Biomass and Bioenergy 178 (2023) 106972

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

# Applying biorefinery concepts for sugarcane straw upcycling using alkaline and enzymatic treatments to produce value-added compounds and bioenergy

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ARTICLE INFO

*Keywords:*
Sugarcane straw biorefinery
Biotransformation
Lignin nanoparticles
Bioenergy

ABSTRACT

The objective of this study was to develop a sugarcane straw biorefinery that targets the valorization of glucan, hemicellulose, and lignin streams generated through sequential and integrated treatments. Complementary biocatalytic strategies include ferulic acid isolation and biotransformation into high-value compounds, such as coniferol (a molecule with broad application in chemical synthesis) using a genetically engineered *Escherichia coli* strain (BL21-pROB) and xylooligosaccharides (XOS) using a feruloyl esterase and xylanase chimeric enzyme (XynZ). This biorefinery scheme also considers lignin recovery for solid biofuel and lignin nanoparticle synthesis. The evaluation of integration scenarios showed that a mild-alkaline pretreatment followed by enzymatic hydrolysis with XynZ and subsequent biotransformation with BL21-pROB strain, saccharification, and fermentation steps allowed up to 85% (w/w) of sugarcane straw plant cell wall component conversion into coniferol, XOS, fermentable sugars, bioethanol, lignin nanoparticles, and solid biofuel.

## 1. Introduction

Advanced biorefineries are moving towards more sustainable and economically feasible practices by addressing major global challenges, including environmentally friendly production of goods and replacement of fossil fuels [1,2]. Strategies and associated requirements for the success of innovative biorefineries include the availability of a sustainable biomass feedstock supply and the improvement of process efficiency and integration through extensive research and development.

Brazil is the world's largest producer of sugarcane and generates large amounts of bagasse and straw from the ethanol and sugar industries. An interesting comparison reveals that about 140.0 kg of sugarcane straw (SCS) is produced for each ton of sugarcane processed, while 250.0 kg of bagasse is produced for each sugarcane ton [3]. Regarding SCS availability, Brazil generates approximately 20.0 million tons of SCS in a year [3].

SCS is a potential low-cost feedstock for obtaining marketable bio-based products (food ingredients, phenolics, and chemicals) and biofuels other than power and heat within integrated biorefineries [1]. SCS is typically composed of green tops and dry leaves and consists primarily of glucan (~30–45% w/w), hemicelluloses (~20–30% w/w), and lignin (~15–30% w/w) [1]. The pretreatment step is crucial for overcoming SCS recalcitrance and separating the main components, dictating the economic feasibility of the overall process to obtain the desired products and applications [4].

Among the marketable bioproducts that can be produced from SCS, ferulic acid (FAC) and its derivatives are considered fine chemicals which are good candidates for boosting the industrial implementation of large-scale sustainable and efficient sugarcane biorefineries. FAC is mainly produced by the classical Perkin reaction from fossil-based

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https://doi.org/10.1016/j.biombioe.2023.106972
Received 5 July 2022; Received in revised form 28 July 2023; Accepted 8 October 2023
Available online 12 October 2023

benzaldehyde using hazardous chemicals, such as metallic catalysts, even though it can be isolated from plant cell walls through alkaline saponification or biologically by employing feruloyl esterases [5]. Moreover, FAC occurs naturally at approximately 1% (w/w) in certain grasses, such as sugarcane miscanthus and switchgrass [2].

FAC-derivative molecules, particularly coniferol (COL), have a high market value (US$ 400 per g). COL is used to synthesize various chemicals, such as pinoresinol (a hypoglycemic agent), dihydroconiferyl alcohol, coniferyl acetate, and iso-eugenol [6]. The successful production of COL from wheat straw has been demonstrated after biocatalytic treatment using a xylanase/feruloyl esterase from *Clostridium* sp. (XynZ), which released 26 mg/L FAC from plant biomass, followed by 100% conversion yield performed by a whole-cell bioconversion based on a carboxylic acid reductase from *Nocardia* sp. (NiCAR.), and *Coptotermes gestroi* aldo-keto reductase (CgAKR-1) [5].

SCS is also an attractive source of bioactive compounds such as xylooligosaccharides (XOS) obtained from its xylan fraction [7–9]. XOS are sugar oligomers composed of xylose units and can be used for food and pharmaceutical purposes, such as in dietary fibers, because of their prebiotic effects [10]. Hemicellulolytic enzymes can be used to obtain XOS [11,12]. Given its market price, it may be worth considering XOS, a co-product in an SCS biorefinery, because its prices vary from $ 25/kg to $ 50/kg depending on its purity level (70%–95%) [13].

Moreover, lignins are often obtained from lignocellulosic biomass via alkaline treatment or as a residue of biomass saccharification, and they can be employed to generate heat and power and utilized for the synthesis of lignin nanoparticles (LNPs). These LNPs can be used as high-value biological additives for polymers, nanocomposite films, reinforcing materials, nanocarriers, emulsifiers, antioxidants, and bio-nanocomposite catalysts, as suggested by recent studies [14,15].

Thus, several opportunities can be explored to maximize profitability in biorefineries, based on upgrading the biomass phenolic fractions and the pentose and hexose hydrolysates to generate bio-based products. Nonetheless, most technologies behind these chemicals are under development, and their commercial feasibility is uncertain. Thus, the objective of this study was to propose a biorefinery using SCS as feedstock, aiming at the valorization of different currents generated through sequential and integrated treatments of this biomass. These complementary currents included (i) FAC isolation and biotransformation to COL using a previously constructed *Escherichia coli* strain called BL21-pROB, which expresses NiCAR and CgAKR-1 [5]; (ii) XOS obtained from the soluble hemicellulose fraction after pretreatment using a recombinant feruloyl esterase and xylanase chimeric enzyme (XynZ) [16]; and (iii) lignin recovery for solid biofuel and synthesis of LNPs.

## 2. Materials and methods

### 2.1. Feedstock preparation

The SCS used in this work, composed of green tops and dry leaves, was kindly provided by Mill Ferrari (São Paulo, Brazil). SCS particles were ~1.0 cm in length and 1.0 mm in thickness. The recovered SCS (~7% (w/w) moisture content) was mainly composed of glucan (39.0 ± 1.0%, w/w), acetyl-arabinoxylan (27.6 ± 0.4%, w/w), and total lignin (21.5 ± 0.9%, w/w) on a dry matter (DM) basis [11].

### 2.2. SCS fractionation

The scheme for biomass processing pretreatment is illustrated in Fig. 1a. SCS was subjected to mild alkaline pretreatment (SCS-A) or XynZ treatment (SCS-XynZ). In the latter process, the recovered solid fraction after SCS-A was treated sequentially with XynZ (SCS-A-XynZ). All SCS-pretreated solids were subjected to saccharification to evaluate the pretreatment effect on fermentable sugar release, whereas liquid fractions were treated for separating lignin through acid precipitation and sequentially extracted using ethyl acetate to isolate FAC in the organic fraction, and XOS or hemicellulose in the aqueous fraction (Fig. 1b). These processes are described below.

#### 2.2.1. SCS mild-alkaline pretreatment (SCS-A)

The mild-alkaline pretreatment parameters were based on previous studies as it has demonstrated the potential to solubilize hemicellulose and lignin while concurrently yielding a glucan-rich pulp [17]. One kg (DM) of SCS was treated with 0.5 M NaOH in a solid: liquid ratio of 10% (m/v) at 60 °C in an oven without agitation for 24 h. Next, the mixture was strained through a nylon bag to separate the liquid from the solid fraction. The solid fraction was washed with the same volume of water as the initial volume of added water, generating ~10 L of alkaline liquor. The recovered solid was washed and dried in an air-forced oven at 30 °C overnight and used further for saccharification, as shown in Fig. 1b.

#### 2.2.2. XynZ treatment (SCS-XynZ and SCS-A-XynZ)

The enzymatic treatment of SCS and SCS-A was performed using purified xylanase (XynZ) from *Clostridium thermocellum* ATCC 27405, which is composed of a xylanolytic and a C-terminal domain with feruloyl esterase activity [16]. Before XynZ-treatment, 1.0 kg (DM) of SCS and SCS-A was autoclaved for 20 min at 121 °C using separately 9.0 L of tap water suspension. Next, the solids were recovered through a nylon bag and buffered with 1 M sodium phosphate buffer (final concentration 0.1 M, pH 6.4) with a final volume of 10 L. XynZ-treatment was performed in a 20.0 L flask using 0.5 g purified enzyme (21 U/mg and 0.4 U/mg for xylanase and feruloyl esterase activity, respectively) at 50 °C for 24 h at 50 rpm in an orbital rotary shaker. Subsequently, the whole

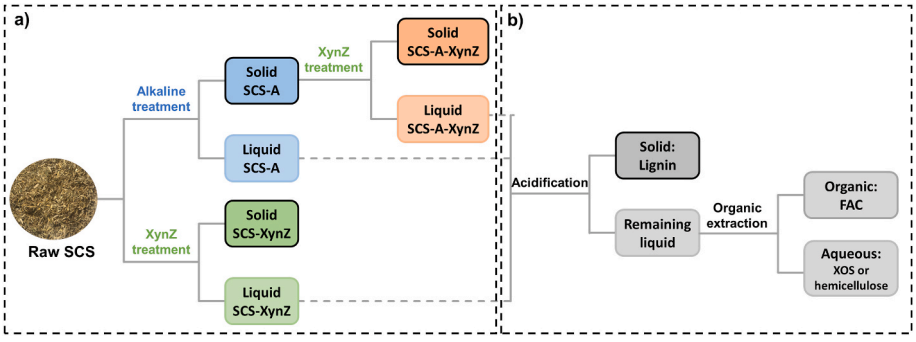


**Fig. 1.** Pretreatment, enzymatic treatment (a), and fractionation strategies (b) employed for sugarcane straw (SCS) valorization. All liquid fractions were submitted to the same fractionation processes. Legend: solid and liquid SCS-A, solid and liquid fractions recovered after SCS submitted to mild-alkaline pretreatment; solid and liquid SCS-XynZ, solid and liquid fractions recovered after SCS submitted for enzymatic treatment with XynZ; solid and liquid SCS-A-XynZ, solid and liquid fractions after SCS submitted for mild-alkaline pretreatment followed by enzymatic treatment with XynZ.

mixture was strained through a nylon bag to separate the supernatant from the solid fraction.

#### 2.2.3. Lignin precipitation and recovery

The pH of the alkaline liquor produced after SCS-A treatment and the supernatants after XynZ treatment (SCS-XynZ and SCS-A-XynZ) was adjusted to 2.0 using 37% HCl (w/w) (Fig. 1b) and the solution was centrifuged at 3000×*g* for 15 min. The unwashed solid fractions, consisting mainly of high molecular weight lignins, were dried in an oven at 60 °C and weighed. As described in subsequent sections, lignin was used for nanoparticle synthesis and energy density measurements.

#### 2.2.4. Phenolic compound extraction (FAC and derivatives)

After lignin precipitation, liquid fractions from the SCS treatments underwent fractionation using liquid-liquid extraction. Pure ethyl acetate was used as the extraction solvent, with a volume ratio of 1:1. The mixture was vortexed for 5 min to ensure thorough mixing, followed by overnight incubation at room temperature (20 °C). Subsequently, we used a separation funnel to isolate the organic and aqueous phases. The organic phase, enriched with FAC, was separated. The organic fraction was concentrated using a rotary evaporator set in a water bath at 70 °C. The resulting concentrate, rich in FAC, was the precursor for biocatalytic COL production. For chemical analysis, the concentrate was re-suspended in methanol. Further details of this analysis are provided in the following sections.

### 2.3. Saccharification and fermentation

Solid fractions recovered from SCS-A, SCS-XynZ, and SCS-A-XynZ treatments (Fig. 1a) were hydrolyzed with the Cellic CTec® 2 commercial enzymatic cocktail (Novozymes) with 130 Filter Paper Units (FPU)/mL. Briefly, 10 g (DM) of each biomass was hydrolyzed with 25 mg per gram of substrate (approximately 20 FPU/g of biomass) at 2.5% solid content (m/v) in citrate buffer (50 mmol/L, pH 5), and a final reaction volume of 0.4 L incubated at 50 °C, 200 rpm for 48 h [17]. Hydrolysis reactions were performed in triplicate. The yeast *Saccharomyces cerevisiae* (Santa Adelia) was used for fermentation. A pre-inoculum composed of 10 g/L of yeast, 20.0 g/L of peptone, and 20 g/L of glucose was prepared in 20 mL media in 50 mL Falcon tubes, and the yeast was cultivated at 30 °C and 150 rpm for 12 h. Fermentation was performed using 20 mL of pre-inoculum centrifuged cells in 50 mL Falcon tubes using 20 mL of the enzymatic hydrolysates as substrate (containing variable amounts of g/L glucose). The assays were performed under the same conditions as the inoculum preparation, and samples were collected after 6 h of fermentation [18].

### 2.4. LNP synthesis

Colloidal LNPs were prepared using the solvent shifting procedure by dissolving the acid-insoluble lignin recovered from the alkaline liquor in 5 mL of dimethyl sulfoxide (DMSO) at 20 mg/mL (DM) and dripping the solution into 100 mL of deionized water, under constant stirring (100 rpm) at 20 μL/min [19]. After 1 h, the mixture was dialyzed using a cellulose membrane (Spectra/Por 2 Standard RC Dry Dialysis Tubing, 12−14 kDa, Spectrum Laboratories, USA) against distilled water for 3 days (distilled water was replaced every 8 h). The final concentration of LNPs in the suspension was determined gravimetrically in triplicate.

### 2.5. COL production by FAC biotransformation

To convert FAC obtained from SCS biological or chemical treatments to COL, the *Escherichia coli* strain BL21-pROB coexpressing a carboxylic acid reductase from *Nocardia iowensis*, an aldo-keto-reductase from *Coptotermes* gestroi according to Tramontina et al. (2020) [5]. The phenolic extract rich in FAC was obtained via mild alkaline pretreatment, or XynZ treatment was added to the media at a final concentration of 5 mM. Samples were taken after 24 h to evaluate COL production.

### 2.6. Chemical analysis

The contents of cellulose, hemicellulose, lignin, extractives, and ashes were determined following the standardized methodologies set forth by the National Renewable Energy Laboratory (NREL) [20,21]. Glucose, cellobiose, xylose, arabinose, acetic acid, formic acid, furfural, and hydroxymethylfurfural (HMF) concentrations were determined by high-performance liquid chromatography (HPLC) in an Agilent 1200 equipment equipped with a refraction index detector and using a BIO-RAD HPX87H ion exchange column, at 45.0 °C, with 5 mmol/L $H_2SO_4$ as the mobile phase. Ash content was gravimetrically determined. The content of extractives was determined using a Soxhlet extractor with a 1:1 solution of cyclohexane and ethanol.

Hemicellulose (acetyl-arabinoxylan) present in the liquid fraction of SCS-A hydrolysate was quantified by hydrolyzing 5 mL of this liquid in an autoclave for 1 h. Before this hydrolysis, the pH was adjusted to 1 with a 72 w/w% solution of $H_2SO_4$, and the hemicellulose quantity was ascertained based on the levels of acetyl, xylose, and arabinose [11].

XOS characterization, including all pentoses released in liquid media, was executed using high-performance anionic exchange chromatography (HPLC-PAD) following the method described by Brenelli et al. (2020) [11] To integrate the peaks, the Megazyme® analytical standards (Bray, County Wicklow, Ireland) were employed, namely: xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6).

FAC and COL were quantified by LC/MS by the method outlined by Tramontina et al. (2020) [5]. The purity of each FAC-rich extract was determined by comparing the measured concentration obtained from the LC/MS analysis to the total weight of the respective dried extract.

### 2.7. Energy content measurement

Untreated SCS, enzymatic hydrolysis residue (EHR), and lignin recovered after acid precipitation were subjected to a standard bomb calorimeter analysis (Parr™ 6400 Automatic Isoperibol Calorimeter) for energy content evaluation. All samples were dried in an oven at 30 °C until the MC was below 5%, milled to less than 0.5 mm, and compressed into pellets using a hydraulic pelletizer before being weighed. The heat content was determined by burning the samples with excess oxygen at 430 psi (30 bar) in a sealed steel bomb.

### 2.8. LNP characterization

The LNPs size distribution and zeta potential in aqueous suspension were measured using a Zetasizer Nano ZS-3600 (Malvern Instruments Ltd, Malvern, UK). The LNPs were diluted in Milli-Q® water for 1 h before the measurements. The LNP dispersion was analyzed by transmission electron microscopy (TEM) using a Carl Zeiss LIBRA 120 instrument with a tungsten filament operating at 120 kV. Before the analysis, the dispersions were diluted to 0.05 wt %, and a drop of the LNP-containing sample (5 μL) was added to a copper grid and dried in a desiccator.

### 2.9. Statistical analysis and data presentation

All experiments were conducted in triplicate, and data are presented as mean ± standard deviation. To determine statistically significant differences between datasets, we applied the student's t-test, with a significance threshold set at $p < 0.05$. All data analysis and figure generation were performed using OriginPro software.

## 3. Results and discussion

The SCS biorefinery configuration described in this study was

designed to maximize the utilization of the major plant cell wall components (glucan, hemicellulose, and lignin) and produce highly valuable molecules (XOS, FAC, and COL), simple chemicals (monosaccharides and bioethanol), colloidal LNPs, and lignin for bioenergy.

A mild alkaline pretreatment was chosen to overcome SCS recalcitrance because it promotes lignin solubilization and the selective breakdown of alkali-labile ester linkages along with monomeric hydroxycinnamates, releasing free phenolics, such as FAC, without degrading carbohydrates. In addition, this pretreatment increases the porosity and surface area of plant cell wall fibers, thereby enhancing enzymatic hydrolysis [22]. Moreover, as supported by prior studies, we selected mild-alkaline pretreatment based on its well-established efficacy in lignocellulosic biomass pretreatment for advancing biorefinery concepts [5].

The high molecular fragments of lignins were recovered by acidification of the alkaline liquor, whereas the remaining soluble fraction was subjected to organic solvent extraction to obtain a concentrated extract rich in FAC, which was composed mainly of hemicellulose derivatives (arabinoxylan). The BL21-pROB strain was used to uptake FAC as a precursor and convert it to COL through a cascade of whole-cell/one-pot biological reactions. In previous studies [23], the XynZ enzyme was used as a biocatalyst to produce XOS from sugarcane bagasse (SCB). Herein, we directly tested the XynZ enzyme activity in both untreated SCS and SCS-A (Fig. 1).

### 3.1. FAC extraction and bioconversion to COL

After subjecting SCS to a mild alkaline treatment (SCS-A), XynZ treatment (SCS-XynZ), and combined treatment (SCS-A-XynZ), the resulting products in the concentrated organic fraction were analyzed for their FAC content (Fig. 2a). The mild-alkaline pretreatment was significantly more effective in releasing FAC from SCS (2.3 ± 0.1 g/kg initial SCS) compared to XynZ-treatment (0.2 ± 0.01 g/kg initial SCS). The combined treatment (mild-alkaline pretreatment followed by XynZ hydrolysis) resulted in a slightly lower yield (0.1 ± 0.01 g/kg ± initial SCS), as most of the FAC was extracted during the first step, and a small amount of ferulates remained in the substrate for XynZ hydrolysis.

The values obtained for the FAC overall extraction yields were 0.23%, 0.01%, and 0.01% (w/w of the original SCS) for SCS-A, SCS-XynZ, and SCS-A-XynZ, respectively, in agreement with previous studies using SCB or other types of grasses as feedstock [5,24–26]. Xu et al. (2005) obtained yields of ~0.4% (w/w) FAC from SCB using a strong alkaline treatment [27]. Later, Mandelli et al. (2014) obtained 0.1% (w/w) of total phenolics from chemically pretreated SCB (peroxide-HAc) after enzyme treatment with XynZ [23]. In this study, it is likely that a substantial portion of the esterified hydroxycinnamic acids, in particular FAC, was not entirely saponified by mild alkali under the conditions tested and remained ester-linked to both the solubilized lignin fraction and the non-solubilized lignin-hemicellulose fraction.

It is worth mentioning that other pretreatment and hydrolysis-related parameters, such as temperature, enzyme dosage, and choice of solvent, may influence the yields from hydroxycinnamic acid extraction. Thus, the methodology proposed in this study can be further optimized to increase FAC production.

The purity of the FAC obtained was 8.2 ± 2.0% for SCS-A, 15.6 ± 3.1% for SCS-XynZ, and 22.3 ± 2.9 for SCS-A-XynZ. The enzymatic step after mild alkaline treatment of SCS favored the purity of the resulting FAC extract once the undesired products were removed during solid-liquid separation in the first alkaline step.

Biotransformation with the pROB strain using TB media supplemented with 5 mM (final concentration) of naturally extracted FAC resulted in 83.7 ± 4.1% and 96.1 ± 2.1% conversion into COL from SCS-A and SCS-XynZ, respectively (Fig. 2a). Further, 1.8 ± 0.1% g and 0.2% ± 0.01% g of COL (derived from 1 kg of SCS) were produced by SCS-A and SCS-XynZ schemes, respectively. The concentrated fraction from SCS-A-XynZ could not be converted into COL because the alkaline step had already extracted most of the FAC. Therefore, under the conditions tested, the SCS-A scheme was the best option for FAC and COL production from the SCS (Fig. 2a).

### 3.2. XOS production and hemicellulose recovery

Fig. 2b shows the amount of XOS (from DP2 to DP5) released after mild alkaline pretreatment (SCS-A) and enzymatic treatment (SCS-XynZ). XynZ alone produced 0.5 ± 0.0 g of total XOS from 1 kg of SCS, whereas combined mild-alkaline treatment and enzymatic hydrolysis increased the amount of XOS released from SCS to 1.7 ± 0.1 g. Thus, the SCS-A-XynZ scheme favored the release of XOS, with a 3.7-fold improvement compared to the enzymatic treatment alone (Fig. 2b). The improved performance of SCS-A-XynZ may result because of the greater access of XynZ to hemicellulose subsequent to partial lignin removal after mild alkaline treatment.

A total of 160 ± 8 g of hemicellulose derivatives was quantified in the aqueous fraction subsequent to organic extraction (Fig. 1) after the mild alkaline pretreatment, indicating that the lignin-carbohydrate complexes were solubilized. The hemicellulose stream can be used for various applications, such as furfural or levulinic acid production [28]. The mild-alkaline pretreatment showed the best results concerning using the hemicellulosic fraction of SCS in biorefinery concepts; furthermore, this pretreatment facilitated XynZ action and improved the XOS production yield (Fig. 2b).

Literature comparisons regarding using xylanases for XOS production are difficult because each study used specific saccharification conditions, varying the substrate, pH, temperature, enzyme type, and loading [11,29]. Xylans from grasses contain substituent compounds as decorations in the polymer main chain; thus, XOS production can require additional accessory enzymes, including α-arabinofuranosidases and acetyl xylan esterases [30].

Nearly 45% of the XOS released from SCS was composed of X2 and X3, representing 80% of the total XOS produced after the SCS-A-XynZ

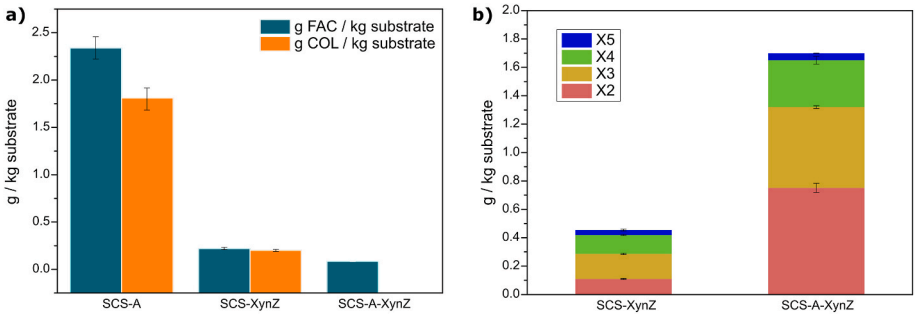


**Fig. 2.** (a) The amount of FAC (g/kg of SCS) obtained after organic extraction and COL (g/kg of SCS) produced after the whole-cell biotransformation using the pROB strain; (b) XOS profile (DP from 2 to 5) produced from the substrates SCS-XynZ and SCS-A-XynZ (2.5%, w/v) after enzymatic treatment with XynZ. Error bars represent the standard deviation (SD) of two biological replicates.

procedure (Fig. 2b). In agreement with previous studies, XynZ hydrolysis after mild alkaline treatment has the advantage of releasing predominantly short and unbranched XOS [23]. In addition, the mild alkaline pretreatment likely promoted partial deacetylation, favoring enzyme activity on xylan and the release of unbranched XOS [31].

Chemical hydrolysis and autohydrolysis generate XOS with high DP (up to 6), and enzymatic hydrolysis produces low-DP XOS (mainly dimers and trimers). Enzymatic hydrolysis is the preferred method for XOS production because chemical hydrolysis can generate undesired and toxic compounds from plant biomass [11]. Low-DP XOS are known for their high prebiotic activity, making them more suitable for pharmaceutical and food applications [11].

### 3.3. Recovery of insoluble lignin aiming energy generation and production of LNPs

An amount of 0.2 ± 0.02 kg lignin and derivatives per kg of SCS was recovered from alkaline black liquor after acid precipitation, and only 0.03 ± 0.01 kg per kg SCS after XynZ-treatment (SCS-XynZ) (see Supplementary Material). Because lignin was mainly extracted during the mild alkaline treatment, the SCS-A-XynZ treatment generated negligible amounts of lignin after acid precipitation (results not shown).

The lignin recovered after alkaline extraction by acid precipitation could be used as a solid biofuel in biorefineries because it generates 22.4 ± 0.02 MJ/kg of lignin (Table 1). Similarly, the enzymatic hydrolysis residue (solid fraction; composed mostly of lignin) generates 19.6 ± 0.02 MJ/kg of lignin. These values are higher than the energy content for untreated SCS (17.6 ± 0.01 MJ/kg) owing to lignin enrichment. Importantly, this energy content is higher than cellulose and hemicellulose [32–34]. The recovery of lignin for supplying heat and power in biorefineries is advantageous and a more profitable alternative than using the untreated substrate.

Other potential applications for valorizing lignin-rich side streams include the production of colloidal LNPs (Fig. 3). Lignin isolated from sugarcane biomass during pretreatment and fractionation is generally available as a pool of irregular and heterogeneous particles with broad molecular weight distribution, severely affecting its potential applications [32–34]. Thus, nanostructured derivatives, such as LNPs with defined morphological features and controllable properties, broaden lignin usability, circumventing a few problems that hinder their industrial utilization, such as low dispersity and stability in aqueous solutions [33].

In this study, the colloidal LNPs obtained by the solvent-shifting method were characterized by TEM (Fig. 3a and b), and the results indicate an average diamenter of 44 nm (Fig. 3c). The hydrodynamic diameter and zeta potential of LNPs were measured by DLS over time (Fig. 3c), indicating an average hydrodynamic diameter of ~130 nm and zeta potential of ~|38| mV in aqueous suspension (pH 7), which is considered highly stable for colloidal systems (Bhattacharjee, 2016). The difference between the hydrodynamic diameter determined by DLS and the values measured by TEM arises because DLS measures the average diameter of the dispersed particles in water, which are hydrated and consequently larger than the samples measured by TEM [35]. Moreover, during the period analyzed (30 days), slight variations in the zeta-potential and hydrodynamic diameter were observed (Fig. 3c).

As depicted in Fig. 3e, in general, the size of the LNPs increased as the pH increased, whereas the zeta potential decreased. At pH 10, the nanoparticles exhibited a more negative zeta-potential (−45 mV) and increased to nearly 230 nm in size, indicating LNP destabilization [36]. The nanoparticles were remarkably stable from pH 4 to 5, as the size and zeta potential exhibited no apparent changes. pH plays an important role in the particle size of the synthesized LNPs and is an important parameter that dictates its applicability.

**Table 1**
The energy content of the untreated SCS, saccharified SCS and recovered lignin from black liquor after alkaline mild pretreatment.

| Biomass | Energy content (MJ/kg) |
|---|---|
| Untreated SCS | 17.6 ± 0.01 |
| Saccharified | 19.6 ± 0.02 |
| Recovered Lignin | 22.4 ± 0.02 |

### 3.4. Enzymatic hydrolysis of cellulosic solid residues to produce fermentable sugars and ethanol

The digestibility of SCS obtained before and after the proposed treatments (mild alkaline, XynZ, and the combination thereof) and the amount of ethanol produced after fermentation was assessed (Fig. 4). Approximately 52 ± 8 g of glucose was released from 1 kg of untreated SCS after saccharification, compared to 437 ± 2 g and 385 ± 7 g from SCS-A and SCS-A-XynZ, respectively. As expected, SCS-A-XynZ increased the glucose mass released after saccharification up to 8-fold compared with the saccharification of the untreated SCS. This increase indicated that the delignification of XOS and FAC via complementary hydrolysis promoted beneficial glucan exposure, facilitating feedstock saccharification (details in the next section). XynZ treatment applied directly to untreated SCS also increased glucose release, but the results were worse than those of SCS-A and SCS-A-XynZ (Fig. 4).

The amounts of xylose and arabinose produced from SCS-A after saccharification were higher than those of SCS-XynZ and SCS-A-XynZ. The total amount of these pentose sugars was lower than that of glucose produced from all samples (Fig. 4). Therefore, these data suggest that the remaining hemicellulose in SCS-A was more susceptible to hemicellulases present in the Cellic® CTec2 enzyme cocktail than in SCS-XynZ and SCS-A-XynZ samples, perhaps due to the enhanced enzyme accessibility to the substrate surface during hydrolysis.

Regarding ethanol production, fermentation reached the maximum concentration after 6 h compared to the 24 h assay, and all the sugars were consumed after 6h (data not shown). The ethanol concentration was similar to that of enzymatic hydrolysis, in which SCS-A-XynZ presented a higher ethanol yield (146 ± 1 g/kg of SCS), followed by SCS-A (108 ± 2 g/kg of SCS) and SCS-XynZ (93 ± 3 g/kg of SCS). The fermentation of the SCS hydrolysate was not feasible because of the low sugar yields (Fig. 5; see Supplementary Material).

While SCS-A-XynZ yielded a reasonable conversion rate of 78.5% (w/w) ethanol production compared to the theoretical value of the sugar conversion in the hydrolysate. SCS and SCS-XynZ achieved lower sugar concentrations in the hydrolysate and, therefore, lower ethanol titles. In contrast, SCS-A, despite its high yields of sugars, achieved ethanol yield that remained below our expectations (Fig. 4) [37]. Fermentation inhibitors generated during alkaline pretreatment might have influenced this discrepancy, especially for SCS-A, which was not exhaustively washed before the tests [38]. Additionally, the yeast's metabolic shift towards producing cell biomass, glycerol, and acetic acid was not accounted for in this study, which could be another plausible explanation.

### 3.5. Morphological characterization after mild-alkaline pretreatment and XynZ treatment

The co-product yields produced by enzymatic action can also be explained by morphological evaluation (see Supplementary Material). SEM images indicated that untreated SCS showed cellulose fibers covered by parenchymal tissue composed mainly of hemicellulose, lignin, and non-polar waxes, which hindered XynZ and cellulase action from producing XOS and fermentable sugars. After mild alkaline pretreatment (SCS-A), fibers were exposed mainly because of lignin removal, including some fibers detached from the substrate.

Treatment with XynZ applied to SCS (SCS-XynZ) did not significantly change the fiber morphology compared to SCS, which explains the lower yields of XOS and fermentable sugars (see Supplementary Material).

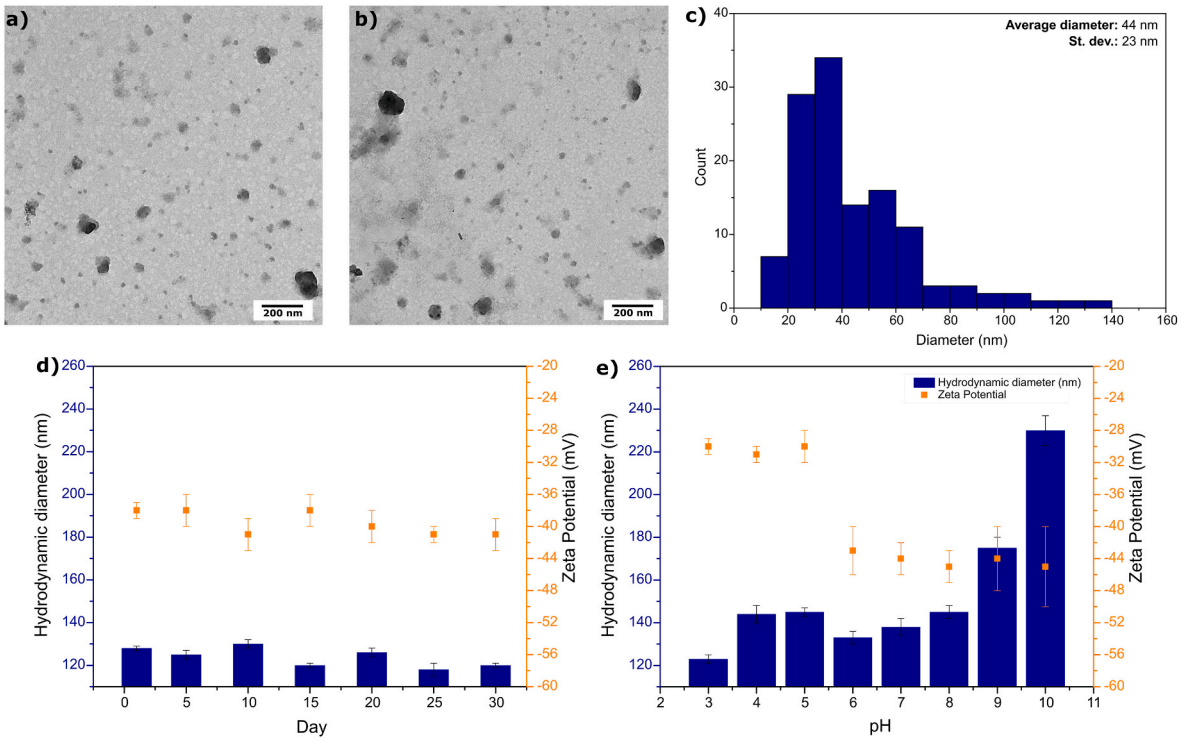


**Fig. 3.** (a–b) TEM images of LNPs; (c) histogram of LNP length distribution; and LNP hydrodynamic diameter and zeta potential by changing (d) time (analysis performed at pH 7); and (e) pH.

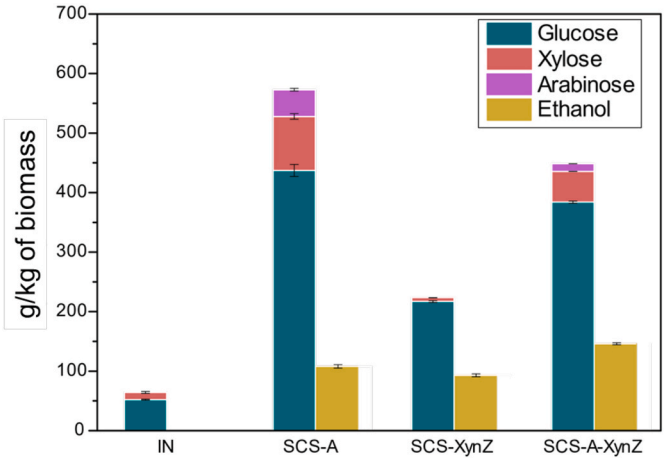


**Fig. 4.** Monosaccharides and ethanol quantification after saccharification and fermentation of SCS. Error bars represent the standard deviation (SD) of two biological replicates.

However, XynZ treatment applied to the alkaline-treated substrate (SCS-A-XynZ) resulted in better yields of XOS, partly because the fibers were more exposed after the mild alkaline pretreatment (see Supplementary Material). Although FAC and a part of hemicellulose were extracted due to mild alkaline pretreatment, the substrate was more suitable for XynZ and cellulase action. This observation corroborates with the higher XOS yield obtained for the SCS-A-XynZ sample. Accordingly, the saccharification results were also related to the morphological changes observed. Similar to XynZ, cellulases have more difficulty accessing cellulose in non-treated substrates because of the native coverage of the fibers.

### 3.6. Biorefinery layout proposal

Based on the insights gained from our research, we propose a biorefinery layout designed to produce FAC, COL, XOS, lignin (including nanoparticles), and fermentable sugars from SCS. Our approach combines mild-alkaline treatment with XynZ enzyme treatment, demonstrating the highest yield across the targeted products. The pathway depicted in Fig. 5 outlines the processing of one metric ton (DM) of SCS. For a more in-depth analysis of the alternative routes explored during our research, detailed mass balances are available in the Supplementary Material.

In the proposed biorefinery scheme (Fig. 5), up to 85% of the original components of the SCS plant cell wall, including glucan, hemicellulose, and lignin content, were converted into bioproducts through sequential fractionation and conversion steps. The mild-alkaline pretreatment enabled efficient sugar release (up to 15-fold) compared to the untreated SCS and generated a phenolic extract containing FAC. The subsequent XynZ treatment of the biomass-derived mild-alkaline process improved the XOS yield, which showed a 3.7-fold increase compared to using XynZ alone. By directing the SCS-A-XynZ cellulosic pulp through saccharification and fermentation processes, we observed a significant enhancement in ethanol productivity compared to the SCS-A route, a remarkable 34.9 ± 2.5% increase in fermentation yields.

As shown in Fig. 5, the chemical compositional analysis indicated that the majority of the lignin, ash, and extractive content of SCS were solubilized, and a glucan-rich pulp was obtained (66 ± 5% w/w) by the combination of mild-alkaline followed by XynZ treatment. In this biorefinery layout, it was possible to recover 170 ± 20 kg of lignin fragments per ton of SCS material. The recovered lignin was used not only as a solid biofuel for the cogeneration of heat and power (CHP) units owing to its high energy content (22.4 ± 0.1 MJ $kg^{-1}$) but also as a platform to obtain LNPs, which are a value-added product with several applications [39]. Moreover, 58% (160 kg per ton of SCS) of the hemicellulose content of SCS was solubilized by the combination of mild-alkaline followed by XynZ treatment. According to previous studies, the hemicellulose content could be further processed in anaerobic digesters for biogas generation, which may be valuable for the biorefinery concept [40].

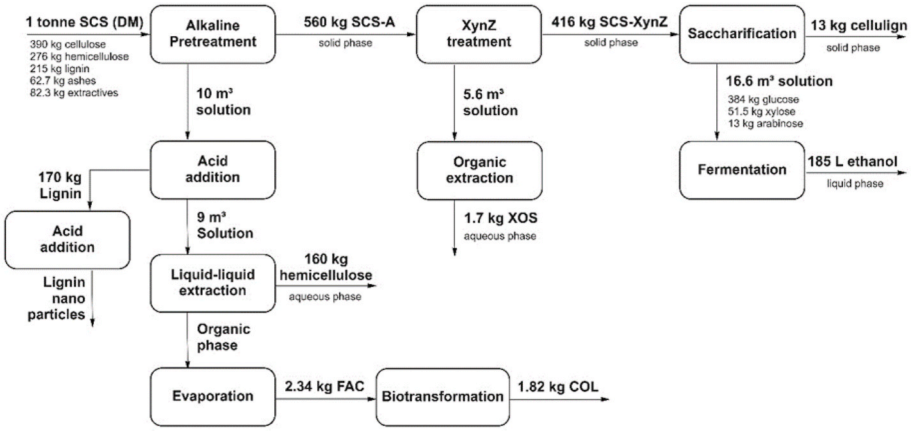


**Fig. 5.** Overall process operations (represented by rounded boxes; the value is the average value obtained) and mass balance of the derived streams (indicated by arrows) are obtained as the best scenario among the biorefinery strategies employed in this work.

From an industrial standpoint, one of the main drawbacks of alkaline pretreatments includes the solubilization of non-sugar biomass constituents, such as ash, proteins, metabolites, and waxes [22], which alter the physical properties of the liquid phase with economic and operational consequences. However, this work demonstrated that acid precipitation and organic solvent extraction could drastically reduce the number of impurities, producing a FAC-enriched extract, which can be further purified via membrane separation or liquid-liquid extraction [41]. The liquid separation may be the best candidate to investigate and optimize in future studies. Depending on the applied solvent, the solvents used in the liquid-liquid operation unit can be readily recovered from the mixtures by distillation [42].

The combination of mild-alkaline followed by XynZ treatment yielded 0.6 ± 0.0% of the original hemicellulose as XOS (1.7 ± 0.1 kg), with a wide range of DP, as a liquid stream. The resulting solid substrate was readily converted into fermentable sugars after a saccharification step, which released 88.6% (364 ± 8 kg) of the glucose found in the original SCS (equivalent to 390 ± 10 kg of cellulose). The subsequent fermentation yielded 78.5% ethanol (146 ± 1 kg) compared to the theoretical value, which is considered reasonable compared to the literature [37]. However, the obtained ethanol titer of 1.5% (w/w) would not permit economically feasible fermentation because the rule of thumb requires a titer of at least 4% [43]. This difference can be addressed by increasing the solid loading in the previous saccharification step, offering the advantage of decreasing the heating utility demands for the operation. A lignin-rich stream (170 ± 20 kg) was also obtained, which could be used as a fuel complement (19.6 ± 0.1 MJ/kg) in a CHP unit.

Although XynZ treatment is a straightforward strategy for FAC release from biomass and is considered environmentally friendly, it resulted in lower FAC and XOS yields compared to alkaline treatment. Furthermore, the combination of mild-alkaline treatment followed by XynZ treatment improved the overall saccharification and fermentation yield compared to other scenarios tested in this study. Additionally, the cost of XynZ production could jeopardize the economic feasibility of the biorefinery scheme proposed in this study.

In this study, by implementing lignin precipitation operations and FAC solvent extraction methods, similar biocatalytic conversion rates were achieved compared to previous studies that used commercial FAC from Sigma, which is produced from fossil fuels [5]. Moreover, the pROB strain was robust against inhibitors that may be present in the SCS hydrolysate obtained after mild alkaline treatment. This may be due to the presence of CgAKR-1, which is a key biocatalytic element in the pROB strain. CgAKR-1 belongs to a class of enzymes proven to protect microorganisms against fermentation inhibitors such as coumaric acid, vanillic acid, and (hydroxymethyl) furfural [44].

It is essential to acknowledge that our research does have a limitation related to the significant amount of water used, which goes against sustainability principles. Thus, in future iterations, exploring methods for recycling and reusing process water to address lower water usage would be beneficial. Additionally, adjustments to the process should be considered to incorporate higher solids loadings, which can help alleviate the amount of water used and improve the overall sustainability of the system. In future works, we expect to conduct a comprehensive life cycle analysis to thoroughly understand the environmental impacts associated with the process.

Moreover, sugar and phenolics loss occurred at various stages during the employed pretreatments and hydrolysis steps due to incomplete conversion of polysaccharides and ferulate esters into biochemicals. However, these losses have been incorporated into the process model, reflected in the calculated yields. It is important to emphasize the importance of adopting a balanced approach that integrates economic viability and sustainability. Such harmonization is essential for the successful development and operation of lignocellulosic biorefineries. In this sense, future research to augment the economic attractiveness of an integrated SCS biorefinery should focus on 1) utilizing biomass with a richer FAC content, such as rice bran and corn husk; 2) improving the efficiency of FAC extraction, preferably by greener and cost-effective methods such as tuning a low-cost feruloyl esterase enzyme source; and 3) optimizing the FAC purification steps by applying advanced partition methodologies. 4) investigating options to reduce water consumption in the process, thus mitigating the environmental impact.

## 4. Conclusion

In this research, we have proposed a comprehensive, integrated biorefinery design for the complete valorization of sugarcane straw (SCS), including its glucan, hemicellulose, and lignin constituents. Our approach has successfully converted up to 85% (w/w) of these components into various bioproducts. The mild-alkaline pretreatment effectively released the FAC and lignin from the SCS, which created a biomass fraction favorable to the enzymatic production of XOS and produced a FAC-enriched hydrolysate that served for COL biosynthesis, lignin-based biofuel or lignin nanoparticles (LNPs). Incorporating these steps with traditional saccharification and fermentation processes allowed sugar release and ethanol production from SCS. While our research presents certain limitations regarding process optimization, water utilization, and lifecycle analysis, it nonetheless provides a substantial foundation for future endeavors to develop integrated biorefinery concepts from SCS. These future initiatives will focus on the efficient transformation of lignocellulosic material into value-added bioproducts, all the while promoting sustainable practices. This study, thus, demonstrates the broad spectrum of possibilities and their potential for advancing a

greener and more sustainable industrial production of chemicals, fuels, and food products.

## CRediT authorship contribution statement

**Robson Tramontina:** Investigation, Validation, Writing - original draft, Writing - review and editing, Visualization, Project administration. **Eupídio Scopel:** Validation, Investigation, Writing - original draft, Writing - review and editing, Visualization. **Lívia Brenelli:** Validation, Writing - review and editing, Visualization. **Guilherme P. Nogueira:** Validation. **Telma T. Franco:** Review, editing, and funding acquisition. **Camila A. Rezende:** conceptualization, validation, and funding acquisition. **Rosana Goldbeck:** Conceptualization, validation, and funding acquisition. **Fabio M. Squina:** Validation, Writing-review & editing, Supervision, Funding acquisition.

## Funding

This work was supported by the Fundação de Amparo à Pesquisa no Estado de São Paulo - FAPESP contract numbers 2021/04254-3 and 2021/04254-3 (RT); 2015/50590-4, 2020/05784-3 and 2022/08958-8 (FMS); 2019/19360-3 (ES); 2018/23769-1 (CAR); 2017/15477-8 (LB); 2015/50612-8 (TTF) and 2019/08542–2 (RG). FMS and RG thank CNPq productivity grants 306279/2020-7 and 307014/2020-7, respectively.

## Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this study.

## Data availability

No data was used for the research described in the article.

## Acknowledgments

INCT-INOMAT for TEM analysis.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2023.106972.

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