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Coupling AFEX and Steam-exploded sugarcane residue pellets with a room temperature $CIII_I$-activation step lowered enzyme dosage requirements for sugar conversion.

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ABSTRACT

In this study, the potential integration of steam explosion (StEx) and ammonia fiber expansion (AFEX) with existing sugar/ethanol mills to form decentralized pre-processing depots was explored. Both StEx and AFEX pretreatment facilitated the production of sugarcane bagasse (SCB) and cane leaf matter (CLM) pellets with significantly higher bulk density, mechanical durability, and hydrophobicity relative to their untreated biomass pellet controls. However, ethanol production from standalone StEx and AFEX-treated SCB and CLM pellets required enzyme dosages greater than 21 mg/g glucan to achieve enzymatic hydrolysis sugar yields of 75% and ethanol titres greater than 40 $g.L^{-1}$. Coupling AFEX-treated SCB or CLM pellets with a room temperature $CIII_I$-activation step using liquid ammonia lowered enzyme dosage

requirements by more than 50% without affecting ethanol titers and production yields (> 300 L per Mg residual dry matter raw dry biomass (RDM)). In contrast, treating StEx-treated pellets with $CIII_I$-activation using liquid ammonia did not result in similar enzyme dosage reductions, due to pseudo-lignin formation, leading to enzyme deactivation and/or lignin blockage that retarded enzymatic hydrolysis at low enzyme dosages. A gross energy conversion assessment revealed that low enzyme dosage (2.96 - 3.95 mg enzyme/g RDM) ethanol and electricity co-production from AFEX and $CIII_I$-activated SCB and CLM can recover up to 73% of the energy in the untreated biomass, compared to 54% recovered by StEx and $CIII_I$-activation. The results from this work suggest that StEx or AFEX based pre-processing depots can produce dense and mechanically durable biomass pellets. The AFEX-treated pellets can be easily upgraded using a room temperature $CIII_I$-activation step at the biorefinery to significantly reduce bioconversion enzymes.

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

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Abbreviations

AFEX, Ammonia Fiber Expansion; ANOVA, analysis of variance; Ci_β, cellulose I_β; $CIII_I$, cellulose $CIII_I$; SCB, Sugar Cane Bagasse; DM, Dry Matter; RDM, Residual Dry Matter; CLM, Cane Leaf Matter; D_{enzyme}, Enzyme dosage; HHV, Higher heating value; HPLC, High performance liquid chromatography; LAP, Laboratory analytical procedure; MBI, Michigan

Biotechnology Institute; P_{enzyme}, enzyme on-site production or purchase cost; StEx, Steam Explosion; XRD, X-ray powder diffraction; $Y_{ethanol}$, ethanol yield; WRV, water retention value.

1. Introduction

Meeting future biofuel production targets that will allow the industry to substantially contribute to global energy and sustainability challenges will require mass mobilization of cellulosic biomass [1-2]. To supply a regional or national bioeconomy, biomass logistics and market structures will need to confront and manage unfavorable biomass characteristics, *i.e.,* low bulk density, geographical dispersion, and variable moisture content and chemical composition [3-4]. 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 [5–8]. Moreover, continuous operation of biorefineries will very likely require large-scale feedstock production and biomass procurement from multiple sources. Thus, to advance large-scale cellulosic bioenergy production, strong farmer and/or biomass grower participation will be essential to establish and secure the biomass supply chain [9]. Recent efforts have been focused on de-coupling the feedstock supply chain from biomass conversion to minimize feedstock supply risk and provide economic incentive to the biomass producer. The objective would be to sell commodity-type and infrastructure-compatible bulk solid biomass intermediates to multiple markets, including the bioenergy industry [6,10-11].

Sugarcane crop residues (including sugarcane bagasse (SCB) and cane leaf matter (CLM)) are major agricultural residues with a global annual production estimated at 800 million metric tons per annum [12]. Sugarcane residues typically benefit from sharing “field-to-sugar mill” feedstock supply and handling infrastructure with the existing sugar production process [13]. Establishing distributed supply chain networks by coupling pre-processing depots with existing

sugar mills presents an opportunity to minimize capital and operating costs by leveraging integration benefits [7,14].

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. In some cases, the sugar mill itself might be expanded to become a biorefinery. The densified biomass can subsequently be blended with other uniform, densified feedstocks and/or be transported long distances to exceptionally large centralized biorefineries, allowing for much lower biomass transportation costs (compared with transportation as bales), and improved plant operation due to more uniform feedstock characteristics. A critical overall result of transporting densified biomass is to take advantage of economies of scale to reduce the cost of biofuels [5,10,14].

The proposed system mimics the existing commodity grain model and facilitates a system whereby sugarcane mill owners would supply conversion-ready intermediates that have favorable physical properties for storage and transportation to multiple markets. With a saturated global sugar market, the use of these sugarcane residues for bioenergy production (*e.g.,* cellulosic fuel such as ethanol, farm/centralized biogas, bioelectricity, and heat), or other commodity markets (*e.g.,* animal feed operations, biochemicals) presents a variety of alternative models for adding economic value to sugarcane residues [9]

Previous work has shown that well-studied technologies including steam explosion (StEx) and ammonia fiber expansion (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) [15–19]. However, the requirement of high enzyme dosages (~ 25 mg protein/g glucan) to achieve high carbohydrate-to-

sugar conversions (>75%) from pilot-scale StEx-or AFEX-treated sugarcane residues presents a significant limitation for prospective StEx or AFEX-based integrated depots [20].

Previously, da Costa Sousa *et al.,*[21] developed a single-step extractive ammonia (EA) process that removed ~50% of lignin and demonstrated 60% enzyme dosage reduction in high solids loading enzymatic hydrolysis relative to standalone AFEX. This process used liquid ammonia in the presence of low amounts of water (~10 % of the total biomass weight) to combine the benefits of cleaving lignin-carbohydrate crosslinks via ammonolysis, the selective extraction of lignin, and re-arrangement of the native crystalline cellulose I_β (CI_β) to form the highly digestible allomorph cellulose III_I ($CIII_I$) [22]. However, drawbacks of the EA process include the requirement of external heating, high pressure operating conditions (~86 bar) and high ammonia-to-biomass loadings (3:1 w/w) and high temperature (120^{o}C) which translated into high capital and operating costs requirements.

Liquid ammonia is known to facilitate the transformation of CI_β to $CIII_I$ even at room temperature [23–25], hence, there is an opportunity to exploit this effect to convert the crystalline allomorph cellulose present in AFEX or StEx-treated biomass pellets to $CIII_I$ to reduce enzyme dosage requirements while maintaining 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 and operating costs required to form $CIII_I$ [26]. The process of transforming the crystalline allomorph of native CI_β to $CIII_I$ using liquid ammonia at room temperature and low pressure is herein called '$CIII_I$-activation.'

In the present work, we explore a biorefinery concept wherein StEx- and AFEX pretreatment technologies are performed 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 (Fig. 1). These 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/StEx pretreatment on the physical and mechanical properties of SCB and CLM produced using a single-pass pilot-scale pellet mill and compared these properties with literature-reported values for compacted stockpiles of SCB, CLM bales, and corn grain. Thereafter, we evaluated the impact of upgrading StEx and AFEX-treated pellets using a $CIII_I$-activation process using anhydrous liquid ammonia at room temperature (25^{o}C) to reduce the enzyme dosage required for efficient high solids loading enzymatic hydrolysis and fermentation of SCB and CLM. Lastly, we performed a gross energy conversion assessment to evaluate the overall recovery of the inlet feedstock heat of combustion in the form of ethanol and the electricity equivalent energy for the low enzyme dosage StEx/AFEX coupled with $CIII_I$-activation scenario. This work breaks new ground by showing how fungible sugarcane residue pellets might supply prospective biorefineries based on uniform, easily stored, and transported biomass pellets. This study highlights the importance of feedstock supply chain development, whilst simultaneously reducing overall processing costs of producing biofuel.

2. Experimental

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 [20]. The chemical compositions of these two biomass materials were 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 described previously by Mokomele *et al.,*[27]. SCB and CLM were simultaneously pretreated at Michigan Biotechnology Institute, Lansing, Michigan in separate pretreatment vessels but within 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 set at 50°C (Grieve Corporation, IL) to prevent biomass spoilage. Steam explosion was conducted at Stellenbosch University in a 19 L automated batch pilot-scale unit (IAP, GmBH, Graz, Austria) equipped with a 100 L blowdown tank and a steam generator. The StEx pretreatment protocol and pretreatment conditions applied for SCB and CLM were described elsewhere [20]. Unwashed StEx SCB and CLM samples were dried to 15% moisture content in a convection oven at 35 °C prior to pelletization.

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 [28]. 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 to 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 consistent pellet formation. The cooled pellets were dried at 45 °C in a convection oven to less than 10% moisture and subsequently stored at 4 °C in heat-sealed bags until use.

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.,*[21](Fig. S1). 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. Room temperature anhydrous liquid ammonia was gravimetrically loaded into the tubular reactors to an ammonia to biomass ratio between 0.5 g NH_3/g DM to 3:1 g NH_3/g DM. We found that 0.75 g NH_3/g DM was enough to submerge the StEx or AFEX-treated pellets and subsequently produce $CIII_I$. Immediately after loading ammonia, the reactors were heated to room temperature (25°C) and allowed to soak for 180 min. to ensure $CIII_I$-formation. For the duration of the pretreatment at 0.75g NH_3/g DM, the reactor pressure fluctuated between 9 and 12 bar (131 and 174 psi). After the pretreatment time had elapsed, the reactor was heated to 40°C and maintained at that temperature for 10 minutes before an overhead-valve at the top of the reactor was opened to release ammonia gas. The temperature was slightly raised from 25°C to 40°C to facilitate ammonia release from pretreated biomass. 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. The $CIII_I$-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 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 30 min residence time [29]. 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 powder diffraction (described below).

2.4. Physical and mechanical properties of pellets

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 caliper (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 fraction of fines caused by ineffective pelletization or pellet disintegration at the pellet mill outlet was measured by sieving 500 grams of the pellets collected at the pellet outlet during pseudo-steady state operation through a No.7 size wire-cloth test sieve 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 Seedbro pellet durability tester (Seedbro Equipment Company, Des Plaines, IL, USA). Briefly, 500g of fines-free pellets were tumbled in a dust-tight metal box for 10 minutes at 50 rpm and then sieved through a No.3.5 size (sieve opening of 5.66 mm) wire-cloth test sieve to remove the generated fines (ASTM Standard E11-87). The average diameter of the pellets produced was in the range 6.5-7mm, hence, this sieve size was appropriate to retain all the intact pellets. 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.,* [16].

2.5. Proximate analysis, ultimate analysis, and calorific value of pellets

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-treated, StEx-treated samples according to ASTM method 1131. Pellet elemental analysis was conducted using a Vario EL Cube elemental analyzer (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.

2.6. X-Ray powder 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.,* [21]. Briefly, approximately 0.5 g 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. The 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°.

2.7. Low solids loading enzymatic hydrolysis

Low solids loading enzymatic hydrolysis was conducted to determine the digestibility of StEx- and AFEX-treated SCB and CLM pellets compared to $CIII_I$-activated StEx/AFEX 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-treated SCB and CLM consisted of previously optimized combinations of three different commercial fungal enzyme preparations: Cellic® CTec3, Cellic® HTec3 and Pectinex Ultra-SP [20]. The enzyme activity was measured using different substrates. The exo-cellulase activity was measure using Avicel (depolymerized alphacellulose) substrate, endo-cellulase activity was measure using carboxymethyl cellulose (CMC) substrate and hemicellulose activity was measured using Oat spelt xylan (data not shown) using reported protocol [52]. 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 Cooperative Inc., Ithaca, NY, USA). All the enzyme loadings used in the manuscript were based on mg enzymes/gram of glucan. 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 [30].

2.8. 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 compared 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 min. 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 [25]. 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 [20]. Overall mass balances were conducted using a protocol previously published by Gunawan *et al.,* [31].

2.9. Experimental design for high solids loading and fermentation

A statistical approach was undertaken to determine the minimum enzyme dosage requirements for StEx and AFEX pellets to reach target 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-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 S1. 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 on 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 and xylose yield of 75% and an ethanol concentration of 40 $g.L^{-1}$.

Sugar yields and ethanol concentrations resulting from standalone StEx or AFEX-treated SCB and CLM were compared with those of $CIII_I$-activated AFEX/StEx pellets performed at enzyme dosages of 15, 10, and 7.5 mg/g glucan, solids loading that corresponded to 10% polymeric glucan and xylan, and enzymatic hydrolysis residence time of 72 h.

2.10. 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 whether enzymes are produced on-site or off-site, the enzyme dosage used during hydrolysis, and the ethanol yield obtained after fermentation. For comparing the effect of $CIII_I$-activation of StEx/AFEX pellets on the enzyme cost contribution, the enzyme cost per liter of ethanol produced was estimated by Equation (1)[31-32]:

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

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

2.11. Gross energy conversion assessment

The efficiency of converting the energy equivalent to the heat of combustion of untreated SCB and CLM pellets into ethanol and electricity equivalent energy from standalone StEx/AFEX and $CIII_I$-activated StEx/AFEX 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 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.

2.12. 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 insignificant.

3. Results and Discussion

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-treated SCB and CLM pellets are presented in Table 1. StEx-treated pellets were characterized by increased cellulose and Klason lignin content, primarily due to the solubilization of hemicelluloses during pretreatment [20]. Consequently, StEx-treated pellets were slightly greater in fixed carbon and HHV relative to the untreated controls [33-34].

In contrast, AFEX-treated pellets were characterized by higher nitrogen content and a carbohydrate composition like the untreated controls. For AFEX-treated biomass, the additional nitrogen content is probably chemically linked to the biomass due to ammonolysis reactions that cleave the lignin-carbohydrate complex (LCC) largely responsible for biomass recalcitrance [36]. Furthermore, the Klason lignin content of AFEX-treated pellets 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, hydroxycinnamic acids) during the characterization of the biomass composition [37].

Photographs of the untreated, StEx-treated and AFEX-treated sugarcane residue pellets and corn grain are presented in Fig. 2, with the corresponding physical and mechanical properties presented in Table 2.The geometric mean diameter and height of the biomass pellets were 6.8 ± 0.2 mm and 17.4 ± 3.72 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. Both 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 from 4 to 14-fold relative to their loose (non-densified) controls ($P < 0.05$). The bulk densities of StEx and AFEX-treated

SCB and CLM pellets were slightly lower than those reported for corn grain (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 piles of SCB (100 kg/m^3), respectively [37-38]. 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 [19,39]. Further, AFEX and StEx pretreatment facilitated the production of pellets with durability indexes greater than 98.2%, potentially minimizing additional dry matter loss as fines and limiting the explosion risks that are typically associated with handling, transporting, and storing low durability biomass pellets [41].

Improvements in pellet durability due to AFEX or StEx pretreatment have previously been linked to several factors: (i) the activation of lignin as an intrinsic binder through the modification of the lignin-carbohydrate cross-links during pretreatment, (ii) increasing the lignin content of the biomass (in the case of StEx), (iii) the redistribution of lignin from interior part of plant cell wall towards the outer cell wall during pretreatment, (iv) the reduction of the lignin glass transition temperature, and (v) the presence of a plasticizer (*e.g.* water) during pelletization [16,19]. Finally, both 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 [19] 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 [41,42]. 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 both facilitate the production of denser, durable, and hydrophobic SCB and CLM pellets that can potentially reduce the biomass storage footprint, reduce transport and handling costs, and enable commodity scale distribution of sugarcane residues [10]. Moreover, with consistent physical properties like corn grains, these dense pellets have the potential to be integrated into standardized, high efficiency, and high-volume grain handling infrastructures [9]. Hence, provided these pellets are conversion-ready, they can potentially minimize supply chain risks by enabling biomass transport from remote areas and increasing plant operation reliability due to narrower operation specifications [3]. Since sugarcane residues are seasonal, producing stable, dense SCB and CLM pellets provides a simple way of reducing the storage footprint of these residues for year-round availability.

3.2 Minimum enzyme dosage for ethanol production from StEx and AFEX-treated sugarcane residue pellets

A statistical minimization approach was undertaken to determine the minimum enzyme dosage required to achieve sugar yields greater than 75% and a minimum final ethanol titer of 40 $g.L^{-1}$ from StEx and AFEX treated pellets. To this end, quadratic regression models were derived from a Box-Behnken design of experiments to describe the effect of a wide range of enzymatic hydrolysis parameters on four response variables, *viz.* 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 predictability ($R^2_{predicted}$). The refined regression equations, residual plots, ANOVA, contour plots, and model validations for each model are presented in Fig. S3. 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.

The contour lines representing the range of enzyme dosages and solids loadings that correspond to a minimum of 75% combined glucose and xylose yield and an ethanol titer of 40 $g.L^{-1}$ are presented in Fig. 3. The contour line intersection region (shaded area) represents the range of enzyme and solids loading combinations that lead to combined glucose and xylose yields of 75% and ethanol concentrations greater than 40 $g.L^{-1}$, respectively. At these predefined enzymatic hydrolysis and fermentation targets, the minimum enzyme dosage requirements for 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 because high solids loadings require higher enzyme dosages to maintain the same sugar yield [3,16,43-44]. 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 [43-44]. On the other hand, 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 to achieve the sugar yield and ethanol titer targets for both AFEX and StEx pretreated pellets.

3.3 Upgrading of AFEX/StEx pellets with CIII$_I$-activation

Due to high enzyme dosage requirements for standalone StEx or AFEX pretreated sugarcane residues, we investigated the potential upgrading of StEx and AFEX-treated SCB and CLM pellets using a low pressure, room temperature, and low ammonia-to-biomass loading CIII$_I$-activation process. Formation of CIII$_I$ from CIII$_I$-activated StEx/AFEX-treated SCB and CLM pellets was confirmed qualitatively by comparing their XRD spectra to CIII$_I$-controls prepared from microcrystalline cellulose (Avicel PH-101) (Fig. 4a-d). 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° [21,23,45-46]. Conversely, samples of StEx or AFEX pellets resulted in spectra like microcrystalline Avicel PH-101, indicating that no CIII$_I$ is formed during AFEX- or StEx pre-treated sugarcane residues.

The CIII$_I$-activation step submerges cellulose fibers of lignocellulosic biomass in liquid ammonia, allowing ammonia molecules to form hydrogen bonds with hydroxyl groups from cellulose, resulting in the formation of an ammonia-cellulose I complex [47–49]. The allomorph cellulose 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. The rewiring of the hydrogen bond network allows the cellulose chains to be more hydrophilic with increased water accessibility [21,36]. Coupling AFEX and StEx with a CIII$_I$-activation step enhanced the low solids loading hydrolysis yields by 9-21% and 33-44% relative to AFEX and StEx, respectively (Fig. 4e, f). CIII$_I$ formation using liquid ammonia has been shown to improve the synergistic effects of endo-cellulases and exo-

cellulases, subsequently enhancing cellulose de-polymerization rates by up to 3-fold and enabling enzymatic hydrolysis yields beyond those achieved by cellulose I_β and II allomorphs [4,36]. The improved enzymatic binding and/or activity by cellulolytic enzymes has also been reported to be due to $CIII_I$ allomorph being hydrophilic relative to CI_β. 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 enzymes such as cellulases and hemicellulases [50].

Unlike the EA process which requires high ammonia-to-biomass loadings (3 to 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 (174 psi) at room temperature, significantly lower than those pressures required for AFEX, StEx, and EA pretreatment (Fig. S2). Low temperature and pressure systems are generally advantageous for industrial ammonia-based processes where process safety and reduced costs are important considerations. Moreover, for AFEX-treated sugarcane residues, $CIII_I$-activation consumed only 1.5 mg nitrogen/g DM, with the remainder of the ammonia potentially recovered using the same technologies employed for AFEX pretreatment (Fig. S4). 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.

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 sugar and ethanol yields achievable from $CIII_I$-activated AFEX and StEx

pellets were studied using lower enzyme dosages. Images of $CIII_I$-activated AFEX and StEx-treated SCB are presented in Fig. S1b. As shown in Fig. 5 a, b, upgrading AFEX-treated SCB and CLM pellets with $CIII_I$-activation reduced enzyme dosage required to achieve the same combined glucose and xylose yields relative to AFEX-treated pellets from 25 mg/g glucan to 10 mg/g glucan. At 10 mg/g glucan (5.9–5.6 g enzyme protein/kg RDM), coupling AFEX with $CIII_I$ resulted in ethanol yields greater than 300 L/Mg RDM, like that achieved by AFEX-treated pellets using an enzyme dosage of 25 mg/g glucan ($P > 0.05$) (Fig. 5 e, f). As with the EA process, augmenting AFEX with $CIII_I$-activation combines the benefits of ammonolysis of cell wall esters during AFEX pretreatment and modification of glucan chain packing (or cellulose polymorph) during $CIII_I$-activation to enhance substrate digestibility even under enzyme-limited conditions. Further, the surface of $CIII_I$ is likely more hydrophilic than CI_β, hence $CIII_I$-activation may also help minimize the effect of the solid loading. Nonetheless, an additional 62-87 kg/Mg RDM of the recovered sugars from $CIII_I$-activated AFEX SCB and CLM pellets were in oligomeric form (data not shown), representing a further 36-49 L of ethanol per Mg RDM that can be produced with better enzyme cocktails [51-52].

In contrast, augmenting StEx pretreated sugarcane residue pellets with $CIII_I$-activation did not achieve similar enzyme reductions as observed for AFEX-treated pellets (Fig. 5 c, d). At 10 mg/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 25 mg/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 re-deposition of pseudo-lignin compounds on the cell wall surface. We previously confirmed the

presence of pseudo-lignin hydroxyl, carbonyl, and aromatic functional groups in StEx-treated SCB and CLM (pretreated at the same conditions as this work) by FTIR analysis [53]. These pseudo-lignin-type moieties have been demonstrated to limit efficient hydrolytic biomass deconstruction [54]. Recent work has shown that lignin from hydrothermally pretreated corn stover, wheat straw, and *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 [55].

In contrast, Pielhop *et al.,* (2017) found that re-polymerized lignin-like structures from auto hydrolysis pretreated spruce wood significantly intensified enzyme adsorption to lignin and accelerated enzyme deactivation [56-57]. Hence, results from this work suggest that even though $CIII_I$ was formed in the StEx pellets, this potentially more digestible cellulose could not be easily accessed by the hydrolytic cellulases probably due to substrate blockage by lignin, cellulase deactivation by re-polymerized pseudo-lignin at the cell wall surface, and/or enzyme inhibition by soluble products (*e.g.,* oligosaccharides, phenolic compounds, furan derivatives, aliphatic acids) generated from pretreatment [20]. This problem might be overcome by redesigning the StEx pretreatment severity conditions prior to pelletization and the $CIII_I$-activation step. For instance, low severity StEx to facilitate easy pellet formation might 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 [21].

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 biorefinery capital costs and increases

process complexity [33]. Klein-Marcus Hamer and co-workers estimated that on-site enzyme production would cost approximately $10.4/kg enzyme, which compares well with the cost of amylase enzymes purchased by the corn ethanol industry (~$25/kg enzyme) [32] but significantly higher than cellulase enzyme costs often assumed in literature [33,58–61].

Since the enzyme cost contribution (on a $/L EtOH basis) depends on the enzyme dosage (kg protein/Mg RDM), the ethanol yield (L EtOH/Mg RDM), and the enzyme production or purchase costs ($/kg protein), reducing the enzyme dosage from 25 kg/Mg glucan to 10 kg/Mg glucan as enabled by $CIII_I$-activation of AFEX-treated SCB and CLM pellets could potentially reduce the overall 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 U.S. $10.40/kg protein) (Fig. 6).

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 U.S. $0.41/L to U.S. $0.17/L. With affordable enzyme dosages and enzyme cost contributions currently estimated at 2 mg protein/g RDM and U.S. $0.066/L, coupling AFEX with $CIII_I$-activation process lowers the required enzyme dosages to about 3 to 4 mg protein/g RDM (or 10 – 7.5 mg/g glucan) and subsequently lowers enzyme cost contribution sensitivity to the cost of on-site enzyme production [62]. This result provides a basis for future techno-economic evaluations to determine whether additional capital and operating costs necessary for adding the $CIII_I$-activation step at centralized biorefineries would justify the enzyme cost savings achieved by reducing enzyme dosage requirements enabled by modifying the native cellulose to its $CIII_I$ allomorph.

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 3. Mass balances for each process included in Table 3 are available in Fig. 5S. The gross energy conversion efficiency for AFEX and AFEX and $CIII_I$-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 20.7 – 22.9 GJ per dry Mg of lignin residues or 8.4 – 9.5 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). If 1 GJ of lignin residue HHV generates a theoretical equivalent of 277.8 kWh of electricity [59-60], a boiler efficiency of 80% and a turbo generator efficiency of 85% [59], the combustion of AFEX and AFEX and $CIII_I$ lignin residues can potentially generate an electricity equivalent of 1576–1795 kWh per Mg raw dry sugarcane residues or 2750 –3304 kWh per Mg of ethanol produced. Depending on the size of the biorefinery, local regulations, and the price of bioelectricity, the produced electricity would supply the energy demand of the biorefinery with any excess electricity sold to the local or national grid [62-63]. Overall, ethanol and electricity production from high enzyme dosage standalone AFEX or low enzyme dosage AFEX plus $CIII_I$ activation recovered approximately 71 – 73% of the heat of combustion of the inlet sugarcane residues.

The ethanol production energy efficiency for standalone StEx and StEx and $CIII_I$ 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 and $CIII_I$ lignin residues were within high volatile sub-bituminous B grade coal range. With lignin residue yields of 0.30 – 0.36 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 and $CIII_I$ lignin residues. The corresponding combined ethanol and electricity production gross energy conversion efficiencies for standalone StEx and StEx and $CIII_I$ were in the range of 53 – 59%, significantly lower than those achieved by standalone AFEX or AFEX and $CIII_I$.

3.6 Other potential markets for standalone StEx and AFEX sugarcane residue pellets

In addition to supplying a biofuel market, StEx- and AFEX sugarcane residue pellets have the potential to be fungible commodities across multiple near-term deployment bioenergy and commodity markets. StEx or AFEX sugarcane residue pellets can be introduced to the animal feed market as recent research has shown that both pretreatments significantly enhance the ruminant animal feed value of SCB and CLM relative to untreated controls [27]. Mor and co-workers have recently demonstrated that when AFEX treated wheat straw pellets substituted 50% of wheat straw in Karan-Fries cattle diets, up to 42% increase in dry matter intake and 18% increase in milk energy was observed after a 77 day feed trial [67]. Furthermore, AFEX-treated SCB and CLM have also demonstrated high biogas potential in anaerobic digestion due to their favorable biodegradability and C/N ratios [53]. Hence, AFEX-treated SCB and CLM pellets 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. Lastly, StEx-treated SCB can be traded as bioenergy feedstocks with relatively low ash content for heat and bio-power generation using mature technologies such as combustion and gasification [68].

4. Conclusions

For biofuels to make a meaningful impact on national/global energy and sustainability goals, large-scale biomass mobilization and commoditization systems must be established. In this work, we demonstrated that integrating StEx or AFEX based depots with sugar mills can produce dense and conversion-ready sugarcane residue pellets with bulk densities and mechanical durability like corn grain, thereby enabling effective storage and long-distance biomass transportation. Coupling AFEX-treated SCB and CLM pellets with a room temperature $CIII_I$-activation step reduced the enzyme dosages by more than 50% (to 3.95 – 2.96 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 relative to AFEX-treated pellets. Higher lignin content in StEx-treated sugarcane residues is one possible reason for reduced sugar yield during enzymatic hydrolysis under low enzyme dosage conditions. For future large-scale sugarcane-based biorefineries with uniform feedstock supply systems, this work provides 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 described, for the first time, the benefits of upgrading AFEX-treated pellets using a low-pressure $CIII_I$-activation process using liquid ammonia to reduce enzyme loading during hydrolysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Captions:

Fig. 1. Integrating StEx or AFEX based pretreatment depots to sugar mills for uniform feedstock supply biofuel production systems to service large-scale cellulosic ethanol biorefineries.

Fig. 2. Illustration of the untreated, AFEX pretreated, StEx pretreated SCB and CLM pellets relative to 1G ethanol industrial corn grain (a) Untreated SCB, (b), AFEX-SCB, (c) StEx-SCB, (d) Untreated CLM, (e) AFEX-CLM, (f) StEx-CLM and (g) corn grain.

Fig. 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 depicts the combinations of enzyme dosage and solids loading that are required to reach minimum combined glucose and xylose yield of 75% and ethanol concentrations of 40 $g.L^{-1}$. (a) – AFEX-SCB; (b) – StEx-SCB; (c) – AFEX-CLM and (d) – StEx-CLM.

Fig. 4. XRD confirmation of $CIII_I$ formation from microcrystalline cellulose (a, b), $CIII_I$-activated AFEX pellets (c) and $CIII_I$-activated StEx pellets (d). Comparison of low solids loading combined glucose + xylose yields from StEx/AFEX and CIIII-activated StEx/AFEX pellets (e, f).

Fig. 5. Comparing the effect of $CIII_I$-activation on high solids loading enzymatic hydrolysis yields (a, c) and ethanol yield per Mg raw dray feedstock (b, d) at high and low enzyme dosages.

Fig. 6. Estimating the effect of CIIII-activation on the enzyme cost contribution to ethanol production.

Figure 1

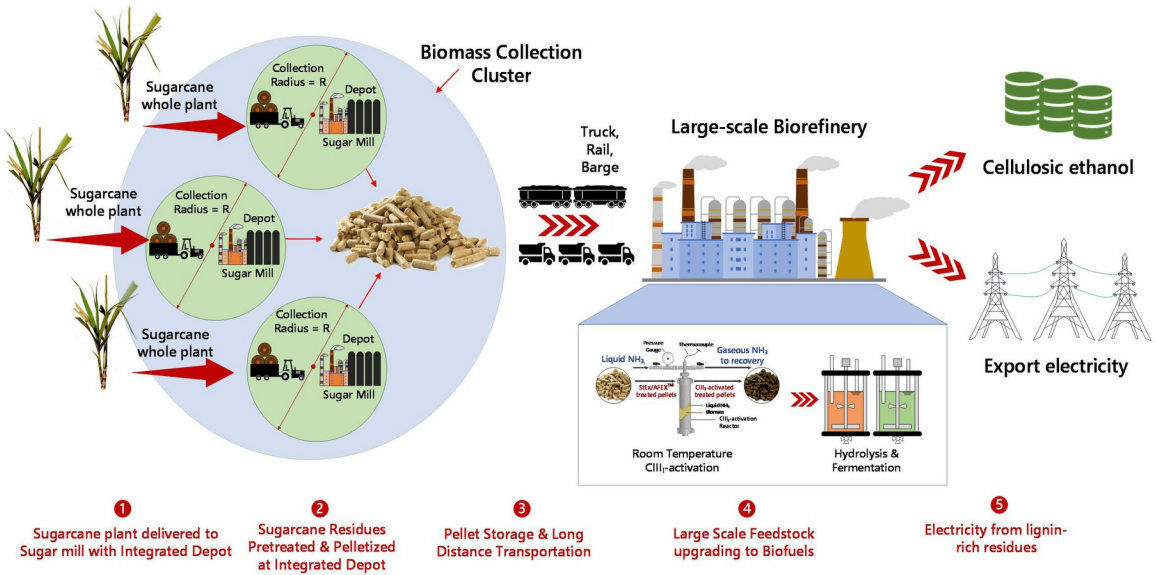

Biomass Collection Cluster
Sugarcane whole plant
Collection Radius = R
Depot
Sugar Mill
Sugarcane whole plant
Collection Radius = R
Depot
Sugar Mill
Sugarcane whole plant
Collection Radius = R
Depot
Sugar Mill
Truck, Rail, Barge
Large-scale Biorefinery
Cellulosic ethanol
Export electricity
Liquid NH3
Gaseous NH3 to recovery
Room Temperature CIIIl-activation
Hydrolysis & Fermentation
1 Sugarcane plant delivered to Sugar mill with Integrated Depot
2 Sugarcane Residues Pretreated & Pelletized at Integrated Depot
3 Pellet Storage & Long Distance Transportation
4 Large Scale Feedstock upgrading to Biofuels
5 Electricity from lignin-rich residues

Figure 2

Figure 3

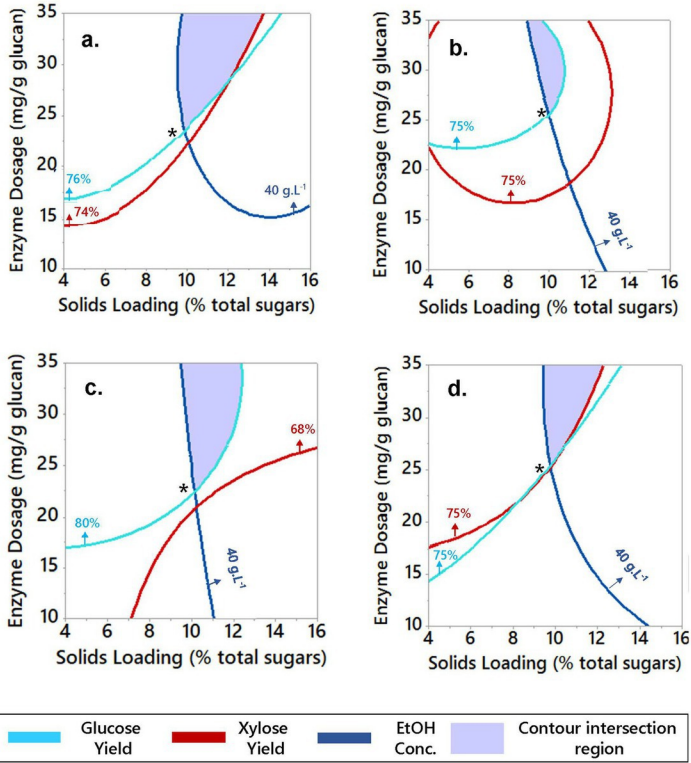

a.
b.
c.
d.
Enzyme Dosage (mg/g glucan)
Solids Loading (% total sugars)
76%
74%
40 g.L-1
75%
75%
40 g.L-1
80%
68%
40 g.L-1
75%
75%
40 g.L-1
Glucose Yield
Xylose Yield
EtOH Conc.
Contour intersection region

Figure 4

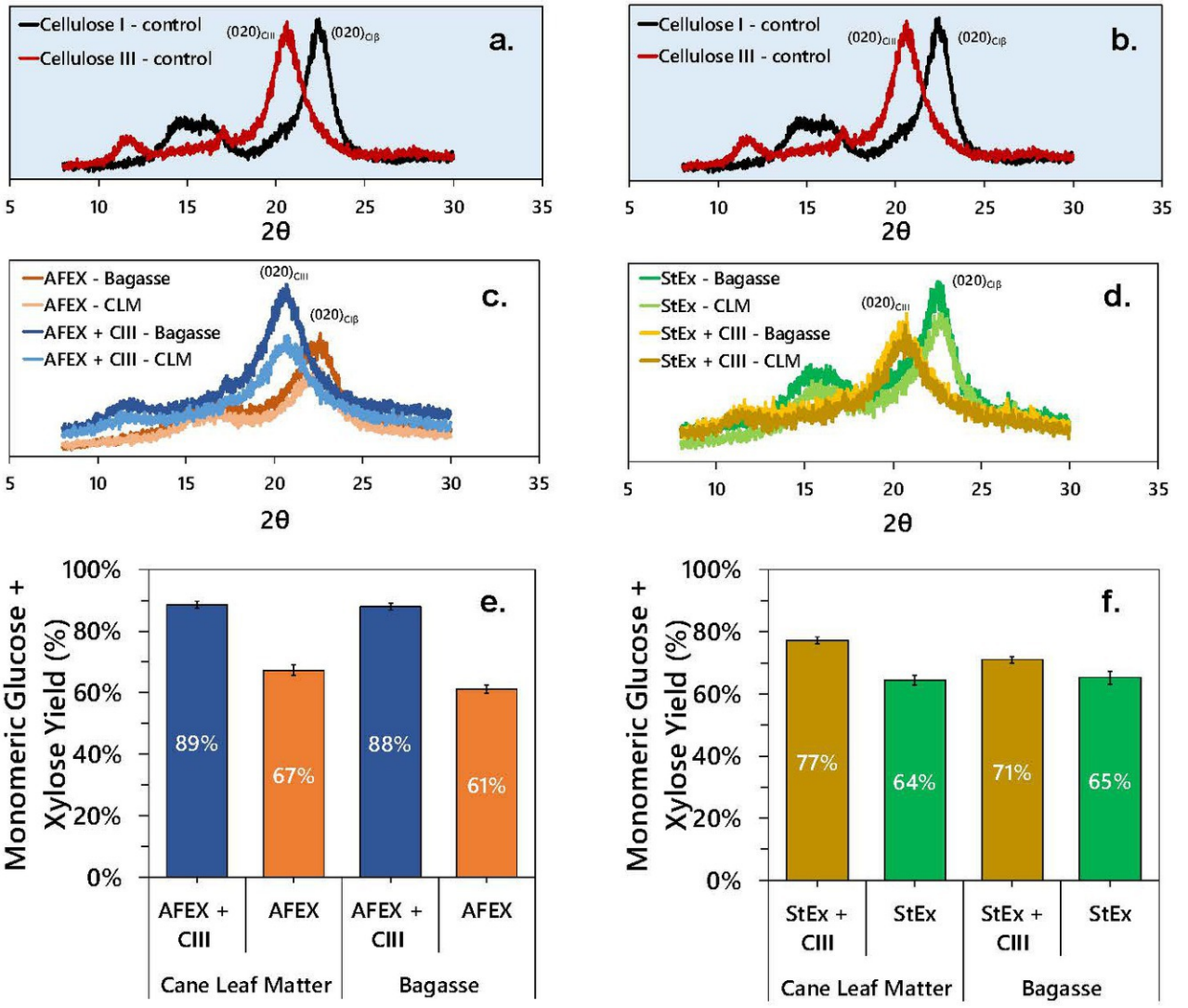

Cellulose I - control
Cellulose III - control
(020)CIII
(020)CIβ
a.
b.
2θ
AFEX - Bagasse
AFEX - CLM
AFEX + CIII - Bagasse
AFEX + CIII - CLM
c.
StEx - Bagasse
StEx - CLM
StEx + CIII - Bagasse
StEx + CIII - CLM
d.
Monomeric Glucose + Xylose Yield (%)
100%
80%
60%
40%
20%
0%
89%
67%
88%
61%
e.
AFEX + CIII
AFEX
Cane Leaf Matter
Bagasse
77%
64%
71%
65%
f.
StEx + CIII
StEx

Figure 5

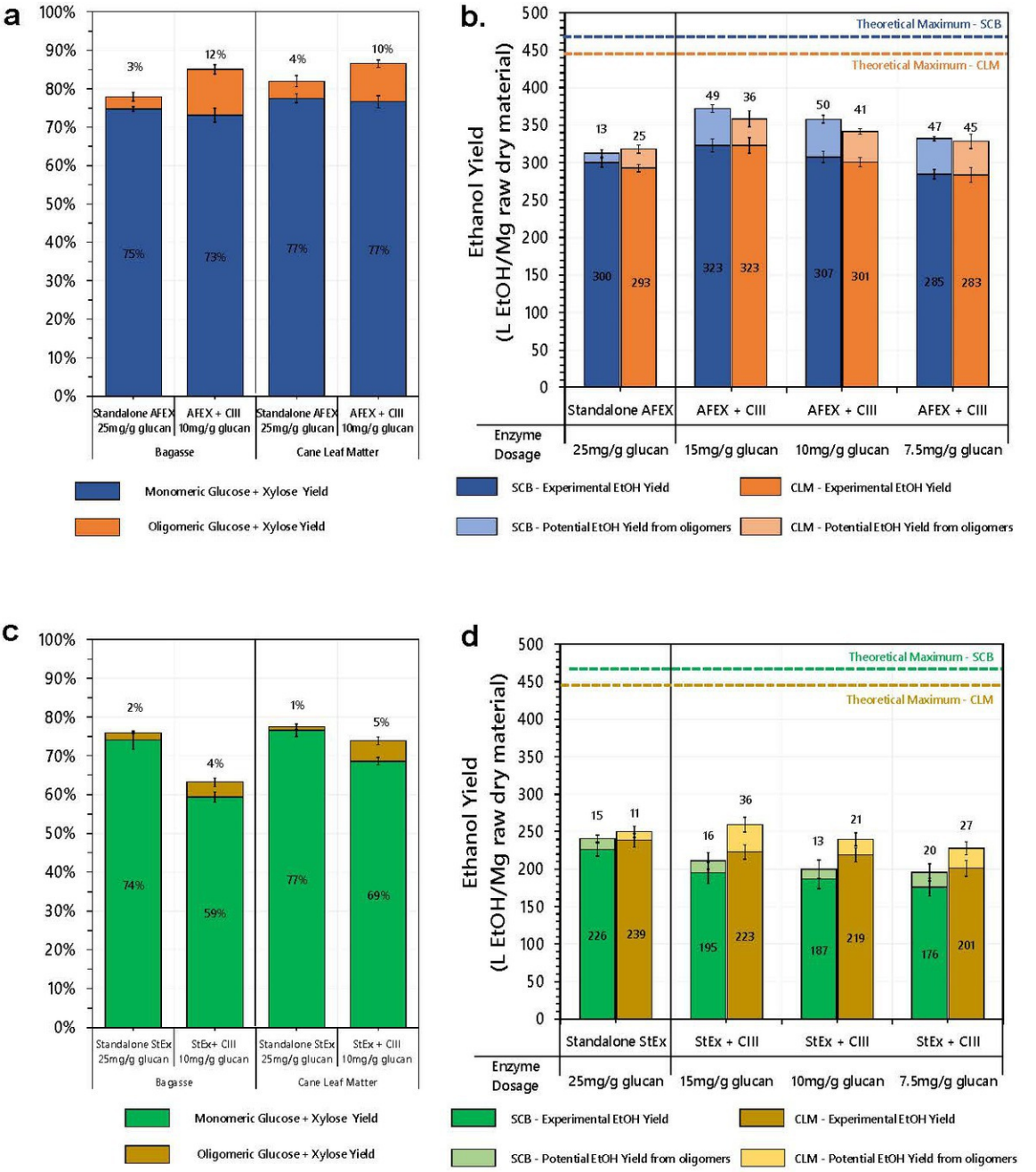
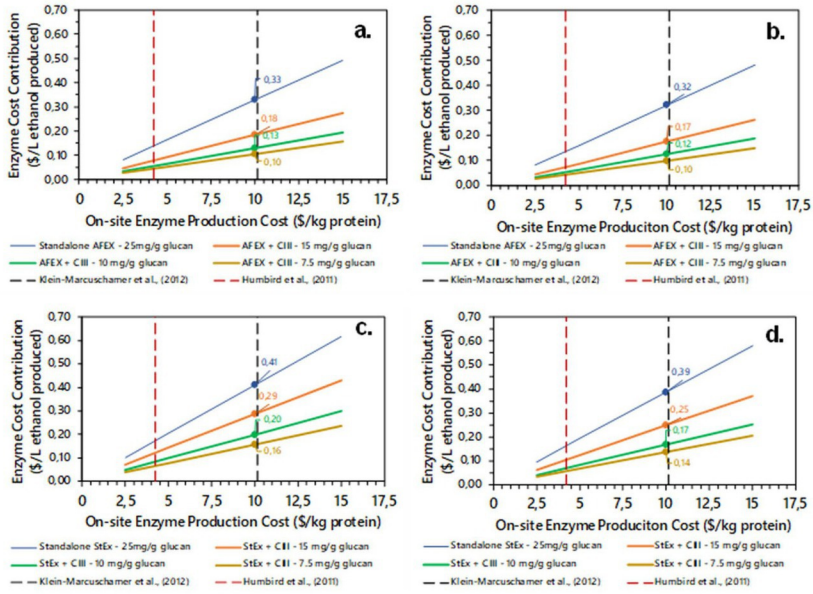

a
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
3%
12%
4%
10%
75%
73%
77%
77%
Standalone AFEX 25mg/g glucan
AFEX + CIII 10mg/g glucan
Standalone AFEX 25mg/g glucan
AFEX + CIII 10mg/g glucan
Bagasse
Cane Leaf Matter
Monomeric Glucose + Xylose Yield
Oligomeric Glucose + Xylose Yield
b.
Ethanol Yield (L EtOH/Mg raw dry material)
500
450
400
350
300
250
200
150
100
50
0
Theoretical Maximum - SCB
Theoretical Maximum - CLM
13
25
49
36
50
41
47
45
300
293
323
323
307
301
285
283
Standalone AFEX
AFEX + CIII
AFEX + CIII
AFEX + CIII
Enzyme Dosage
25mg/g glucan
15mg/g glucan
10mg/g glucan
7.5mg/g glucan
SCB - Experimental EtOH Yield
CLM - Experimental EtOH Yield
SCB - Potential EtOH Yield from oligomers
CLM - Potential EtOH Yield from oligomers
c
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2%
4%
1%
5%
74%
59%
77%
69%
Standalone StEx 25mg/g glucan
StEx+ CIII 10mg/g glucan
Standalone StEx 25mg/g glucan
StEx + CIII 10mg/g glucan
Bagasse
Cane Leaf Matter
Monomeric Glucose + Xylose Yield
Oligomeric Glucose + Xylose Yield
d
Ethanol Yield (L EtOH/Mg raw dry material)
500
450
400
350
300
250
200
150
100
50
0
Theoretical Maximum - SCB
Theoretical Maximum - CLM
15
11
16
36
13
21
20
27
226
239
195
223
187
219
176
201
Standalone StEx
StEx + CIII
StEx + CIII
StEx + CIII
Enzyme Dosage
25mg/g glucan
15mg/g glucan
10mg/g glucan
7.5mg/g glucan
SCB - Experimental EtOH Yield
CLM - Experimental EtOH Yield
SCB - Potential EtOH Yield from oligomers
CLM - Potential EtOH Yield from oligomers

Figure 6


a.
b.
c.
d.
Enzyme Cost Contribution ($/L ethanol produced)
On-site Enzyme Production Cost ($/kg protein)
On-site Enzyme Produciton Cost ($/kg protein)
0,33
0,18
0,13
0,10
0,32
0,17
0,12
0,10
0,41
0,29
0,20
0,16
0,39
0,25
0,17
0,14
Standalone AFEX - 25mg/g glucan
AFEX + CIII - 15 mg/g glucan
AFEX + CIII - 10 mg/g glucan
AFEX + CIII - 7.5 mg/g glucan
Klein-Marcuschamer et al., (2012)
Humbird et al., (2011)
Standalone StEx - 25mg/g glucan
StEx + CII - 15 mg/g glucan
StEx + CII - 10 mg/g glucan
StEx + CII - 7.5 mg/g glucan

Tables

Table 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 ($P < 0.05$)

Table 2: Physical and mechanical properties of untreated and pretreated sugarcane residues pellets

Biomass	Pretreatment	Pellet Dimensions (mm) ‡		Bulk Density, ρ_{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 [29] for round CLM bales; [2] Purchase et al., [30]; [3] Boac *et al.,* [56],

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

Table 3: Energy conversion assessment for ethanol and electricity co-production from high enzyme dosage standalone StEx/AFEX™ pellets and low enzyme dosage $CIII_l$-activated StEx/AFEX™ SCB and CLM pellets

Parameter	Standalone				Standalone + $CIII_l$-activation			
	AFEX™ - SCB	**AFEX™ - CLM**	**StEx - SCB**	**StEx - CLM**	**AFEX™ - SCB**	**AFEX™ - 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 (kg EtOH/Mg RDM)	235.7 ^{A,B}	231.0 ^{B}	178.8 ^{D}	188.3 ^{C}	242.6 ^{A}	239.6 ^{A}	153.8 ^{E}	175.9 ^{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 [52]. 1 GJ of biomass calorific value was assumed to be equivalent to 277.8 kWh of electricity [51]. 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 ($P < 0.05$)