanol productivity only surpassed by *S. cerevisiae* LF1, the results from this work suggest that CelluX™ 4 is



one of the more promising industrial xylose-fermenting yeast strains for the efficient conversion of both glucose and xylose in inhibitor-laden hydrolysates derived from autohydrolysis based pretreatment technologies such as StEx.

### 8.3.4 *Simultaneous saccharification and co-fermentation of steam exploded and non-detoxified sugarcane bagasse*

Based on the yeast screening results, both *S. cerevisiae* TP-1 and *S. cerevisiae* CelluX™ 4 were selected as the two best performing strains and henceforth used to evaluate ethanol production from StEx-SCB whole slurry compared to AFEX™-SCB biomass in PSSCF. PSSCF runs were performed to evaluate whether the presence of unhydrolysed solids in tandem with the pretreatment derived inhibitors would impact the xylose fermentation capacity of TP-1 and CelluX™ 4 as was previously reported for *S. cerevisiae* 424A (LNH-ST) [361]. For the PSSCF experiments, enzymatic hydrolysis was performed using a more industrially relevant enzyme dosage of 20 mg/g glucan (~8 mg/g untreated dry bagasse) using mixtures of CTec2, HTec2, and Pectinex Ultra-SP. The sugar, ethanol, and acetate profiles during PSSCF with TP-1 and CelluX™ 4 are shown in Figure 8.4.

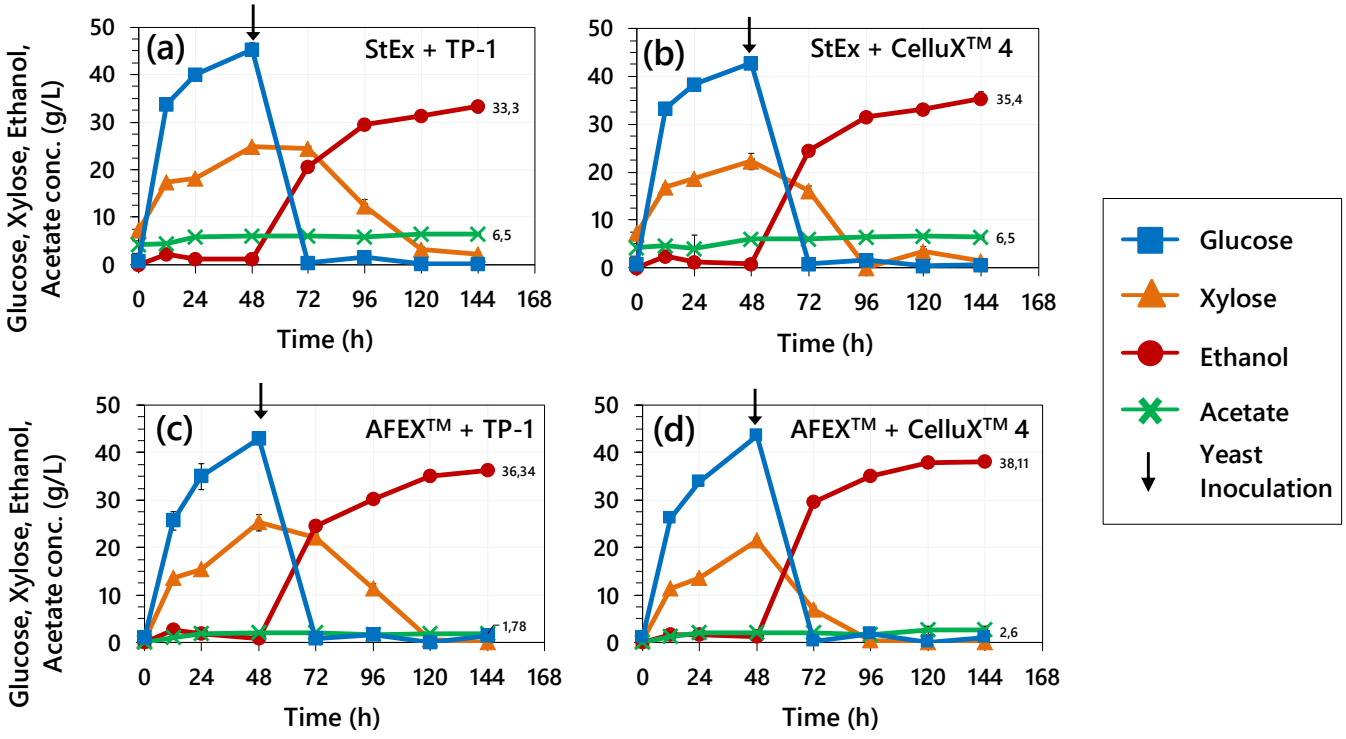


**Figure 8.4:** Comparison of PSSCF of AFEX™-treated SCB solids and StEx-treated SCB whole slurry using *S. cerevisiae* TP-1 and *S. cerevisiae* CelluX™ 4. All the PSSCF experiments were carried out with a 48 h pre-saccharification at 50 °C followed by a simultaneous saccharification and co-fermentation period of 96 h at 35 °C using a total solids loading of 15% (w/w), inoculum of 1.8 g CDW.$L^{-1}$, and enzyme dosage of 20 mg protein per gram glucan. Arrows illustrate the time when the yeast cells were inoculated.



After 48 h pre-saccharification at the relatively low enzyme loading, the reaction mixtures from both the StEx-SCB whole slurry and the AFEX™-treated SCB solids contained glucose and xylose concentrations that were marginally higher than 40 $g.L^{-1}$ and 20 $g.L^{-1}$, respectively (Figure 8.4 (a,c)). Conversely, the acetic acid concentrations in the AFEX™ reactions flasks were about 3-fold lower than the StEx whole slurry flasks. This was primarily due to the dominant ammonolysis reactions that cleave the ester-linked *O*-acetyl groups in hemicellulose to form acetamide instead of acetic acid during AFEX™ pretreatment [341].

After the inoculation of TP-1 into the both StEx-SCB whole slurry and AFEX™-SCB experiments, glucose was rapidly consumed within 24 h, with the final ethanol concentration reaching 33 and 34$g.L^{-1}$ after 144 total reaction time. Xylose was consumed to near completion (< 1.5 $g.L^{-1}$ residual xylose) after 120 h for both experiments, indicating that most the monomeric xylose that was simultaneously released by the hemicellulases from the soluble oligomers or the insoluble xylan was consumed the transformant strain TP-1. Similarly, PSSCF carried out with CelluX™ 4 resulted in the near complete glucose and xylose consumptions in the both AFEX™-SCB and StEx-SCB whole slurry experiments. Moreover, the xylose consumption rate and the final ethanol concentrations from CelluX™ 4 were marginally higher than those achieved with TP-1 ($P < 0.05$), suggesting that the presence of the unhydrolysed solids might not have a significant impact on the xylose fermentation capacity of both TP-1 and CelluX™ 4.

Like the hydrolysate fermentation experiments, both TP-1 and CelluX™ 4 demonstrated strong furfural and 5-HMF detoxification phenotypes in the PSSCF experiments StEx-SCB whole slurry experiments, with both furan aldehydes completely assimilated within 24 h after inoculation (Figure 8.5). These results suggest that the concentrations of these furan aldehydes in SCB that was steam exploded at a pretreatment severity of 3.94 (200 °C, 10 min) are likely below the inhibition concentrations for the transformant strain TP-1 and CelluX™ 4.



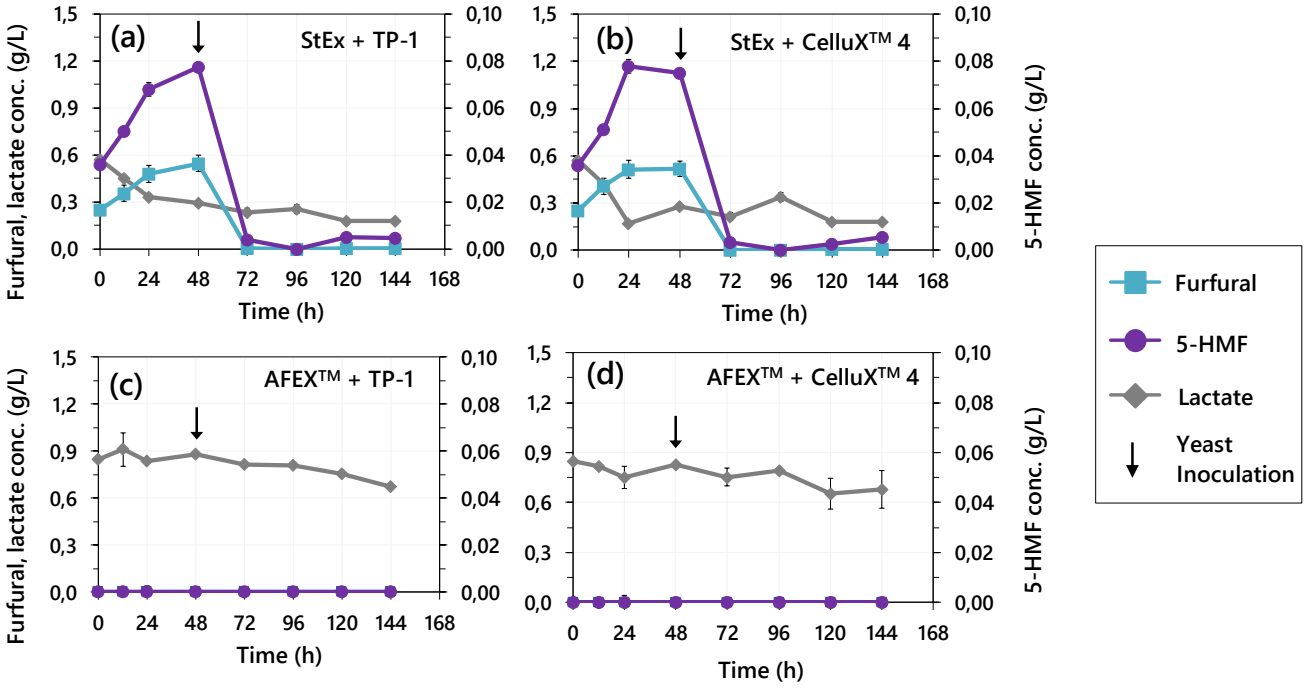


**Figure 8.5:** Furan aldehydes and lactic acid profiles during PSSCF of AFEX™-treated SCB solids and StEx-treated SCB whole slurry. Arrows indicate the time point when the yeast cells were inoculated.

However, the overall yields from the non-optimized PSSCF strategy used in this work might have been reduced by biomass recalcitrance or inhibition of the hydrolytic enzymes by pretreatment derived inhibitors that are present in the reaction medium [151,170,280]. In particular, soluble oligosaccharides, the aliphatic acids, soluble phenolic compounds, pseudo-lignin compounds that are bound to the unhydrolysed solids, and ethanol generated from the fast glucose fermentation phase have all previously demonstrated strong enzyme activity deactivation and/or non-productive binding effects, which become even more pronounced at during low enzyme loading hydrolysis conditions [152]. Redesigning and optimizing the substrate feeding and enzyme addition strategies in PSSCF have recently been demonstrated to reduce the effect of these inhibitory compounds from PSSCF and generate higher overall process yields [362–365]. Nonetheless, even in the presence of potential enzyme inhibition, the results of this study demonstrate that the use of the transformant TP-1 and CelluX™ 4 for StEx-treated whole slurry PSSCF alleviates microbial inhibition as a reason for achieving low ethanol yields from StEx-pretreated sugarcane residues.



### 8.3.5 *Mass balances for PSSCF process*

The ethanol yield per tonne untreated SCB provides a metric for quantifying the combined effect of biomass recalcitrance, enzyme inhibition and microbial inhibition for various pretreatment, hydrolysis, and fermentation process combinations. The results from the PSSCF experiments using AFEX™- and StEx-treated SCB and the inhibitor tolerant yeast strains TP-1 and CelluX™ 4 were used to generate process mass balances and to estimate the overall ethanol yield per tonne untreated SCB (Figure 8.6). Owing to the absence of significant carbohydrate degradation, AFEX™ only increased the nitrogen content of the pretreated biomass by approximately 1.5 kg per 100 kg of untreated SCB. In contrast, StEx resulted in the recovery of 97% and 76% of polymeric glucan and xylan in the whole slurry relative the glucan and xylan content in the untreated biomass, respectively, demonstrating a 3% and 24% loss of glucan and xylan, respectively, due to sugar degradation during StEx pretreatment. However, the sugar loss to degradation obtained in this study was lower than that observed in **CHAPTER 4**, primarily due to the lower pretreatment severity used to during StEx pretreatment in this study [5].

Under industrially relevant solids loading and limited enzyme loading conditions, the PSSCF of AFEX™-treated SCB using TP-1 and CelluX™ 4 produced ethanol yields of 50 and 54% relative to the theoretical maximum estimated from the polymeric glucan and xylan input to the PSSCF process, respectively. These ethanol yields correspond to overall process yields of 234 and 251 L of ethanol per tonne untreated SCB, respectively. These ethanol yields achieved for PSSCF of AFEX™-treated SCB using both TP-1 and CelluX™ 4 were lower than those reported in **CHAPTER 4**. However, the lower ethanol yields were not due to inadequate yeast performance but primarily due to the higher AFEX™ pretreatment severity, the use of older cellulase and hemicellulase generation (CTec2 & HTec2 vs CTec3 & HTec3), and lower enzyme dosages used in this study relative to the study presented in **CHAPTER 4**. Nonetheless, the PSSCF of StEx-treated SCB whole slurry generated ethanol yields of 208 and 224 L of ethanol per tonne using TP-1 and CelluX™ 4, respectively. These ethanol yields were



lower compared to those achieved from the PSSCF of AFEX™-SCB, suggesting that carbohydrate loss due to degradation, recalcitrant biomass or enzyme inhibition during the PSSCF of the whole slurry could be decisive factors that need to be further considered to close the gap in yields between AFEX™-SCB and StEx-treated SCB whole slurries.

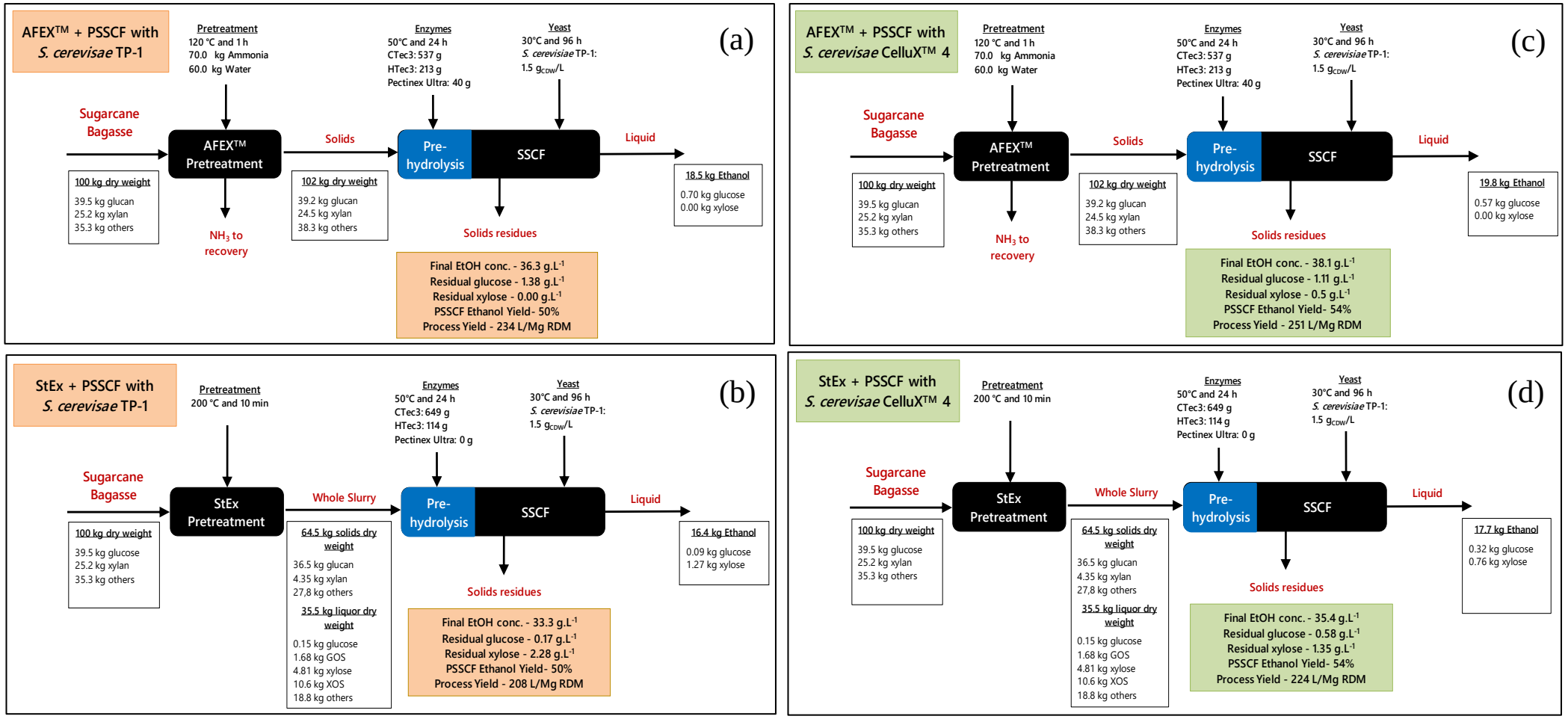


**Figure 8.6:** Comparison of the mass balances and overall ethanol yield per tonne untreated sugarcane bagasse for PSSCF of AFEX™-treated and StEx-treated SCB using *S. cerevisiae* TP-1 and *S. cerevisiae* CelluX™ 4.



### 8.3.6 *Comparing PSSCF performance of TP-1 and CelluX$^{TM}$ 4 with literature*

To establish commercially viable cellulosic biorefineries, a major goal for cellulosic ethanol production is to achieve efficient conversion of both hexose and pentose sugars to ethanol (high yield per unit untreated feedstock), high ethanol concentrations from fermentation (> 40 g.L$^{-1}$), high volumetric productivity and low enzyme loadings. A comparison of selected literature reported processes evaluating industrially relevant ethanol production from sugarcane bagasse is presented in Table 8.3.

The ethanol yields for the PSSCF of the StEx-treated SCB whole slurry using CelluX$^{TM}$ 4 (224 L.Mg RDM$^{-1}$) were higher than those previously reported by Mokomele *et al.,* [341] using the recombinant yeast strain *S. cerevisiae* 424A (LNH-ST) in SHCF (204 L.Mg RDM$^{-1}$). As previously discussed, the degradation products in the StEx-SCB whole slurry and the fermentation metabolites from the glucose-consumption phase limited the xylose-uptake by *S. cerevisiae* 424A (LNH-ST) thus achieving lower ethanol yields even at the higher enzyme loadings used in that study. In contrast, the PSSCF of the StEx-SCB whole slurry using TP-1 achieved ethanol yields that were equivalent to those reported by Mokomele *et al.,* [341] (205 L.Mg RDM$^{-1}$) and also higher than those reported by Mesa *et al.,* [366], due to the efficient fermentation of the fermentable glucose and xylose released by the hydrolytic enzymes. Furthermore, higher volumetric ethanol productivities were achieved by the PSSCF experiments relative to the SHCF of StEx-treated SCB whole slurries.

Literature reported ethanol yields for AFEX$^{TM}$-treated SCB range from 272 – 324 L.Mg RDM$^{-1}$ (Table 8.3). The PSSCF of AFEX$^{TM}$-SCB using both CelluX$^{TM}$ 4 and TP-1 produced ethanol yields of 234 and 251 L/Mg RDM, which were lower than this literature reported range. These results suggest that either SHCF is a more efficient process relative to PSSCF for AFEX$^{TM}$-treated SCB or that the PSSCF process is limited by biomass recalcitrance at low enzyme loadings.



**Table 8.3:** Examples of literature reported ethanol production from sugarcane bagasse at industrially relevant solids loadings

| Biomass | Pretreatment | Configuration | Solids Processing Option | Total Enzyme dosage & Enzyme cocktails | Solids Loading (%, w/w) | Fermentation microorganism | EtOH Yield (L/ton RDM) | EtOH conc. (g/L) | EtOH prod. (g/L/h) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Sugarcane bagasse † | Dilute Acid | SSF | Unwashed solids | 25mg /g pretreated solids (CTec2, HTec2) | 16% | *S. cerevisiae* MH-1000 | n.a | 51 | 0.414 (solids) | [72] |
| Sugarcane bagasse | AFEX™ | SHCF | No washing or detoxification | 32 mg / g RDM (Spezyme SP, Novozyme 188, Multifect Xylanase) | 15% | *S. cerevisiae* 424A (LNH-ST) | 272 | 34 | 0.128 | [46] |
| Sugarcane bagasse ‡ | AFEX™ | SHCF | No washing or detoxification | 8 mg / g RDM (CTec3, HTec3, Pectinex Ultra-SP) | 15.5% | *S. cerevisiae* 424A (LNH-ST) | 275 | 41 | 0.219 | [35] |
| Sugarcane bagasse | AFEX™ | SHCF | No washing or detoxification | 10 mg / g RDM (CTec3, HTec3, Pectinex Ultra-SP) | 15.5% | *S. cerevisiae* 424A (LNH-ST) | 324 | 44 | 0.229 | [341] |
| Sugarcane bagasse | Steam explosion | SHCF | Whole slurry, no detoxification | 10 mg / g RDM (CTec3, HTec3) | 15.5% | *S. cerevisiae* 424A (LNH-ST) | 205 | 35 | 0.162 | [341] |
| Sugarcane bagasse | Steam explosion | SHCF + $C_5$-liquor fermentation | Unwashed solids + non-detoxified $C_5$-liquor | 10 mg / g RDM (CTec3, HTec3) | 15.7% | *S. cerevisiae* 424A (LNH-ST) | 231 | 43 (solids), 3.2 (liquor) | 0.299 (solids) | [341] |
| Sugarcane bagasse | Steam explosion | SHCF + $C_5$-liquor fermentation | Washed solids + non-detoxified $C_5$-liquor | 10 mg / g RDM (CTec3, HTec3) | 15.9% | *S. cerevisiae* 424A (LNH-ST) | 235 | 48 (solids), 3.2 (liquor) | 0.364 (solids) | [341] |
| Sugarcane bagasse ψ | Green liquor & Ethanol | SSCF | Washed solids | 20 FPU CTec2/g glucan + 25 IU Novozyme 188/g glucan | 15% | *S. cerevisiae* CSC + *S. cerevisiae* TSC | n.a | 68 | 0.71 | [367] |
| Sugarcane bagasse | Dilute Acid + Organosolv | PSSF | Washed solids | 47 FPU/g RDM (Celluclast 1.5) | 16% | *S. cerevisiae* No. 1701 | 194 | 37 | 0.77 | [366] |
| Sugarcane bagasse ‡ | AFEX™ | PSSCF | No washing or detoxification | 8 mg / g RDM (CTec2, HTec2, Pectinex Ultra-SP) | 15% | *S. cerevisiae* TP-1 | 234 | 36 | 0.250 | This study |
| Sugarcane bagasse ‡ | AFEX™ | PSSCF | No washing or detoxification | 8 mg / g RDM (CTec2, HTec2, Pectinex Ultra-SP) | 15% | *S. cerevisiae* CelluX™ 4 | 251 | 38 | 0.263 | This study |
| Sugarcane bagasse | Steam explosion | PSSCF | Whole slurry, no detoxification | 8 mg / g RDM (CTec2, HTec2) | 15% | *S. cerevisiae* TP-1 | 204 | 33 | 0.229 | This study |
| Sugarcane bagasse | Steam explosion | PSSCF | Whole slurry, no detoxification | 8 mg / g RDM (CTec2, HTec2) | 15% | *S. cerevisiae* CelluX™ 4 | 224 | 35 | 0.246 | This Study |

**Symbols: †** - hemicellulose-rich liquor from pretreatment was separated from the slurry and not fermented. **‡** - Pilot-scale AFEX™ pretreatment at low severity; **ψ** – Lignin-rich liquor separated from slurry

**Abbreviations**: **EtOH** – ethanol; **PSSF**: pre-hydrolysis simultaneous saccharification and fermentation; **SHCF** – separate hydrolysis and co-fermentation; **SSF** – simultaneous saccharification and fermentation; **$C_5$-liquor**: hemicellulose-rich liquor separated from the pretreatment whole slurry; **RDM** – raw (untreated) dry material; **CTec2** – Cellic® CTec2; **CTec3** – Cellic® CTec3; **HTec2** – Cellic® HTec2; **HTec3** – Cellic® HTec3;



## 8.4 Conclusions

The efficient co-fermentation of both hexose and pentose sugars from pretreated lignocelluloses in the presence of multiple microbial stresses such as ethanol and pretreatment derived inhibitors, remains a critical step towards enabling establishing economically viable cellulosic biofuel production. In this study, we evaluated the glucose and xylose fermentation capability of four XI-pathway industrial recombinant yeast strains on steam exploded and non-detoxified SCB whole slurry hydrolysates. *S. cerevisiae* TP-1 and Cellux™ 4 demonstrated high acetate resistance and furan aldehyde and phenolic aldehyde detoxification phenotypes, resulting in near complete combined glucose and xylose conversion (> 96%) and high ethanol concentrations (> 50 $g.L^{-1}$) from the fermentation of StEx-treated and non-detoxified SCB whole slurry hydrolysates. Under industrially relevant PSSCF solids loadings and low enzyme dosages, both *S. cerevisiae* TP-1 and Cellux™ 4 facilitated the consumption of nearly all the glucose and xylose released by the hydrolytic enzymes, from inhibitor-laden StEx-treated SCB whole slurries and inhibitor deficient AFEX™-treated SCB biomass. Ultimately, the results of this work demonstrate that the yeast strains TP-1 and CelluX™ 4 are robust strains that can efficiently convert both glucose and xylose from inhibitor-laden StEx whole slurries into ethanol, thereby significantly decreasing the gap in recoverable ethanol yields between AFEX™ and StEx pretreatment.



# CHAPTER NINE:

# CONCLUSIONS & RECOMMENDATIONS

With a saturating global sugar market, sugarcane residues are widely considered as key biomass resources that could present a variety of alternative models that could potentially contribute to adding economic value to the sugarcane industry. In this study, for the first time, we performed a systematic comparison of the potential of StEx and AFEX™ as mature pretreatment technologies to valorize sugarcane bagasse and cane leaf matter into biofuel (ethanol) feedstocks, animal feeds, and anaerobic digestion co-feeds. The novel contributions proposed in this study were presented in **CHAPTER 3**. This chapter presents the most significant findings of these contributions and proposes recommendations for future works.

## 9.1 Summary of main findings from research contributions

### 9.1.1 *Contribution 1: Comparing the ethanol production potential of StEx- and AFEX™-treated sugarcane residues in a sugarcane biorefinery*

A side-by-side comparison of the high solids loading enzymatic hydrolysis and fermentation efficiency of StEx- and AFEX™-treated SCB and CLM was performed using optimized combinations of hydrolytic enzymes (Cellic® CTec3, Cellic® HTec3 and Pectinex-Ultra) and *S. cerevisiae* 424A (LNH-ST) as the xylose-fermenting ethanologen (**CHAPTER 4**). Both StEx and AFEX™ required enzyme dosages greater than 20 mg/g glucan to achieve monomeric sugar yields greater than 75% from high solids loading enzymatic hydrolysis (Figure 9.1 a,b). For StEx pretreatment, separating and washing StEx pretreated SCB or CLM slurry alleviated some enzyme inhibition due to pretreatment derived products, end-product inhibition, or non-productive binding to lignin. In contrast, sugar yields from AFEX™-treated sugarcane residues were limited by the production of more than 15% oligosaccharides under lower enzyme loading conditions, indicating that oligosaccharide formation could become a major contributor to lower ethanol yields under enzyme limited conditions. Using a moderate enzyme dosage of 25 mg/g glucan, AFEX™ pretreatment generated the highest ethanol yields of 324 and 316L



per Mg RDM for SCB and CLM, respectively, the highest ethanol yields reported in literature from sugarcane residues (Figure 9.1,c). In contrast, aggregated effect of sugar loss during pretreatment, enzyme inhibition during enzymatic hydrolysis and microbial inhibition during the fermentation of the $C_5$-liquor and whole slurry hydrolysate, limited the ethanol yields from StEx treated SCB and CLM to the range 205 to 257 L per Mg RDM.

From ensuing mass balances, the highest ethanol yields per hectare sugarcane cultivation area were estimated at 4496 and 3416 L per hectare for biorefineries using AFEX™- or StEx-treated sugarcane residues, respectively. A single factor sensitivity analysis revealed that the amount of sugarcane residues available to the StEx- or AFEX™-centred sugarcane biorefinery and the enzyme dosage used presented the greatest effects on the ethanol yields recovered per unit sugarcane cultivation area (Figure 9.1,d). However, it was also evident that ethanol yields from StEx could be significantly improved by increasing the xylose fermentation efficiency from either the whole slurry hydrolysate or the $C_5$-liquor using more inhibitor tolerant ethanologens. In contrast, the conversion of the oligosaccharides formed during enzymatic hydrolysis into fermentable sugars was identified as a key area to improve ethanol yields from AFEX™-treated sugarcane residues.

Ultimately, AFEX™ proved to be a more effective pretreatment method for maximising ethanol yields from sugarcane residues relative to StEx when the lab strain *S. cerevisiae* 424A (LNH-ST) was used as the ethanologen. The identification of auxiliary enzyme activities and the use of robust xylose-fermenting ethanologens were identified as key areas for improving ethanol yields from AFEX™- or StEx-centred 2G sugarcane biorefineries. However, future techno-economic and life-cycle analysis are necessary to determine which pretreatment technology would be best-suited for integrating into existing South African sugar mills.



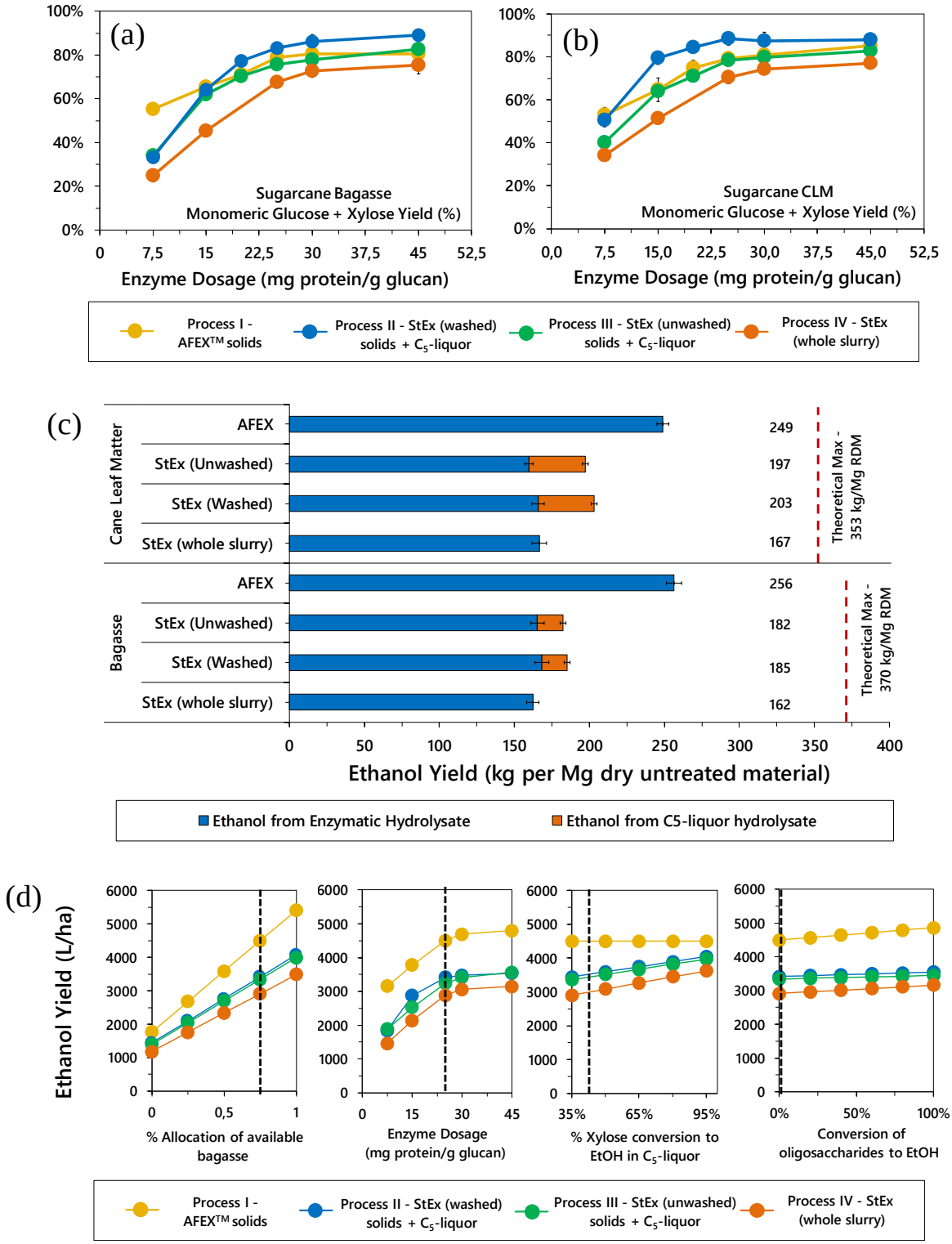


***Figure 9.1:*** Summary of main research findings from contribution 1. (**a,b**) Effect of process configuration on enzyme dosage requirements from StEx- and AFEX™-treated SCB and CLM; (**c**) experimental ethanol yields per Mg untreated biomass for StEx and AFEX™ treated sugarcane residues, (**d**) sensitivity analysis to identify primary process bottlenecks to maximising ethanol yields per unit cultivation area.



### 9.1.2 *Contribution 2: Using steam explosion and AFEX™ to produce animal feeds and biofuel feedstocks in a biorefinery based on sugarcane residues*

In this contribution, we studied the compared the potential use of AFEX™ and StEx to simultaneously produce sugarcane residues with enhanced animal feed value and ethanol production potential. AFEX™ increased the non-protein nitrogen content of sugarcane residues by a minimum of 230% relative to untreated controls, with more than 70% of the nitrogen chemically-linked onto the AFEX™-treated biomass being quantified as degradable by cattle rumen microorganisms. Furthermore, the *in-vitro* true digestibility (IVTD) and metabolizable energy (ME) of AFEX™-treated sugarcane residues were improved by up to 69% and 26% relative to untreated controls ($P < 0.05$), respectively, demonstrating a significant improvement in the animal feed value of SCB and CLM (Figure 9.2,a). Several potential ruminant neuro-toxic Maillard reaction compounds were quantified from AFEX™-treated sugarcane residue extracts, with their concentrations being proportional to the pretreatment severity (temperature and $NH_3$ loading) and soluble sugar content before pretreatment (Figure 9.2,b). In contrast, insignificant changes to the nitrogen content were quantified for StEx-treated sugarcane residues. However, StEx pretreatment improved the IVTD and ME of the sugarcane residues by 54% and 7%, respectively, also demonstrating a significant improvement in the feed value of SCB and CLM. Unlike the AFEX™-treated sugarcane residues, no Maillard reaction products were quantified in StEx extracts.

Using the same pretreated feedstocks, AFEX™-treated sugarcane residues achieved ethanol yields in the range 187 to 321 L ethanol per Mg untreated biomass under industrially relevant solids and enzyme loadings, with the ethanol yields varying depending on the pretreatment severity employed. These ethanol yields corresponded to the potential recovery of up to 4360 L of ethanol per hectare of sugarcane cultivation area. In contrast, StEx-treated residues produced lower ethanol yields in the range 219 to 255 L per Mg untreated biomass, corresponding to 3368 L of ethanol per hectare of sugarcane cultivation area. Ultimately, this contribution demonstrated that both AFEX™ and StEx

could generate enhanced animal feeds and ethanol feedstocks from sugarcane biorefineries. Hence, the results of this study provide significant insights for future studies evaluating the impact of sustainable intensification of pasture and sugarcane cultivation land for feeding ruminants and producing biofuels.

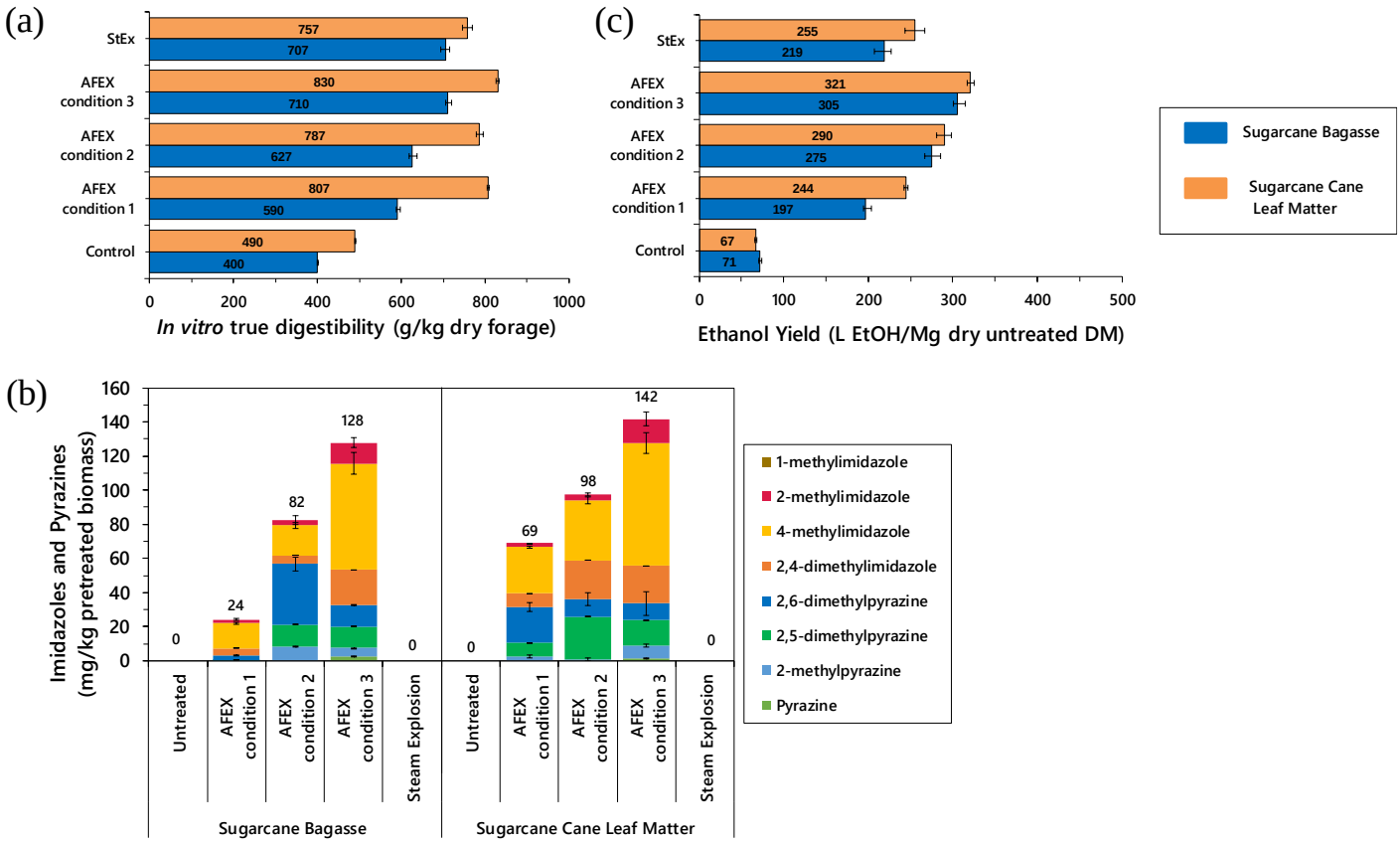


**Figure 9.2:** Summary of the main research findings from contribution 2. The effect of AFEX™- and StEx pretreatment of sugarcane residues on the (**a**) *in-vitro* true digestibility, (**b**) ethanol yield per Mg dry untreated material, and (**c**) the yields of ruminant neuro-toxic Maillard reaction productions (*i.e.* imidazoles and pyrazines).

### 9.1.3 *Contribution 3: Incorporating anaerobic digestion of pretreated sugarcane residues with manure into the livestock-bioenergy production nexus*

The primary aim of this contribution was to investigate and compare the potential benefit of co-digesting StEx- or AFEX™-pretreated sugarcane residues with livestock manure as a bioenergy production and manure management strategy for intensified livestock production farms located in sugarcane dense regions. AFEX™-treated sugarcane residues were characterized by increased nitrogen content, significant cleavage of ether linkages in hemicellulose, and significant cleavage in



ester linkages in lignin-carbohydrate complexes, acetyl groups, and lignin side chains. The combination of the near optimal C/N ratios and the structural modifications of AFEX$^{TM}$-treated sugarcane residues enhanced specific methane yields from SCB or CLM up to 299 L $CH_4$/kg VS, with or without co-digestion with dairy cow manure (DCM) (Table 9.1). StEx enhanced the biodegradability of SCB and CLM by significantly solubilizing hemicellulose, partially cleaving lignin-carbohydrate complexes, and increasing the enzyme accessible area of the pretreated biomass. However, both the untreated and StEx treated sugarcane residues were characterized by high C/N ratios, thereby requiring co-digestion with DCM to achieve sufficient macro- and micro-nutrient balance and therefore high methane yields.

Macro-nutrient analysis of the solid digestates from the co-digestion assays revealed that nitrogen, phosphate, and potassium (N-P-K) contents in the digestate were concentrated to more than 300% relative to that of undigested SCB and CLM. These results suggested that the digestates could potentially replace a fraction of CLM that is typically left on sugarcane fields during green harvesting, therefore enhancing the bioenergy recovery per unit sugarcane cultivation area. In the end, the preliminary results in this contribution suggest that because co-digesting pretreated sugarcane residues with livestock manure enhances methane recovery and the digestate nutrient value, incorporating the anaerobic digestion of pretreated sugarcane residues with livestock manure could provide a more sustainable bioenergy-livestock nexus for livestock and sugarcane dense regions.

***Table 9.1:*** Methane production, energy conversion efficiency and solid digestate fertilizer value for selected mono-digestion and co-digestion substrates

| **Substrate** | **DCM** | **Untreated-SCB** | **Untreated-CLM** | **Untreated-CLM + DCM** | **AFEX-SCB + DCM** | **AFEX-CLM** | **AFEX-CLM + DCM** |
|---|---|---|---|---|---|---|---|
| **DCM/Biomass Ratio** | **100/0** | **0/100** | **0/100** | **75/25** | **50/50** | **0/100** | **50/50** |
| **Digestion C/N ratio** | **15** | **101** | **72** | **35** | **20** | **23** | **19** |
| $CH_4$ Yield | 273 ± 7.9$^{C}$ | 258 ± 8.3 $^{D}$ | 231 ± 4.6 $^{E}$ | 292 ± 6.7 $^{A,B}$ | 299 ± 3.3 $^{A}$ | 292 ± 7.2 $^{A,B}$ | 287 ± 7.7 $^{B}$ |
| Biogas $CH_4$ content (% v/v) | 52 ± 3.4% $^{B}$ | 50 ± 1.8% $^{B}$ | 51 ± 2.0% $^{B}$ | 55 ± 5.0% $^{A}$ | 58 ± 1.6% $^{A}$ | 59 ± 1.6% $^{A}$ | 57 ± 1.2% $^{A}$ |
| **Solid digestate macro-nutrient value** | | | | | | | |
| Total N (kg/Mg dry digestate) | 33.6 ± 1.4 | 13.3 ± 0.3 | 14.4 ± 0.3 | 25.3 ± 1.2 | 24.4 ± 0.9 | 17.5 ± 0.7 | 26.1 ± 1.1 |
| Total P (kg/Mg dry digestate) | 23.1 ± 0.9 | 13.1 ± 0.5 | 15.3 ± 0.6 | 20.3 ± 0.8 | 21.2 ± 0.9 | 15.6 ± 0.5 | 19.1 ± 0.6 |
| Total K (kg/Mg dry digestate) | 13.0 ± 0.4 | 4.8 ± 0.1 | 12.5 ± 0.2 | 12.6 ± 0.3 | 11.5 ± 0.2 | 7.9 ± 0.1 | 11.1 ± 0.2 |



### 9.1.4 *Contribution 4: CIII$_I$-activation of AFEX$^{TM}$ and steam exploded sugarcane residue pellets for low enzyme dosage ethanol production in centralized biorefineries*

**Contributions 1** and **2** revealed that both AFEX$^{TM}$ and StEx pretreatment of SCB and CLM required enzyme dosages higher than 20 mg/g glucan to achieve high combined glucose and xylose yields (> 75%) from high solids loading enzymatic hydrolysis. In this contribution, the principal objective was to investigate the potential use of a room-temperature Cellulose III$_I$-activation process to enhance the digestibility of StEx- or AFEX$^{TM}$-treated sugarcane residue pellets and subsequently lower the enzyme loading requirements for high yield ethanol production. AFEX$^{TM}$ and StEx pretreatment of SCB and CLM facilitate the production of more dense, durable and hydrophobic pellets relative to untreated controls. The bulk densities of StEx and AFEX$^{TM}$-treated SCB and CLM pellets were slightly lower than those reported for corn grains (700-750 kg/m$^3$), but more than 3-fold and 6-fold higher than those for round CLM bales (183 kg/m$^3$) and compacted stockpiles of SCB (100 kg/m$^3$), respectively (Figure 9.3a). The increased hydrophobicity of the pretreated pellets suggested that these pellets would be more aerobically stable (lower dry mass loss due to microbial degradation) relative to untreated controls provided they are stored at moisture levels lower than 15% (wet basis). As a result, densification of pretreated sugarcane residues could potentially decrease the storage footprint and enable simpler handling and transportation logistics relative to SCB stockpiles or even CLM bales.

Coupling AFEX$^{TM}$-treated SCB and CLM pellets with a room temperature CIII$_I$-activation process allowed for the reduction of the enzyme dosage required to achieve ethanol yields greater than 280 L per Mg RDM by more than 60% (from 25 to 7.5 mg protein/g glucan), significantly reducing the enzyme cost contribution per unit volume ethanol produced (Figure 9.3 b,c). Given enzyme costs are projected to contribute up to 15% of the ethanol production costs at enzyme dosages of 20 mg/g glucan, augmenting AFEX$^{TM}$-treated pellets with a CIII$_I$-process could potentially reduce the sensitivity of prospective 2G biorefineries to uncertain enzyme-related costs. In contrast, upgrading StEx-treated



SCB and CLM pellets could only facilitate ethanol yields of 176 and 201 L per Mg raw dry biomass at an enzyme dosage of 7.5 mg protein/g glucan (~ 3 mg/g RDM), respectively (Figure 9.3 d,e). The lower ethanol yields were partially due to lower enzymatic hydrolysis efficiency relative to AFEX™ + $CIII_l$ biomass, with enzyme blockage by lignin, enzyme inhibition by pretreatment derived inhibitors and/or enzyme deactivation by non-productive binding to lignin hypothesized to be the primary mechanisms impeding higher sugar yields.

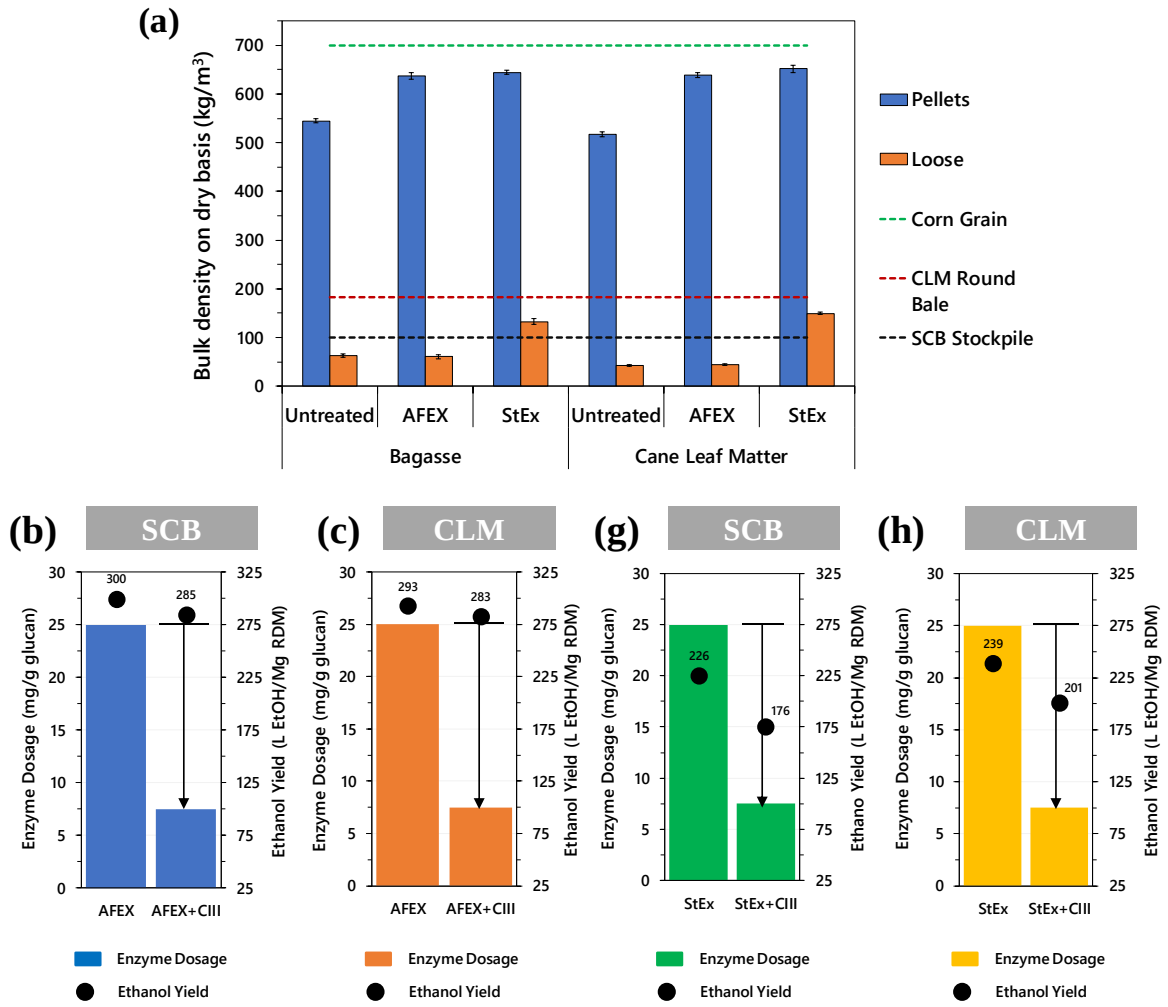


**Figure 9.3: (a)** Comparison of the bulk density of pellets vs. loose SCB and CLM. **(b)** Comparing the effect of $CIII_l$-activation of AFEX™ and StEx-treated SCB and CLM pellets on the ethanol yield per Mg RDM at enzyme dosages of 15, 10, and 7.5 mg/g glucan

Ultimately, this contribution suggests that upgrading AFEX™ pretreated pellets with a $CIII_l$-activation process could be feasible process for reducing the enzyme dosage requirements for large-scale biorefineries adopting uniform feedstock supply systems. However, the results from this work did not evaluate the potential economic impact of having an additional pretreatment step versus the



enzyme-related cost savings. The results of this chapter do however provide a basis for future techno-economic and life cycle analysis to that would determine whether augmenting pre-treated pellets with CIII$_I$-process is a viable processing option for large-scale centralized 2G biorefineries with a uniform feedstock supply chain.

### 9.1.5 *Contribution 5: Evaluating the fermentability of steam exploded and non-detoxified sugarcane bagasse whole slurry using industrial xylose-fermenting Saccharomyces cerevisiae strains*

In **contribution 1**, the efficient conversion of sugars recovered from whole slurry enzymatic hydrolysates and $C_5$-liquors was identified as one of the key bottlenecks for improving ethanol yields from steam exploded sugarcane residues. In this contribution, four xylose-isomerase industrial xylose-fermenting *S. cerevisiae* strains were evaluated and compared for their ability to efficiently convert both glucose and xylose recovered from StEx-treated and non-detoxified whole slurry's. *S. cerevisiae* TP-1 and Cellux$^{TM}$ 4 demonstrated high acetate resistance and furan and phenolic aldehyde detoxification phenotypes, resulting in near complete combined glucose and xylose conversion (> 96%) and high ethanol concentrations (> 50 g.L$^{-1}$) from the fermentation of StEx-treated and non-detoxified SCB whole slurry hydrolysates (Figure 9.4 a-e). The fermentation performance of these two strains was among the more promising industrial xylose-fermenting yeast strains reported in literature for the efficient conversion of both glucose and xylose in inhibitor-laden hydrolysates derived from autohydrolysis based pretreatment technologies such as StEx.

Under a pre-hydrolysis simultaneous saccharification and co-fermentation (PSSCF) configuration at 15% (w/w) solids loading and an enzyme dosage of 8 mg/g RDM, both *S. cerevisiae* TP-1 and Cellux$^{TM}$ 4 facilitated the consumption of nearly all the glucose and xylose released by the hydrolytic enzymes from both AFEX$^{TM}$-treated biomass and inhibitor-laden StEx-whole slurry biomass. Consequently, the PSSCF of StEx-treated SCB whole slurry achieved ethanol yields of 208 and 224 L per Mg raw dry SCB using *S. cerevisiae* TP-1 and CelluX$^{TM}$ 4, respectively. In comparison, the PSSCF of AFEX$^{TM}$ pretreated SCB achieved ethanol yields of 234 and 251 L per Mg raw dry SCB using TP-1 and



CelluX™ 4, respectively. The lower process yields for the StEx-treated SCB whole slurry relative to the AFEX™-SCB reveals the effect of carbohydrate loss due to sugar degradation during StEx pretreatment, the potential inhibition of the hydrolytic enzymes by either degradation products, or enzyme deactivation due to non-productive binding to lignin. Nonetheless, the results of this study demonstrate for the first time that the use of robust and inhibitor tolerant strains such as *S. cerevisiae* TP-1 and CelluX™ 4 for StEx-treated whole slurry PSSCF alleviates microbial inhibition as a reason for achieving low ethanol yields from StEx-pretreated sugarcane residues.

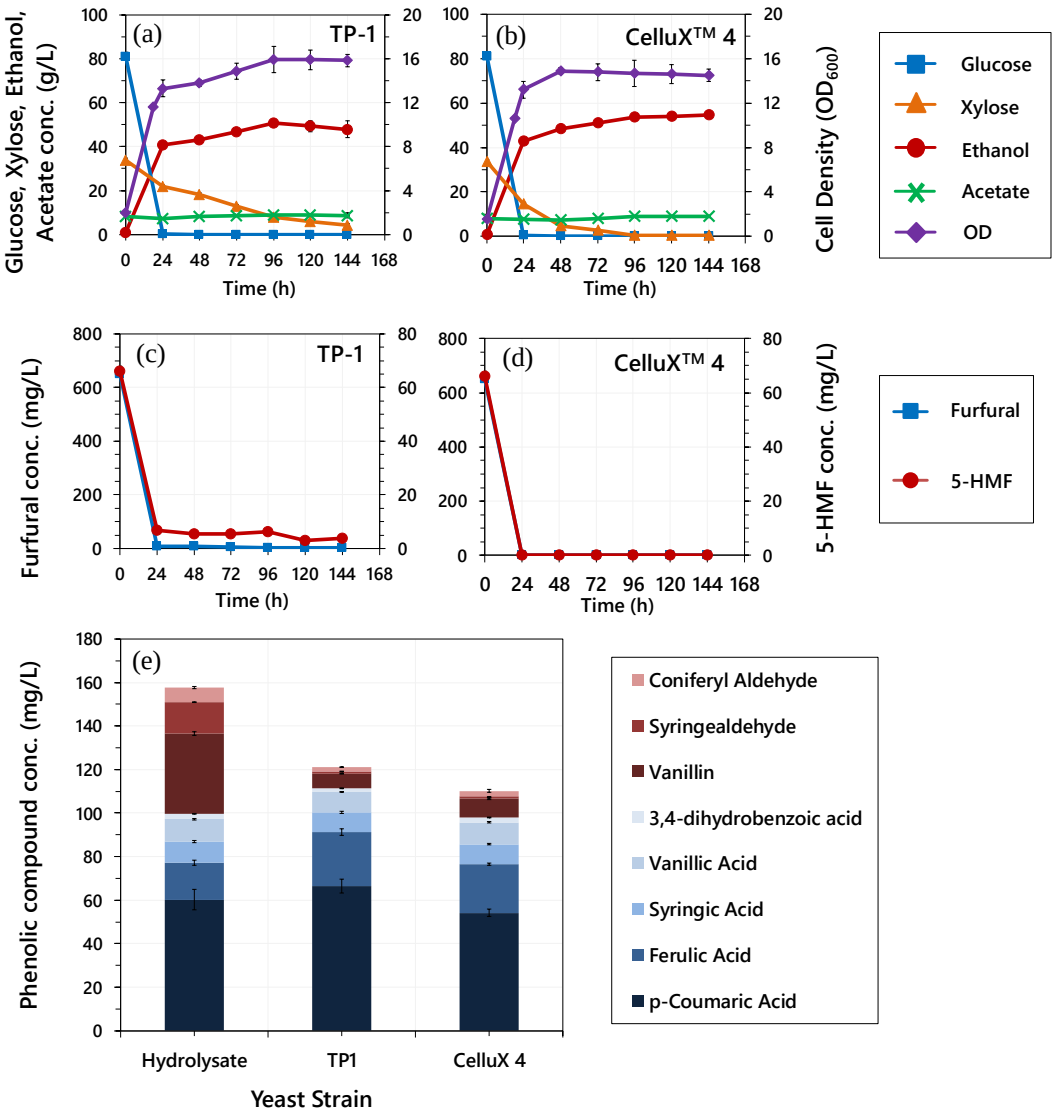


**Figure 9.4:** (**a**) Fermentation profiles for *S. cerevisiae* TP-1 and CelluX™ 4 in StEx-treated and non-detoxified SCB whole slurry hydrolysate; (**b**) Furan aldehyde time profiles; (**c**) Quantification of phenolic compounds before and after fermentation



## 9.2 Conclusions: producing biofuels, animal feeds and/or biogas from AFEX™ or StEx pretreated sugarcane residues

Comprehensive measures of national prosperity such as the United Nations' HDI demonstrate that the more energy a society consumes, the greater that societies opportunities to develop their human potential. Hence, developing sub-Saharan African counties will have to increase their per capita energy consumption without compromising food security to significantly enhance their national human potential. This dissertation investigated and compared the potential for StEx and AFEX™ to produce bio-ethanol, biogas and/or animal feeds from lignocellulosic sugarcane residues. In the context of expanding the sugar industry towards a diversified bioeconomy, the results from this dissertation showed that both AFEX™ or StEx pretreatment successfully enhanced the ethanol production potential, methane production potential, and animal feed value of sugarcane residues, providing alternative models for the sugarcane industry to valorizing sugarcane residues into useful products. For sugarcane and livestock dense regions, annexing either AFEX™ or StEx to existing industrial sites could present additional opportunities to sustainably valorize sugarcane residues for the bioenergy and livestock production sectors, thereby increasing the sugarcane cultivation land use efficiency and contributing to food, feed and bioenergy production.

As shown in Figure 9.5a, AFEX™ or StEx could be adopted as the pretreatment technologies for overcoming the recalcitrance of sugarcane residues in dedicated 2G cellulosic ethanol biorefineries integrated to sugar mills or 1G ethanol distilleries. In **CHAPTERs 4,5** and **8**, it was shown that AFEX™ generates higher ethanol yields relative to StEx pretreatment, with the difference in ethanol yields being dependant on the extent of sugar loss during pretreatment, the whole slurry processing strategy employed, the enzyme dosage, and robustness of the ethanologen used. However, techno-economic modelling using a wide range of enzyme dosages and the higher fermentation yields obtained from the industrial yeast strains would be required to determine the preferred pretreatment method for this scenario.

An alternative strategy to having dedicated 2G biorefineries annexed to every sugar mill is to only integrate the pretreatment units (StEx or AFEX™) to sugar mills and produce dense, durable, hydrophobic, and conversion-ready sugarcane residue pellets. **CHAPTER 7** demonstrated that both AFEX™ and StEx facilitate the production of pellets with superior handling and transportation characteristics relativeto untreated controls. In this scenario, these pellets would be collected from clusters of nearby sugar mills, transported to large-scale centralized 2G biorefineries, thereby creating scale to meet regional or national biofuel demand and/or sustainability targets. Furthermore, should enzyme related costs become unfavourable, the AFEX™ pellets could be upgraded via a room temperature $CIII_l$-activation process to reduce enzyme dosages to approximately 3 mg protein per gram raw dry biomass whilst achieving ethanol yields greater than 280 litres per tonne raw dry biomass (Figure 9.5b). Ultimately, the scale of the prospective biorefineries coupled with techno-economic and life-cycle analyses would be required to determine if the additional processing costs for $CIII_l$-activation justify the enzyme cost reductions allowed by the enhanced digestibility of $CIII_l$-activated sugarcane residues.

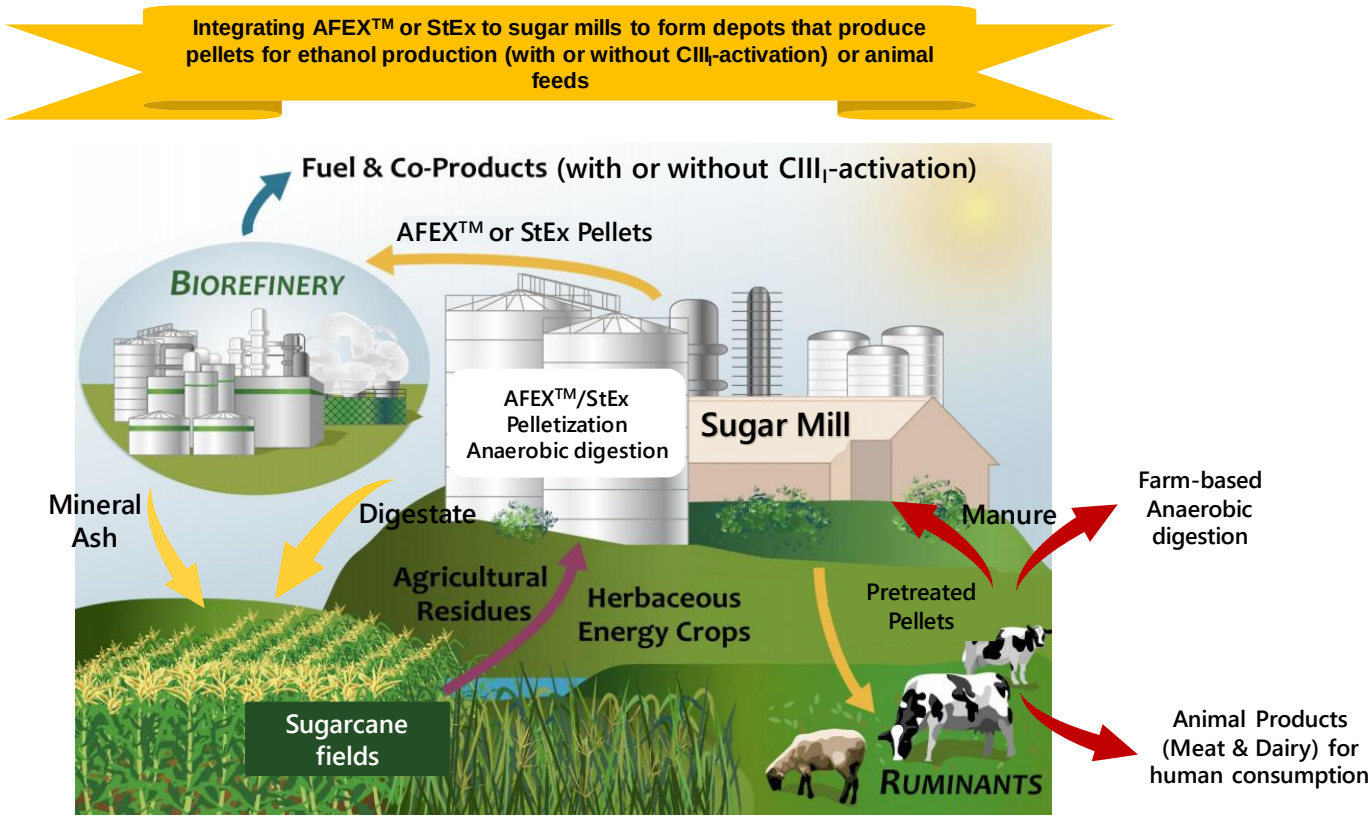


**Figure 9.5:** Integrating AFEX™ or StEx to sugar mills to form depots that produce pellets for ethanol production (with or without $CIII_l$-activation) or animal feeds



In addition to supplying a biofuel market, StEx- and AFEX™ sugarcane residue biomass/pellets can be introduced to the animal feed market as both pretreatments significantly enhance the ruminant animal feed value of SCB and CLM relative to untreated controls (**CHAPTER 5**) (Figure 9.5c). With in-field animal feed trials suggesting that AFEX™-treated residues can substitute up to 30% of corn grains or green grasses in ruminant diets, the potential use of AFEX™-treated residues in animal diets of intensive livestock production systems could present a more efficient biofuel and food production scenario for sugarcane and livestock dense regions.

AFEX™-treated SCB and CLM have also demonstrated high biogas potential in anaerobic digestion due to their favorable biodegradability and C/N ratios (**CHAPTER 6)**. Hence, AFEX™ SCB and CLM biomass can also be used in farm-based or centralized anaerobic digestion plants either as the sole substrate or in co-digestion with livestock manures, to produce energy-rich biogas and digestates that can be used in biofertilizer and soil amendment applications (Figure 9.5d). Alternatively, depending on the proximity of the intensive animal farms to cellulosic biorefineries, the animal manure from these farms could be transported and co-digested with sugarcane residues in AD-based wastewater treatment plants that will form part of the water circuit of prospective cellulosic biorefineries. Furthermore, the integration of anerobic co-figestion could become an effective NPK nutrient recovery and recycling strategy, potentially allowing for lower fertilizer use on sugarcane fields and/or higher CLM recovery for valorization into bioenergy/animal feeds. As a result, the ability to produce both food and bioenergy using StEx/AFEX™-treated crop residues and the potential integration of anaerobic digestion from the livestock sector addresses the “food vs fuel vs livestock waste management” controversy. Furthermore, purified lignin streams from the ethanol production processes can be used as platform feedstocks for aromatics based bioproducts. This lignin generated from crop residues may be the only means to produce biobased aromatic products at scale.

Ultimately, the results of this dissertation provide essential information and insights for future techno-economic and life-cycle analyses that are required to establish the preferred pretreatment



technology and processing strategies to enable economically viable and environmentally sustainable integrated bioenergy and animal feed production from South African sugarcane residues.

## 9.3 Recommendations

### 9.3.1 *Improving enzymatic hydrolysis and fermentation from sugarcane residues*

In **CHAPTER 4**, the identification of auxiliary enzymes, process integration and the use of a suitably hardened xylose-fermenting ethanologens were recommended as key areas for improving ethanol yields from AFEX™ and/or StEx treated sugarcane residues. The identification and blending of auxiliary cellulolytic and hemicellulolytic enzymes with current commercial enzyme cocktails to create unique cocktail combinations tailored for each pretreated substrate could potentially synergistically allow for higher enzymatic hydrolysis efficiency with lower oligosaccharide formation/accumulation. For 2G biorefineries integrated to sugar mills/1G ethanol distilleries, StEx-derived inhibitor laden streams such as the $C_5$-liquor or whole slurry hydrolysates could be mixed with molasses or sugarcane juice prior to fermentation to simultaneously increase the total sugar concentration (and the potential ethanol concentration) of the stream and dilute the concentration of the pretreatment derived inhibitors. Provided mixing these streams does not create inhibitory osmotic stress to the xylose-fermenting ethanologen(s), this could potentially become an effective strategy for minimizing ethanol recovery costs (higher ethanol concentrations fed into distillation) and maximising ethanol yields.

### 9.3.2 *Techno-economic and life-cycle analyses*

The side-by-side comparison of AFEX™ and StEx showed that AFEX™ was able to achieve higher ethanol yields relative to StEx, with sugar loss to degradation, enzyme inhibition and microbial inhibition being the primary areas for the lower ethanol yields from StEx treated sugarcane residues. However, selecting the preferred pretreatment technology is primarily an economic and environmental impact issue. Hence, estimating the cost of ethanol production ($USD/ L ethanol) through techno-economic analysis and environmental impacts through a life-cycle analysis would



provide the necessary basis for comparing 2G sugarcane biorefineries centred on AFEX™ or StEx pretreatment.

### 9.3.3 *Animal feed trials with AFEX™ or StEx treated sugarcane residues*

Although both StEx and AFEX™ were shown to significantly enhance the animal feed potential of sugarcane residues, animal feed trials with StEx- or AFEX™-treated sugarcane residues substituting for concentrates and/or green grasses in animal diets will ultimately provide the best indicator of their animal feed value. These trials would quantify the impact of incorporating these pretreated residues in animal diets on key performance indicators such ruminant weight gain, milk production, beef quality, and milk quality. For AFEX™-treated sugarcane residues, these trials would also determine whether the nitrogenous compounds generated during pretreatment (*e.g.* acetamide, phenolic amides, and Maillard reaction products) would be present in beef/milk at concentrations beyond those stipulated in local animal feed regulatory policies.

In addition, it is also recommended that these animal feed trials also quantify the impact of substituting traditional concentrates and/or green roughages with pretreated sugarcane residues on the entric methane emissions (methane expelled by ruminant through burping) from ruminants such as cattle. Entric methane emissions from livestock are one of the major greenhouse gas emissions contributors, hence the quantification of entric methane emissions would provide essential information regarding the suppression or enhancement of livestock methane emissions due to the incorporation of AFEX™- or StEx-treated sugarcane residues in animal diets.

### 9.3.4 *Scale up anaerobic digestion*

The anaerobic digestion assays used in this work were conducted at small-scale and should be further evaluated at bench-scale in both batch and continuous operation to evaluate the stability of anaerobic digestion using AFEX™-treated or StEx-treated sugarcane residues. In addition, the pretreatment conditions used in preparation of anaerobic digestion substrates were based on optimized conditions for ethanol production. Hence, these conditions might not be optimized for



economical anaerobic digestion. Therefore, it is recommended that the effect of a wide range of AFEX™ and StEx pretreatment conditions on the bench-scale anaerobic digestion efficiency be established. Furthermore, techno-economic models and corresponding life-cycle analysis should also be performed to determine the optimal size of intensified cattle farms to allow for economically viable anaerobic co-digestion of manure and agricultural residues.



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