Sustainable Energy & Fuels

Sustainable strategies to achieve industrial ethanol titers from different bioenergy feedstocks: Scale-up approach for better ethanol yield

Journal:	*Sustainable Energy & Fuels*
Manuscript ID	SE-ART-04-2024-000520.R1
Article Type:	Paper
Date Submitted by the Author:	11-Jun-2024
Complete List of Authors:	Deshavath, Narendra Naik; University of Illinois at Urbana-Champaign, Agricultural and Biological Engineering Woodruff, William; University of Illinois at Urbana-Champaign, Department of Chemical and Biomolecular Engineering Singh, Vijay; University of Illinois at Urbana-Champaign, Agricultural and Biological Engineering

Data Availability Statement: The data generated for this article are provided in the manuscript and the Supplementary Information. Raw data for figures and graphs will be available at CABBI at https://cabbi.bio/datasets/.

Sustainable strategies to achieve industrial ethanol titers from different bioenergy feedstocks: Scale-up approach for better ethanol yield

Narendra Naik Deshavath,[1,2], William Woodruff,[2,3] Vijay Singh[1,2]*

[1]Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

[2]DOE Center for Advanced Bioenergy and Bioproducts Innovation, University of Illinois Urbana-Champaign

[3]Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

***Corresponding author**

Prof. Vijay Singh

E-mail: vsingh@illinois.edu

Tel: +1 (217) 333-9510

Abstract

Hydrothermal pretreatment is a promising approach to lignocellulosic biomass processing for enzymatic hydrolysis and high-yield bioethanol fermentation, as it reduces downstream inhibitor content and the amount of toxic byproducts generated. In this study, the ethanol yield and productivity of an engineered xylose-fermenting strain of *Saccharomyces cerevisiae* were tested on lignocellulosic hydrolysates produced with varying citrate buffer concentration, solid loading, supplemental nitrogen source, and feedstock of origin, and a semi-integrated bioprocess which integrates enzymatic hydrolysis and bioethanol fermentation was developed. The greatest ethanol yield (g_p/g_s) of 0.490±0.008, 0.460±0.001, 0.420±0.002 and 0.410±0.002 were obtained from bioenergy sorghum (BES), *miscanthus* × *giganteus* (MG), energy cane (EC), and oilcane (OC), respectively. In addition, an equivalent of 291 L, 253.54 L, 257.8 L, and 260.3 L of bioethanol were produced per ton of BES, MG, EC, and OC, respectively, by using urea as a nitrogen source in a bioreactor.

Keywords: Hydrothermal pretreatment, lignocellulosic feedstocks, Fermentation, Bioethanol, integrated bioprocessing

1. Introduction

The use of bioethanol produced from lignocellulosic biomass in the transportation sector can reduce carbon emissions and dependence on fossil fuels. However, the cost of enzymes needed for processing lignocellulosic biomass, incomplete hydrolysis of polymeric sugars, and the presence of fermentative inhibitors in the resulting substrate have limited the commercialization of this biorefinery approach.[1] Fermentative inhibitors deter the fermentability of hydrolysates and reduce ethanol yield and productivity.[1] Generally, fermentative inhibitors can be classified into three categories: process-derived, inherent, and supplemented. Process-derived fermentative inhibitors include furfural, 5-hydroxymethylfurfural (5-HMF), levulinic acid, and formic acid. These are formed during acidic and high-temperature pretreatment of lignocellulosic biomass by the breakdown of hexose and pentose sugars.[2] Apart from these, acetic acid, glucuronic acid, ferulic acid, vanillic acid, coumaric acid, benzoic acid, hydroxybenzoic acid, syringaldehyde, etc., are the inherent inhibitors derived during the hydrolysis of lignocellulosic biomass.[2–4] Acetic acid, glucuronic acid, and ferulic acid are the structural constituents of hemicellulose, which are linked with the xylan backbone.[5] Moreover, vanillic acid, coumaric acid, syringaldehyde, etc., are the structural components of lignin.[6] Finally, citrate buffer and sulfate ions are the unavoidable supplemented inhibitors used during the enzymatic hydrolysis of pretreated lignocellulosic biomass. Generally, 50 mM sodium citrate buffer strength is used to maintain the pH of the enzymatic hydrolysis medium between 4.8 and 5.5 [7]; however, it deters microbial metabolic growth in the subsequent fermentation of hydrolysates.[8,9]

Several pretreatment technologies were investigated to deconstruct the complex network of lignocellulosic biomass. Dilute sulfuric acid and alkali are the most widely used inorganic

catalytic agents for the pretreatment of lignocellulosic biomass. Dilute sulfuric acid pretreatment hydrolyzes most hemicellulose fraction and produces pentose sugars but it also forms sugar decomposition products. [10,11] In contrast, alkaline pretreatment dignifies the lignocellulosic biomass, and enhances the cellulose digestibility in the subsequent enzymatic hydrolysis, but it eliminates most of the hemicellulose fraction along with the minor fraction of cellulose. This eventually hampers the overall ethanol yield per ton of lignocellulosic biomass.[12] Moreover, before the enzymatic hydrolysis, considerable quantities of water are required to remove residual acid or base from the pretreated biomass.[13] Several studies also performed enzymatic hydrolysis of dilute sulfuric acid pretreated biomass by directly adjusting the pH at 4.8 to 5.5 with ammonium hydroxide instead of washing.[14] However, it forms sulfate ions that deter microbial metabolic growth, leading to low ethanol yield and productivity.[15] In addition, high levels of ammonium salts in the stillage required a specialized wastewater treatment section in the downstream processing.[14]

Hydrothermal pretreatment methods are an alternative approach that avoids the addition of costly chemical catalysts that complicate downstream processing while still permitting enzymatic access to hemicellulose and cellulose.[17] The incorporation of a downstream disc milling step increases sugar yields, and the effectiveness of this combined approach has been demonstrated and scaled up for bioenergy sorghum processing in a continuous steam explosion reactor.[18-19] The resulting hydrolysate is high in sugars and low in process-derived fermentative inhibitors, and additional process optimization has reduced the required concentration of citrate buffer.[20-21]

This study describes the development and scale-up of a semi-integrated enzymatic hydrolysis and bioethanol fermentation process for pilot-scale continuous hydrothermally

pretreated bioenergy sorghum, *Miscanthus giganteus*, energycane, and oilcane. Through systematic variation of solid loading, buffer concentration, and process conditions, the effect of these parameters on sugar utilization and final ethanol titer and yields was determined.

2. Materials and methods

2.1. Feedstock and its processes

Bioenergy sorghum (BES), *Miscanthus giganteus* (MG), energycane (EC), and oilcane (OC) were used as feedstocks for lignocellulosic biomass production. Bioenergy sorghum and *Miscanthus* were collected from the Energy Farm (Latitude 40.06604° N, Longitude: 88.20836° W) of the University of Illinois at Urbana-Champaign. Energycane and oilcane were collected from the experimental fields (Latitude: 29.40879° N, Longitude: 82.17119° W) of the University of Florida, Gainesville. EC and OC were first processed to extract the juice, and the residue was washed, dried, and hammer milled to reduce the particle size to 1-3 cm, while BES and MG were directly processed via hammer mill (Schutte-Buffalo Industrial Hammer Mill, W-8-H, Buffalo, NY). Samples were collected from OC and EC for compositional analysis using the NREL protocol.[16] The compositions of BES and MG composition have been previously reported.[17,18]

2.2. A pilot-scale continuous hydrothermal pretreatment process

25 kg each of BES, MG, EC, and OC biomass were pretreated in a pilot-scale continuous hydrothermal reactor (AdvanceBio SüPR•2G Hydrolyzer System., Milford, OH, USA). The moisture content of the lignocellulosic feedstocks was first adjusted to 50% (w/w) using a ribbon blender. BES, EC, and OC were pretreated at 190℃ and 10 bar for 10 min, while MG was pretreated at 170℃ and 7.5 bar for 10 min, which was chosen to avoid degradation of anthocyanin, a natural pigment valuable to the cosmetics industry.[18] The pretreated biomass was

dried overnight in a conventional tray drier at 46±3℃ and mechanically refined using a burr mill (Quaker City Grinding Mills, Pottstown, PA; Model 4E) to further reduce the particle size. Compositional analysis of hydrothermally pretreated biomass was performed according to a modified NREL procedure.[19]

2.3. Enzymatic hydrolysis

Fed-batch enzymatic hydrolysis of hydrothermally pretreated biomass was conducted in 500 mL screw cap conical flasks containing 50 g of dry solids per 100 mL of liquid medium, for a 50% (w/v) solid loading. Samples from each feedstock were hydrolyzed using either 50 mM citrate buffer or distilled water as the liquid medium. Initially, 20% (w/v) of solids were loaded in the flask, and additional solids were added to increase the loading to 30%, 40%, and 50% at 6 h, 24 h, and 36 h, respectively. A single dose of cellulase NS22257 (60 mg protein/g cellulose) and hemicellulase NS22244 (20 mg protein/g xylan) (Novozymes North America, Inc., Franklinton, NC, U.S.A.) was added at 20% solid loading. 4 mL of 25% PEG 4000 was added to the hydrolysis medium at 30% solid loading to improve the enzyme-substrate interaction. Aliquots were collected from the reaction mixture (Thermo Scientific, Sorvall Legend Micro 17, Centrifuge) before each addition of 10% solids for sugar quantification, and the sample collected at 30% solid loading was used for GC-MS analysis. The enzymatic hydrolysis reaction was conducted at 50℃ and 185 RPM (Eppendorf, New Brunswick ™, Innova ® 44 Incubator shaker) for 72 h. The percentage of cellulose and xylan hydrolysis were calculated by the following equation (**Eq. 1**):

$$Hyd_{eff.}(\%) = \left[\frac{(C_{sug.} - E_{sug.}) \times (V_L \times CF)}{(S_C \times S_L)} \right] \times 100 \qquad \text{Eq. 1}$$

Where $Hyd_{eff.}$ is the hydrolysis efficiency of cellulose and xylan; $E_{sug.}$ (g/L) is the concentration of sugars in the enzyme blank; $C_{sug.}$ (g/L) is the concentration of cellobiose and glucose or xylose in the hydrolysis medium; V_L is the liquid volume of enzymatic hydrolysis medium; *CF* is the conversion factor (1.10) to account for relative change in volume of hydrolysis medium. S_C (g/g) is the structural carbohydrate (cellulose or xylan) content of pretreated biomass, and S_L is the percentage of solid loading during the enzymatic hydrolysis.

2.4. Fermentation

2.4.1. Microorganism and seed culture preparation

A commercial genetically modified xylose-fermenting *Saccharomyces cerevisiae* strain was used for bioethanol production from hydrolysates.[20] Seed cultures were prepared in screw cap conical flasks containing 25 mL of YPDX medium (10 g/L yeast extract, 20 g/L peptone, 12 g/L glucose, and 8 g/L xylose; the pH was adjusted to 5.6). After inoculation, the cells were grown at 30℃ and 140 RPM (Eppendorf, New Brunswick ™, Innova ® 44 Incubator shaker) for 18 h.[24] Microbial cells were harvested by centrifugation at 8000 RPM (Eppendorf, Centrifuge 5084 R 15 amp version) for 10 min. The pellet was washed with distilled water and resuspended to measure the optical density at 600 nm. 2 mL of seed culture medium at 18 h was used as the inoculum for 100 mL of fermentation medium, for an initial OD_{600}~0.1, which is equivalent to a cell concentration of 3.4 mg/100 mL.

2.4.2. Hydrolysate fermentation

The enzymatic hydrolysates were processed via centrifugation at 8600 RPM for 15 min (Eppendorf, Centrifuge 5084 R 15 amp version) and divided into two equal fractions before the preparation of the fermentation media. The first fraction was supplemented with YP (to a final

concentration of 10 g/L yeast extract and 20 g/L peptone), and the second fraction was supplemented with 4 g/L of urea as a nitrogen source, and each was titrated to a pH of 5.8 with 5N NaOH before being filter sterilized with a 0.2 μm membrane filter. Bioethanol fermentation was conducted in 250 mL screw cap conical flasks containing 100 mL of media. 2 mL of seed culture was added to inoculate the cultures, and they were incubated at 30℃ and 160 RPM agitation for 144 h. Samples of the fermentation medium were collected periodically for HPLC analysis to quantify sugar and ethanol concentrations. Ethanol yield and fermentation efficiencies (F_{effi}) were calculated according to the following equations (**Eq. 2** and **Eq. 3**):

$$Ethanol\ yield\ (g_p / g_s) = \left[\frac{Ethanol\ titer\ (g/L)}{Initial\ suagrs\ (g/L) - residual\ sugar(g/L)} \right] \quad \text{Eq. 2}$$

$$F_{effi}(\%) = \left(\frac{Empirical\ ethanol\ (L)}{Theoretical\ ethanol\ (L)} \right) \times 100 \quad \text{Eq. 3}$$

2.4.3. Semi-integrated bioprocess

For semi-integrated bioprocess (SIB) testing, enzymatic hydrolysis of pretreated biomass and fermentation were carried out in the same vessel without separation of the enzymatic slurry. A preliminary study was conducted in 500 mL screw cap conical flasks containing 100 mL of liquid medium with a 25% (w/v) solid loading in batch mode without citrate buffer. Cellulase NS22257 and hemicellulase NS22244 were loaded into the reaction mixture at 60 mg protein/ cellulose and 20 mg protein/ xylan, respectively. Hydrolysis was performed at 50℃ and 185 RPM for 10 h. The pH was adjusted to 5.8 using 5N NaOH, and the enzymatic slurry was agitated for 2 h at 185 RPM. 0.4 g of urea was added prior to inoculation with 2 mL of seed culture, which gives an initial cell concentration of 3.4 mg/100 mL.

Subsequent tests of this process were performed in a 1 L bioreactor (Sartorius stedim biotech, BIOTAT® Qplus with BioPAT® DCU Tower) with a 500 mL working volume. The sparger and baffles were removed from the reactor vessel to avoid clogging and to facilitate the better mixing of the enzymatic slurry. Hydrolysis was conducted at 50℃ and 500 RPM for 10 h in fed-batch mode, with 50 g dry solids added at 0 h, 3 h, and 6 h (25 g was added for OC instead at this point) for an overall solid loading of 30% (w/v) for BES, MG, and EC, and 25% (w/v) for OC. A single dose of cellulase NS22257 (60 mg protein/g cellulose) and hemicellulase NS22244 (20 mg protein/g xylan) were added to the bioreactor before adding the pretreated biomass. After hydrolysis, the bioreactor was cooled to 30℃ and at 12 h, 2 g of urea was added, and the pH of the enzymatic slurry was adjusted to 5.8 using 5N NaOH prior to inoculation with 10 mL of seed culture, which gives an initial cell concentration of 17 mg/500 mL.

Adjusting the pH with 5N NaOH and addition of inoculum to the fermentation medium dilutes the sugar concentrations, which can be calculated with the following equations (**Eq. 4** and **Eq. 5**):

$$CF = \frac{V_{EH}}{V_{FM}} \quad \text{Eq. 4}$$

$$C_{sug.} = EH_{sug.} \times CF \quad \text{Eq. 5}$$

Where CF is the conversion factor, V_{EH} is the liquid volume of enzymatic hydrolysis medium, V_{FM} is the liquid volume of fermentation medium after adjusting the pH and adding the inoculum. $C_{sug.}$ is sugar concentration in semi-integrated bioprocess, $EH_{sug.}$ is the sugar release (g/L) at a particular solid loading.

2.5. Analytical methods

2.5.1. High performance liquid chromatography

Cellobiose, glucose, xylose, arabinose, formic acid, acetic acid, 5-HMF, furfural and ethanol were quantified using a Water HPLC system (Waters e2695 Separation Module, Waters Corporation, Milford, MA, USA) equipped with a 2414 refractive index (RI) detector (maintained at 30℃), and an Aminex HPX-87H column (300 × 7.8 mm, 9 μm particle size; Bio-Rad Laboratories, Hercules, CA, USA) was used for separation of compounds at 65℃ and a 0.6 mL/min flow rate with 5 mM H_2SO_4 as the mobile phase.

2.5.2. Gas chromatography-Mass spectroscopy

Each 200 μL hydrolysate sample was acidified to a pH of 2 with concentrated (37.4%) HCl and extracted twice with 1 mL ethyl ether. The organic phase was evaporated under nitrogen and methylated by adding 100 μL of methoxyamine hydrochloride (40 mg/mL in pyridine) and heating at 50℃ for 90 min, then adding 100 μL of *N-Methyl-N-(trimethylsilyl)trifluoroacetamide* (MSTFA) and heating at 50℃ for 120 min. Hentriacontanoic acid (1 mg/mL) was used as an internal standard and 30 μL was added prior to derivatization. Samples were analyzed on an Agilent 7890 gas chromatograph with an Agilent 5975 mass selective detector and HP 7683B autosampler (Agilent Inc, Palo Alto, CA, USA). A ZB-5MS (60m×0.32mm I.D. and 0.25μm film thickness) capillary column (Phenomenex, CA, USA) was used, the inlet and MS interface temperatures were held at 250℃, the ion source temperature was held 230℃, and the helium carrier gas flow rate was held at 2.4 mL/min. 1 μL of each sample was injected with a 10:1 split ratio, held for 5 min at 70℃, heated to 310℃ at a rate of 5℃/min, and held for 10 min at 310℃. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in 30-800 m/z scan range. MS peaks were evaluated using the AMDIS 2.71 (NIST,

Gaithersburg, MD, USA) program and metabolites were identified with reference to a custom-built library (484 unique metabolites). To allow comparison between samples, all data were normalized to the internal standard in each chromatogram and the sample dry weight.

2.5.3. Statistical analysis

Separate enzymatic and fermentation (SHF) experiments were conducted in triplicates. Semi-integrated enzymatic hydrolysis and fermentation were conducted in duplicate. Mean and standard deviations were reported. Tukey's test was performed to check the sugar yield similarities between citrate buffer and distilled water as a medium for enzymatic hydrolysis. Regression analysis of sugar yields was performed to predict the actual sugar yield at 25% (w/v) and 30% (w/v) solid loading during the semi-integrated enzymatic hydrolysis and fermentation. Tukey's test and regression analysis were conducted using Origin Pro software (OriginPro, Version 2023. OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Effect of hydrothermal pretreatment on lignocellulosic feedstocks

Hydrothermal pretreatment combined with disc milling is proven to be the most effective way to deconstruct the conglomerate structure of lignocellulosic biomass without greatly impacting the overall composition of structural carbohydrates.[17] After hydrothermal pretreatment, the compositional analysis of the MG, EC, and OC feedstocks identified higher cellulose and xylan contents with a lower fraction of acid-insoluble lignin (**Table 1**). In contrast, a slight decrease in xylan and an increase in acid-insoluble residue was observed in BES. Like all other feedstocks, the cellulose content slightly increased in BES **(Table 1)**. The mean cellulose contents of MG, BES, and OC were not significantly different as determined by Tukey's test

($p<0.05$), however the cellulose content of pretreated EC was higher (Tukey's significant difference level $p=0.05$). The difference in xylan content between MG and EC was not statistically significant ($p<0.05$). Compared to the EC and MG, a higher xylan content was observed in OC, whereas a lower xylan content was observed in BES. However, the overall structural carbohydrate composition (cellulose and xylan) of MG (63.65±1.06%), EC (65.92±0.41%), and OC (64.20±1.35%) were not significantly different ($p\leq0.05$). Overall structural carbohydrate content of BES (57.04±0.13%) was significantly different from that of MG, EC, and OC feedstocks. The acid-soluble lignin fraction was not reported due to interference with reactive furfural and 5-HMF at 240 nm in UV-Vis Spectroscopy. The small change in composition may reflect evaporation and the solubilization of lignin derivatives during pretreatment. These results indicate that hydrothermal pretreatment is an effective method to deconstruct the structure of lignocellulosic biomass irrespective of feedstock origin.

3.2. Enzymatic hydrolysis

Reduced citrate buffer concentrations (5 mM and 0.5 mM) were previously investigated on enzymatic hydrolysis of hydrothermally pretreated bioenergy sorghum and showed similar sugar yields compared to standard citrate buffer (50 mM).[19] The pH of distilled water with 20% (w/v) solid loading of hydrothermally pretreated feedstocks was between 5.2 and 5.8 for all samples, permitting enzymatic hydrolysis. Therefore, fed-batch enzymatic hydrolysis was conducted in distilled water without using citrate buffer for hydrothermally pretreated feedstocks. The resulting cellulose and xylan hydrolysis efficiencies and their corresponding sugar yields were compared with standard citrate buffer (50 mM). According to Tukey's test, no significant difference in sugar yield between the samples hydrolyzed in distilled water as opposed to citrate buffer was observed (**Fig. 1**). The highest sugar yields were observed for OC,

followed by EC, BES, and MG (**Fig. 1**). Yield from MG may have been affected by lowering the pretreatment temperature (170℃), however previous studies report that anthocyanin degradation can occur when the pretreatment temperature increases beyond 170℃.[18] As anthocyanins are emerging value-added compounds that can be used as an eco-friendly alternative to synthetic dyes, their presence as a co-product may increase the economic viability of these biomass conversion processes.[18]

As the solid loading increased from 20% to 50%, the extent of cellulose and xylan hydrolysis decreased (**Fig. S1**). For instance, cellulose hydrolysis decreased to 78.50±0.14% from 98.70±0.41%, and xylan hydrolysis decreased to 88.21±0.19% from 93.69±0.41% by increasing the solid loading of pretreated OC from 20 to 50% (w/v) (**Fig. S1**). The hydrolysis efficiencies of cellulose and xylan were calculated according to **Eq. 1**. This is possibly due to mass transfer limitations and competitive inhibition of the enzymes by lignin derivatives.[4,21,22] Most of the sugars (75.36±1.14 to 233.21±1.81 g/L) were released between 6 h and 48 h, with only a minimal increase in sugar concentration between 48 h and 72 h (**Fig. S2**). As a result, 48 h was identified as the optimal time for harvesting hydrolysate from the enzymatic slurry. The highest sugar concentrations of 195.52±0.77 g/L, 224.41±0.70 g/L, 242.20±1.48 g/L and 254.49±3.85 g/L were achieved at 50% (w/v) solid loading of MG, BES, EC and OC, in either 50 mM citrate buffer or distilled water, indicating that citrate buffer is not necessary for pH control during enzymatic hydrolysis. In the case of OC enzymatic hydrolysis at 50% solid loading, the pH of the hydrolysate was reduced to 4.02 in the citrate buffer condition and 3.31 in the distilled water condition. The use of 50 mM citrate buffer did not restrict the pH change of the enzymatic hydrolysis medium. The intrinsic components of lignocellulosic biomass, such as organic acids and aromatics, were released upon hydrolysis and reduced the media pH. For

instance, an increment of acetic acid concentration during fed-batch enzymatic hydrolysis could be good evidence for pH reduction (**Fig. S3**). Acetic acid is a hydrolysis product of hemicellulose linked to the xylan backbone as an acetate form. Even though the pH of the enzymatic hydrolysis medium reached below 3.5, sugar yields were unaffected without using the citrate buffer. Hydrolysate prepared using distilled water with 50% (w/v) solid loading was used as a medium for subsequent fermentation experiments.

3.3. Fermentation

One of the critical factors for lignocellulosic biorefinery is to attain a high titer of bioethanol from the hydrolysates. This can be possible with a high solid enzymatic hydrolysis. Producing elevated titers (>40 g/L) of bioethanol reduces the distillation cost in the downstream processing of fermentation broth.[23,24] The fermentability of hydrolysates (derived from 50% w/v) was examined for all the pretreated feedstocks. A negligible concentration of sugar decomposition products and excellent enzymatic digestibility of pretreated biomass without citrate buffer are the foremost advantages of hydrothermal pretreatment (**Table 2**). These advantages were further validated in the subsequent fermentation process.

Maximum bioethanol titers of 77.38±0.59 g/L, 72.16 ±2.78 g/L, and 66.47±3.51 g/L were obtained from urea-supplemented BES, EC, and MG hydrolysates, respectively. No ethanol production or sugar consumption was observed in urea-supplemented OC hydrolysate. From YP-supplemented BES, MG, EC, and OC hydrolysates, bioethanol titers of 79.50±2.30 g/L, 69.61±0.31 g/L, 68.28±3.03 g/L, and 59.23±0.66 g/L were achieved, respectively (**Fig. 2**). BES feedstocks demonstrated the highest fermentability, considering the ethanol titer, yield, productivity, and sugar utilization, likely due to lower inhibitor concentrations. The concentrations of acetic acid, formic acid, furfural, 5-HMF, and phenolic compounds were lower

in the BES and MG hydrolysates as compared to EC and OC (**Table 2** and **Fig. 3**). Given the higher inhibitor content, ethanol productivity, as an indicator of fermentability, was below 1 g/L/h for both EC and OC hydrolysates (**Table 3**). Similar concentrations of furfural (0.14±0.01 g/L) and 5-HMF (0.04±0.01 g/L) concentrations were observed in BES and MG hydrolysates (**Table 2**), and the acetic acid concentration in BES (5.34±0.04 g/L) was lower than that in MG (8.86±0.03 g/L) hydrolysate. All phenolic compounds except for ferulic acid, syringic acid, and 3-hydroxycinnamic acid were lower in concentration in BES hydrolysate as compared to MG (**Fig. 3),** and BES hydrolysate was shown to have a higher fermentability, with the highest ethanol productivity (**Table 3**).

The lowest ethanol titer and productivity were observed in the YP-supplemented oilcane hydrolysate. However, industrial titers of bioethanol were produced using either urea or YP as a nitrogen source in the BES, MG, and EC hydrolysates. Ethanol productivity is a crucial indicator for evaluating the role of YP and urea in hydrolysate fermentability. YP-supplemented hydrolysates showed excellent fermentability compared to urea (**Table 3**). Amino acids, peptides, vitamins, fats, and growth hormones are rich in yeast extract and peptone, which enhances yeast metabolism during bioethanol production.[25] Even in OC hydrolysate, 59.23±0.66 g/L ethanol titer was produced with a productivity of 0.41±0.05 g/L/h in the presence of higher concentrations of acetic acid (13.07±0.25 g/L), formic acid (1.73±0.01 g/L), furfural (0.55±0.01 g/L), and 5-HMF (0.57±0.02 g/L) (**Table 2**). In addition, GC-MS analysis of hydrolysates revealed that the OC hydrolysate contains higher concentrations of phenolic compounds than other hydrolysates (**Fig. 3**). The phenolic compounds play a vital role in plant deference mechanisms, which protect the plants from the infestation of pests.[26,27] The current study revealed that phenolic compound concentrations were a lot higher in oilcane and energy cane.

Different pests quickly damage the crop since these two are highly edible, sugary feedstocks. Therefore, the plants develop a defense mechanism to avoid pest infestation by cell wall thickening and lignification.[28,29] Lignin is a three-dimensional methoxylated polyphenolic compound. These lignin derivatives (phenolics) are released during the deconstruction of lignocellulosic biomass. As seen in **Fig. 3**, most phenolic concentrations were lower in MG and BES because these two crops are specially grown for bioenergy applications and do not contain an edible sugary substance, unlike the energy cane and sugarcane. Therefore, inherent lignocellulosic compounds like phenolics and acetic acid are major contributors to impede hydrolysate fermentability since the hydrothermal pretreatment generated a negligible concentration of sugar decomposition products such as furfural, 5-HMF, and formic acid, which are far below for microbial inhibition limits (**Table 2**). Previous results have shown that in the presence of 2 g/L furfural or 5 g/L HMF, *S. cerevisiae* growth on glucose is reduced to 10% and 11% of the control.[30] However, after 32 h of incubation, *S. cerevisiae* was able to produce 49% of the control ethanol yield in the presence of 5 g/L 5-HMF, indicative of partial acclimatization to the presence of inhibitors, although to a lesser extent than certain other yeast species.[30] In this study, 50% (w/v) solid loading derived enzymatic hydrolysates contained 0.14 g/L to 0.55 g/L of furfural, 0.04 g/L to 0.57 g/L of 5-HMF, and 0.51 g/L to 1.73 g/L of formic acid, which are far below the reported inhibitory limits of *S. cerevisiae*. For example, a combination of 4.8 g/L acetic acid, 9.9 g/L formic acid, 23.3 g/L levulinic acid, 1.2 g/L furfural, and 1.26 g/L 5-HMF was found to reduce the ethanol yield of *S. cerevisiae* grown on synthetic hydrolysate to 0.35 g_p/g_s, and a combination of 10 g/L acetic acid and 3 g/L furfural also reduced growth rates.[31, 32] As these concentrations are much higher than those observed in this study, sugar decomposition products were not expected to have a significant effect on fermentability of these hydrolysates.

Studies of individual phenolic compounds in *S. cerevisiae* have reported 0.5 to 1.5 g/L vanillin, 0.5 to 1.5 g/L hydroxybenzaldehyde, and 1.5 g/L of ferulic acid, syringaldehyde, and coumaric acid as inhibitory concentrations that reduced microbial growth and ethanol production.[30,32,33] In this study, concentrations of acetic acid, ferulic acid, syringic acid, and 3-hydroxycinnamic acid were higher in MG hydrolysate compared to BES hydrolysate, and the fermentability of MG was noted to be lower than that of BES, indicating that certain combinations of inhibitors may have a greater effect on the fermentability of hydrolysates (**Table 3**). The elevated concentration of fermentative inhibitors deters the hydrolysate fermentability but does not lead to microbial death. As shown in **Table 2**, among all the hydrolysates, the highest concentrations of sugar decomposition products (0.55±0.07 g/L furfural, 0.57±0.02 g/L 5-HMF, and 1.73±0.01 g/L formic acid), acetic acid (13.07±0.25 g/L) and phenolic compounds (**Fig. 3**) were observed in the OC hydrolysate, however, an engineered *S. cerevisiae* acclimatized for 96 h and produced an ethanol titer of 59.23±0.66 g/L between 96 h and 144 h fermentation (**Fig. 2d**). This indicates that the substantial concentrations of inhibitors can hinder microbial growth but are not detrimental (death) to microbes. It has been reported that a wild-type *S. cerevisiae* CBS 1200 (Centraalbureau voor Schimmelcultures, Delft, The Netherlands) tolerated 15 g/L acetic acid in fermentation medium and produced 62% of ethanol, and growth of *S. cerevisiae* was noted to be 56% when compared to the control.[30] Another study reported that commercial strains such as *S. cerevisiae* CAT-1 (Catanduva—Ribeirão Preto, Brazil) and JP1 (Santa Rita—Paraíba, Brazil) used for bioethanol production in Brazil have shown complete inhibition at 10 g/L acetic acid.[33] The above-mentioned reports suggest that acetic acid inhibition limits may vary based on strains that are isolated from different geographical regions.

The second conclusion is that hydrolysate fermentability can be enhanced by supplementing the nutrient-rich solution to boost microbial growth against the inhibitory effect. However, the cost of urea is much lower, and as such it is a better candidate for scale-up. Even with YP supplementation, 20.80±3.19 to 75.74±0.26 g/L of residual xylose remained unconsumed at the end of fermentation. Moreover, 30% of hydrolysate was unrecoverable through centrifugation of enzymatic slurry, resulting in 106.82±0.59 g to 138.74±1.20 g sugars per kg of pretreated biomass. This ultimately lowers overall ethanol yields per kg of biomass. Complete conversion of lignocellulosic sugars into bioethanol is also one of the important criteria for lignocellulosic biorefinery. A sustainable lignocellulosic bioprocess can be possible by converting all available sugars into bioethanol.

3.4. Semi-integrated bioprocess

The semi-integrated bioprocess (SIB) was conducted in batch mode at 25% (w/v) solid loading with urea as a nitrogen source to reduce overall costs and inhibitor concentrations. It can be observed that 110.30±0.68 g/L of sugar was obtained from BES after 10 h of hydrolysis, consisting of 68.06±0.87 g/L of glucose, 37.19±0.22 g/L of xylose, and 5.32±0.02 g/L cellobiose. Corresponding sugar concentrations from MG, EC, and OC hydrolysate were 116.48±0.26 g/L, 114.29±2.60 g/L, and 125.70±0.16 g/L. Fermentation of BES, MG, and EC hydrolysate resulted in complete glucose and xylose consumption and maximum ethanol titers of 54.50±0.41 g/L, 48.49±0.63 g/L, and 64.80±1.91 g/L, respectively. Xylose was not completely utilized during the fermentation of OC hydrolysate, which produced 60.78±3.95 g/L of ethanol excluding 11.82±5.55 g/L of residual xylose (**Fig. 4**). When using urea as a nitrogen source, ethanol production did not occur from OC hydrolysate derived from 50% (w/v) solid loading. Reducing the solid loading (25% w/v) in SIB greatly improved the ethanol titers (60.78±3.95

g/L) from OC using urea as a nitrogen source. This could be due to the reduction of inhibitor concentration by reducing the hydrolysis solid loadings. At 50% (w/v) solid loading, 1.73±0.01 g/L formic acid, 13.07±0.25 g/L acetic acid, and 0.55±0.07 g/L furfural and 0.57±0.02 g/L 5-HMF (Table 2) were observed in the enzymatic hydrolysate. Also, as shown in Table 6, at 30% (w/v) solid loading, 1.02±0.01 g/L formic acid, 8.62±0.02 g/L acetic acid, 0.40±0.01 g/L furfural, and 0.10±0.01 g/L 5-HMF were observed in the OC hydrolysate. At 25% (w/v) solid loading of OC, 0.63±0.01 g/L formic acid, 7.50±0.12 g/L acetic acid, 0.35±0.01 g/L furfural, and 0.05±0.01 g/L 5-HMF were observed in the fermentation medium.

Ethanol yields for BES, MG, EC, and OC were 0.500±0.008 gp/gs, 0.500±0.025 gp/gs, 0.570±0.025 gp/gs, and 0.530±0.003 g_p/g_s, respectively. The ethanol yields for OC and EC hydrolysate were greater than the theoretical maximum for the measured sugar concentrations, likely due to continued hydrolysis and release of sugars in the enzymatic slurry during the fermentation process. It was not possible to quantify the amount of sugar released during the fermentation, however, a regression analysis was used to predict the sugar concentration at 25% (w/v) solid loading based on the amounts sugar released between 20% and 40% (w/v) solid loadings in the fed-batch enzymatic hydrolysis **(Fig. 5**). In the fed-batch enzymatic hydrolysis of OC at 30% (w/v), 179.56±1.08 g/L (experimental result) sugar yield was obtained, which strongly agrees with the regression analysis result of 179.50 g/L (**Fig. 5**). According to this analysis, sugar concentrations of 131.80 g/L (BES), 103.65 g/L (MG), 134.32 g/L (EC), and 148.84 g/L (OC) would be available at the start of fermentation accounting for dilution by 5N NaOH (for pH adjustment) and the inoculum (**Eq. 4** and **Eq. 5**). The adjusted ethanol yields were 0.420±0.003 g_p/g_s, 0.470±0.003 g_p/g_s, 0.430±0.010 g_p/g_s, and 0.450±0.006 g_p/g_s for BES, MG, EC, and OC, respectively. Ethanol yields were significantly higher in the semi-integrated

bioprocess of OC and EC hydrolysate compared to separate hydrolysis and fermentation, although titers were lower. However, all feedstocks were able to support industrially viable ethanol titers.

In all conditions, complete utilization of glucose was observed between 24 h and 48 h, with consumption rates of 2.83±0.03 g/L/h, 1.64±0.07 g/L, 1.61±0.03 g/L, and 1.34±0.01 g/L/h for BES, MG, EC, and OC hydrolysates, respectively. The engineered *S. cerevisiae* co-utilized glucose and xylose, however, glucose consumption rate was higher than xylose. Literature suggests that higher glucose concentration competitively inhibits the xylose uptake.[34] Complete xylose utilization was observed for BES, MG, and EC hydrolysates only, with corresponding consumption rates of 0.75±0.01 g/L/h, 0.72±0.01 g/L/h, and 0.48±0.01 g/L/h. Slower xylose utilization rates increase the fermentation time and reduce the efficiency of the bioconversion process relative to a purely glucose substrates, and this challenge remains to be addressed by improved strain design. In the case of semi-integrated bioprocess however, the xylose utilization rate was higher than that of separate hydrolysis and fermentation (**Table 4**).

Due to the complete consumption of xylose along with glucose in the SIB shake flask study, a scale-up of semi-integrated bioprocess in a 1L bioreactor was conducted at 30% (w/v) solid loading for BES, MG, and EC to improve the ethanol titers. For the OC, 25% (w/v) solid loading was chosen to permit the utilization of a greater portion of the xylose within the fermentation medium since 11.82±5.55 g/L xylose remained after SIB in the shake flask. Higher ethanol titers were observed at the larger scale for BES (73.59±1.79 g/L) and MG (57.88±0.25 g/L) hydrolysate, with complete utilization of glucose and xylose. For EC, there was no significant change in titer, while for OC, there was a reduction, and in both cases, xylose was not completely consumed (**Fig. 6**). The increase in solid loading of EC also increased inhibitor

concentrations in the hydrolysate, particularly in the case of phenolic compounds (**Fig. 3**). Most of the ethanol was produced by 72 h, while a smaller amount was produced over the period from 72 h to 144 h. The highest ethanol productivities were observed in semi-integrated bioprocess compared to SHF (**Table 5**). The slower utilization rate of xylose relative to glucose extends the latter period as some or all of the remaining xylose is consumed, compounding toxicity effects from inhibitors and accumulated ethanol. Ethanol yields of 0.490±0.008 g_p/g_s, 460±0.001 g_p/g_s, 0.420±0.001 g_p/g_s, and 0.410± 0.002 g_p/g_s were obtained from BES, MG, EC, and OC hydrolysates in bioreactors, respectively. These values were similar to those observed in shake flasks, with a higher value for BES. Therefore, performing the semi-integrated process at lower solid loading reduces the fermentative inhibitor concentration (**Table 6**) and results in improved xylose consumption, ethanol yield, and productivity using urea as the cheapest nitrogen source without compromising the industrial ethanol titers.

3.5. Mass balance

For better understanding, mass balance was reported per kg of pretreated biomass. In the separate hydrolysis and fermentation, with the 74.33±0.22% cellulose and 90.28±0.39% xylan hydrolysis efficiencies at 50% solid loading, BES yielded 18.72±0.16 g of cellobiose, 257.44±0.68 g of glucose and 132.80±0.58 g of xylose from 408.7 g cellulose and 161.8 xylan. After the enzymatic hydrolysis, 70% of hydrolysate recovered from the enzymatic slurry, which contained 13.10±0.11 g of cellobiose, 180.21±0.47 g of glucose, and 92.96±0.40 g of xylose. In subsequent fermentation of hydrolysate, 134.82±0.52 mL of ethanol was produced per kg of pretreated BES, accounting for 0.42±0.01 g_p/g_s ethanol yield and with the 33 g of residual xylose remained after the fermentation. Similarly, 102.69±0.70 mL was produced from MG and EC with the corresponding residual xylose of 46.69 g, and 79.7g remained after fermentation. No

ethanol production was observed in the oilcane hydrolysate supplemented with urea as a nitrogen source (**Fig. 7a**).

In the semi-integrated bioprocess, with hydrolysis efficiencies of 87.67±0.69% for cellulose and 97.09±0.84% for xylan at 30% w/v solid loading, BES yielded 20.45±0.13 g of cellobiose, 305.28±2.45 g of glucose and 142.82±1.24 g of xylose. Ultimately, 291±2.23 mL ethanol produced per kg of BES, with the ethanol yield of 0.49±0.008 g_p/g_s **(Fig. 7b)**. On a larger scale, this would produce 291 L/Ton of bioethanol. Similar calculations for MG, EC, and OC yield 253.54 L/Ton, 257.8 L/Ton, and 260.3 L/Ton, respectively.

Conclusion

Performing enzymatic hydrolysis in distilled water without citrate buffer and substituting urea for yeast extract and peptone as the nitrogen source during ethanol fermentation did not significantly reduce titer and yield, and as a result, these cost-saving measures can be incorporated into future bioprocesses. Of the four feedstocks tested, bioenergy sorghum produced the best-performing substrate, accounting for ethanol yields, titer, and complete utilization of available sugars. Sugarcane-derived feedstocks in particular presented challenges in terms of titer and incomplete sugar utilization, likely due to their inhibitor content. The semi-integrated hydrolysis and fermentation bioprocess demonstrated that high ethanol yields can be obtained at larger scales and that multiple processes can be combined in a single bioreactor unit.

CRediT authorship contribution statement

Narendra Naik Deshavath: Conceptualization, Methodology, Investigation, data interpretation and writing – original draft. **William Woodruff:** Writing- review & editing of original draft. **Vijay Singh:** Data validation, Supervision, Writing – review & editing.

Acknowledgment

This work was funded by the DOE Center for Advanced Bioenergy and Bioproducts Innovation (U.S. Department of Energy, Office of Science, Biological and Environmental Research Program under Award Number DE-SC0018420). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the U.S. Department of Energy. The authors thank CABBI for providing the bioenergy feedstocks for this research. The authors also thank the Integrated Bioprocessing Research Laboratory (IBRL) at the University of Illinois for assistance with the pilot-scale processing of CABBI feedstocks, Kristen K. Eilts for assisting with HPLC analysis, and Roy J Carver Biotechnology Center at UIUC for GC-MS analysis for phenolic compounds detection.

Conflicts of Interest

The authors declare that they have no competing financial or personal interests.

References

1 J. Baeyens, Q. Kang, L. Appels, R. Dewil, Y. Lv and T. Tan, *Prog. Energy Combust. Sci.*, 2015, **47**, 60–88.

2 G. Wan, Q. Zhang, M. Li, Z. Jia, C. Guo, B. Luo, S. Wang and D. Min, *J. Agric. Food Chem.*, 2019, **67**, 10116–10125.

3 T. Pisithkul, T. B. Jacobson, T. J. O'Brien, D. M. Stevenson and D. Amador-Noguez, *Appl. Environ. Microbiol.*, 2015, **81**, 5761–5772.

4 L. Qin, W. C. Li, L. Liu, J. Q. Zhu, X. Li, B. Z. Li and Y. J. Yuan, *Biotechnol. Biofuels*, 2016, **9**, 1–10.

5 N. N. Deshavath, V. D. Veeranki and V. V. Goud, in *Sustainable Bioenergy*, eds. M. Rai and A. Ingle, Elsevier, 1st edn., 2019, pp. 1–19.

6 B. A. McKinley, S. N. Olson, K. B. Ritter, D. W. Herb, S. D. Karlen, F. Lu, J. Ralph, W. L. Rooney and J. E. Mullet, *PLoS One*, 2018, **13**, 1–20.

7 A. Arora and D. J. Carrier, *ACS Sustain. Chem. Eng.*, 2015, **3**, 2423–2428.

8 N. N. Deshavath, V. V. Goud and V. D. Veeranki, *Fuel*, 2021, **287**, 119545.

9 N. N. Deshavath, V. V. Goud and V. D. Veeranki, *J. Environ. Chem. Eng.*, 2021, **9**, 105696.

10 N. N. Deshavath, M. Mohan, V. D. Veeranki, V. V. Goud, S. R. Pinnamaneni and T. Benarjee, *3 Biotech*, , DOI:10.1007/s13205-017-0752-3.

11 N. N. Deshavath, S. Mahanta, V. V. Goud, V. V. Dasu and S. R. P., *J. Environ. Chem. Eng.*, , DOI:10.1016/J.JECE.2018.08.002.

12 H. Chandel, P. Kumar, A. K. Chandel and M. L. Verma, *Biomass Convers. Biorefinery*, , DOI:10.1007/s13399-022-02746-0.

13 L. Tao, D. Schell, R. Davis, E. Tan, R. Elander and A. Bratis, *NREL 2012 Achievement of Ethanol Cost Targets : Biochemical Ethanol Fermentation via Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*, 2014.

14 and A. A. D. Humbird, R. Davis, L. Tao, C. Kinchin D. Hsu, *Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover*, 2011.

15 E. Casey, N. S. Mosier, J. Adamec, Z. Stockdale, N. Ho and M. Sedlak, *Biotechnol.*

Biofuels, 2013, **6**, 1–10.

16 A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. C. Nrel, *Determination of Structural Carbohydrates and Lignin in Biomass Determination of Structural Carbohydrates and Lignin in Biomass*, 2012, vol. 2011.

17 M. H. Cheng, B. S. Dien, D. K. Lee and V. Singh, *Bioresour. Technol.*, 2019, **289**, 121663.

18 S. Banerjee, R. Singh, K. Eilts, E. J. Sacks and V. Singh, *J. Clean. Prod.*, 2022, **369**, 133508.

19 N. N. Deshavath, B. S. Dien, P. J. Slininger, Y. Jin and V. Singh, *Fermentation*, 2023, **9**, 1–19.

20 A. Juneja, B. Noordam, H. Pel, R. Basu, M. Appeldoorn and V. Singh, *Bioresour. Technol.*, 2021, **320**, 124380.

21 J. Zhang, D. Chu, J. Huang, Z. Yu, G. Dai and J. Bao, *Biotechnol. Bioeng.*, 2010, **105**, 718–728.

22 M. Jin, C. Gunawan, N. Uppugundla, V. Balan and B. E. Dale, *Energy Environ. Sci.*, 2012, **5**, 7168–7175.

23 C. Huang, A. J. Ragauskas, X. Wu, Y. Huang, X. Zhou, J. He, C. Huang, C. Lai, X. Li and Q. Yong, *Bioresour. Technol.*, 2018, **250**, 365–373.

24 G. Shen, X. Yuan, S. Chen, S. Liu and M. Jin, *Renew. Energy*, 2022, **186**, 904–913.

25 S. Raposo, A. Constantino, F. Rodrigues, B. Rodrigues and M. E. Lima-Costa, *Appl. Biochem. Biotechnol.*, 2017, **181**, 827–843.

26 S. Kaur, M. K. Samota, M. Choudhary, M. Choudhary, A. K. Pandey, A. Sharma and J. Thakur, *Physiol. Mol. Biol. Plants*, 2022, **28**, 485–504.

27 A. R. War, M. G. Paulraj, T. Ahmad, A. A. Buhroo, B. Hussain, S. Ignacimuthu and H. C. Sharma, *Plant Signal. Behav.*, 2012, **7**, 1306–1320.

28 E. Miedes, R. Vanholme, W. Boerjan and A. Molina, *Front. Plant Sci.*, 2014, **5**, 1–13.

29 V. Ninkuu, J. Yan, Z. Fu, T. Yang, J. Ziemah, M. S. Ullrich, N. Kuhnert and H. Zeng, *J. Fungi*, , DOI:10.3390/jof9010052.

30 J. P. Delgenes, R. Moletta and J. M. Navarro, *Enzyme Microb. Technol.*, 1996, **19**, 220–225.

31 S. Larsson, E. Palmqvist, B. Hahn-Hägerdal, C. Tengborg, K. Stenberg, G. Zacchi and N. O. Nilvebrant, *Enzyme Microb. Technol.*, 1999, **24**, 151–159.

32 E. Palmqvist, H. Grage, N. Q. Meinander and B. Hahn-Hägerdal, *Biotechnol. Bioeng.*, 1999, **63**, 46–55.

33 C. E. V. F. Soares, J. C. Bergmann and J. R. M. de Almeida, *Brazilian J. Microbiol.*, 2021, **52**, 575–586.

34 M. W. Lau and B. E. Dale, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 1368–1373.

Figures

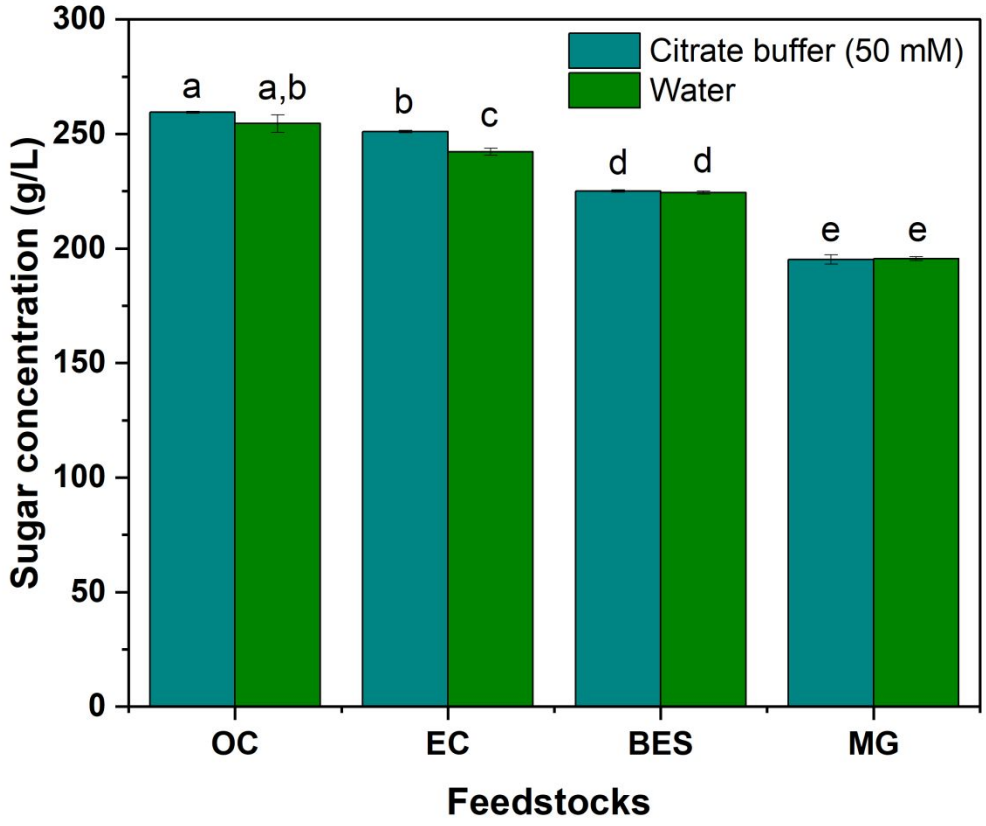


Fig. 1 Negligible role of citrate buffer in enzymatic hydrolysis of hydrothermal pretreated biomass. Statistical analysis was performed using one-way ANOVA, followed by Tukey's test. Note: Bars labeled with the same letter (a, a; b, b; etc.) did not have significantly different sugar concentrations ($p < 0.05$).

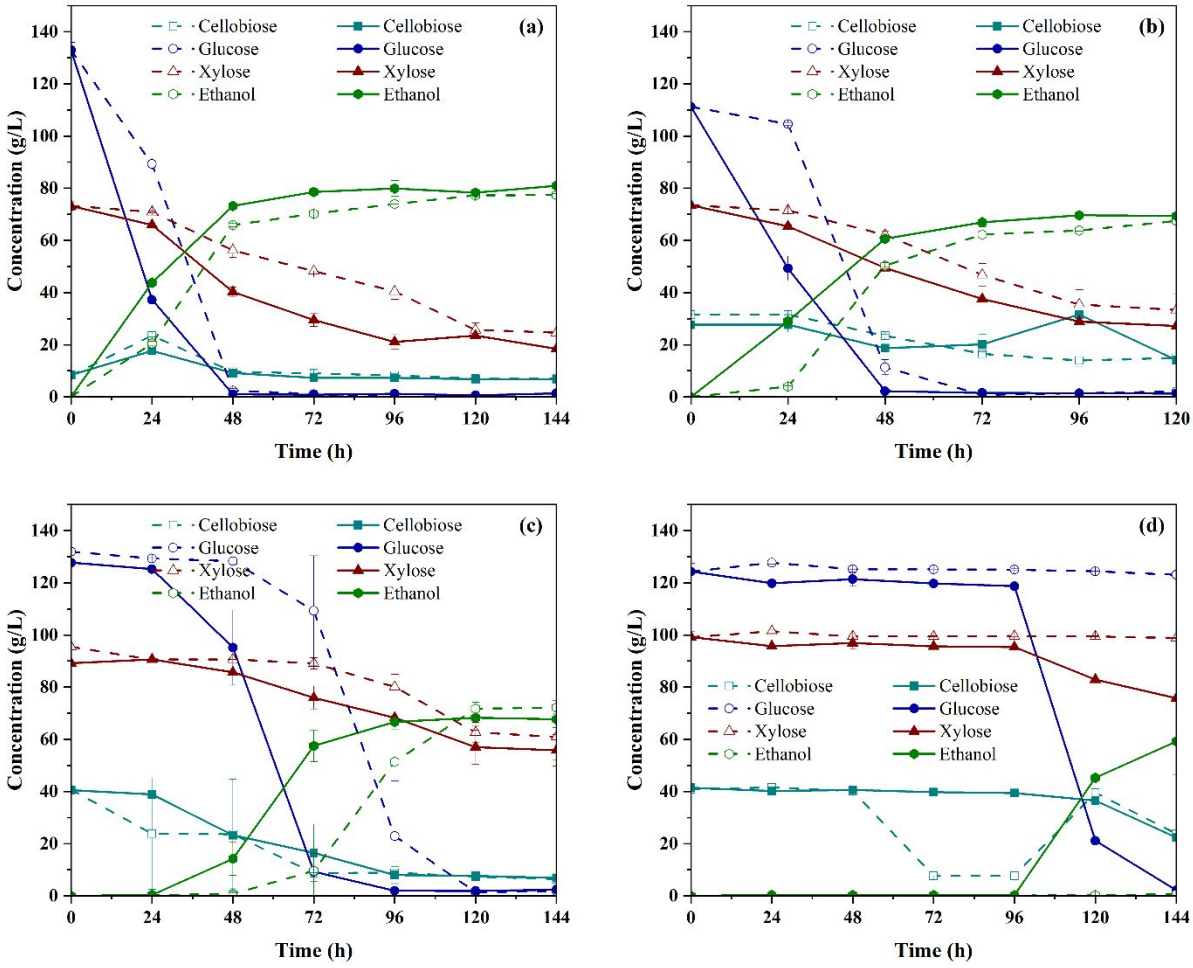


Fig. 2 Fermentation of a) BES, b) MG, c) EC, and d) OC hydrolysates derived from 50% (w/v) solid loading. Sugar consumption (glucose and xylose) and ethanol production profiles of engineered *S. cerevisiae* in YP-supplemented solution are marked with solid lines and those for cells grown on urea-supplemented hydrolysates are marked with dashed lines.

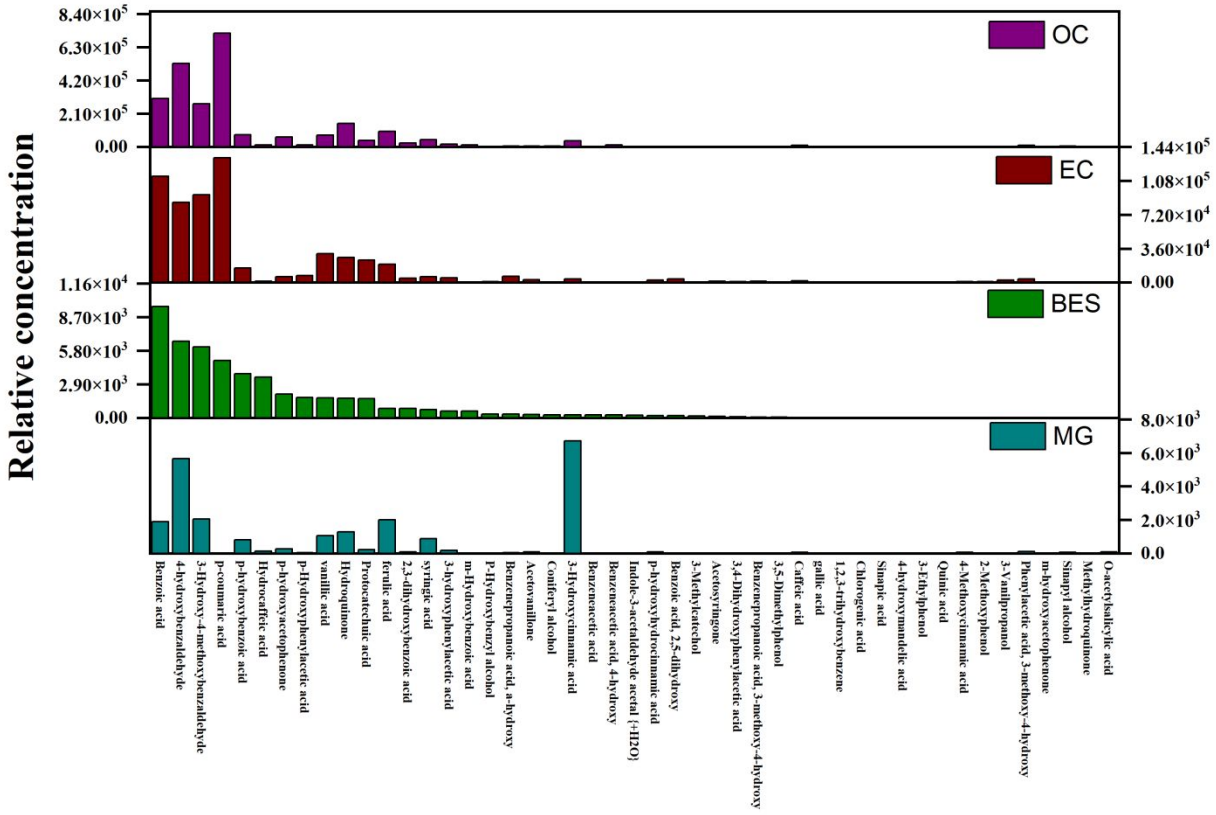


Fig. 3 Phenolic compound profiles of MG, BES, EC, and OC hydrolysates derived from enzymatic hydrolysis at 30% (w/v) solid loading.

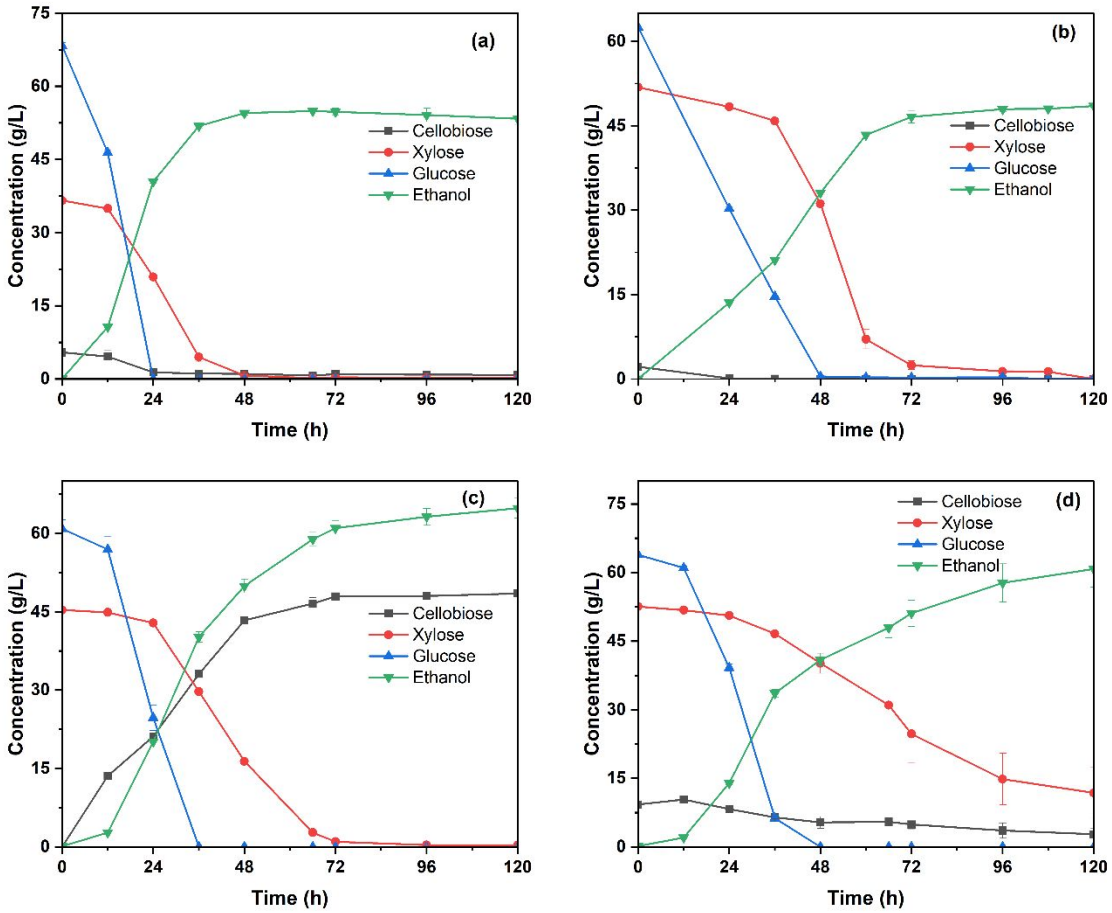


Fig. 4 Sugar consumption and ethanol production profiles from a shake flask study of the semi-integrated bioprocess for bioethanol production from hydrothermal pretreated bioenergy feedstocks **a**) BES, **b**) MG, **c**) EC, and **d**) OC.

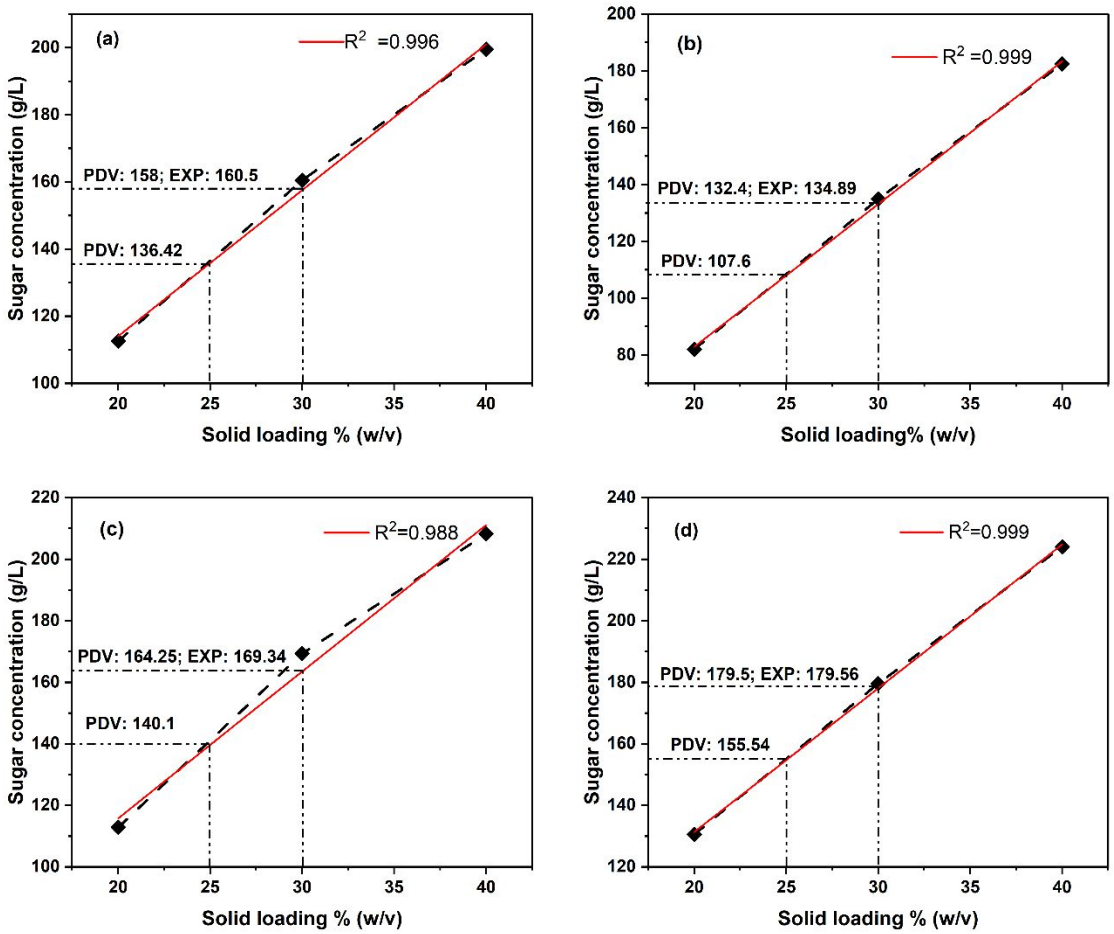


Fig. 5 Regression analysis of sugar yields between 20 to 40% (w/v) solid loading for a) BES, b) MG, c) EC, and d) OC. PDV stands for predicted value, and EXP stands for experimental value. These PDV values were obtained by plotting sugars released during the enzymatic hydrolysis of hydrothermal pretreated bioenergy feedstocks against the solid loading. These regression results were used to predict sugar release during the SIB as the inoculum was added prior to the completion of enzymatic hydrolysis.

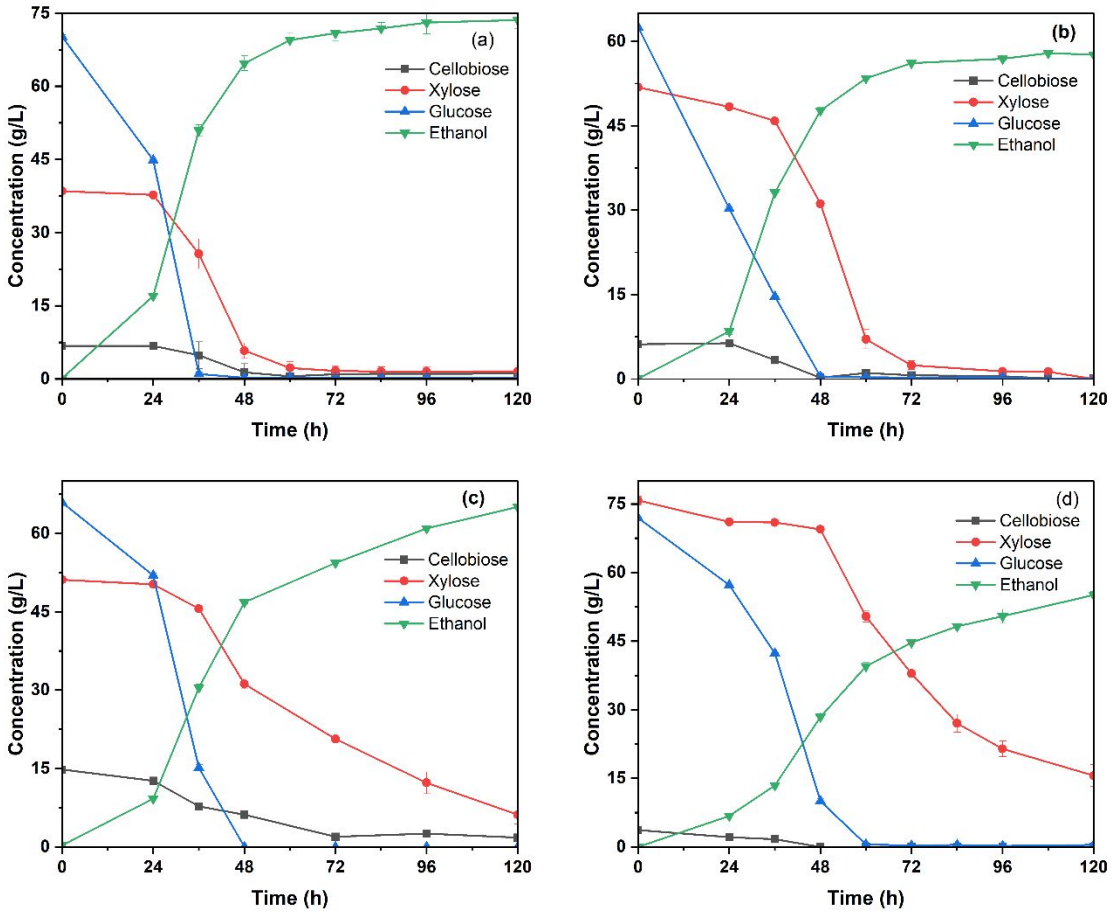
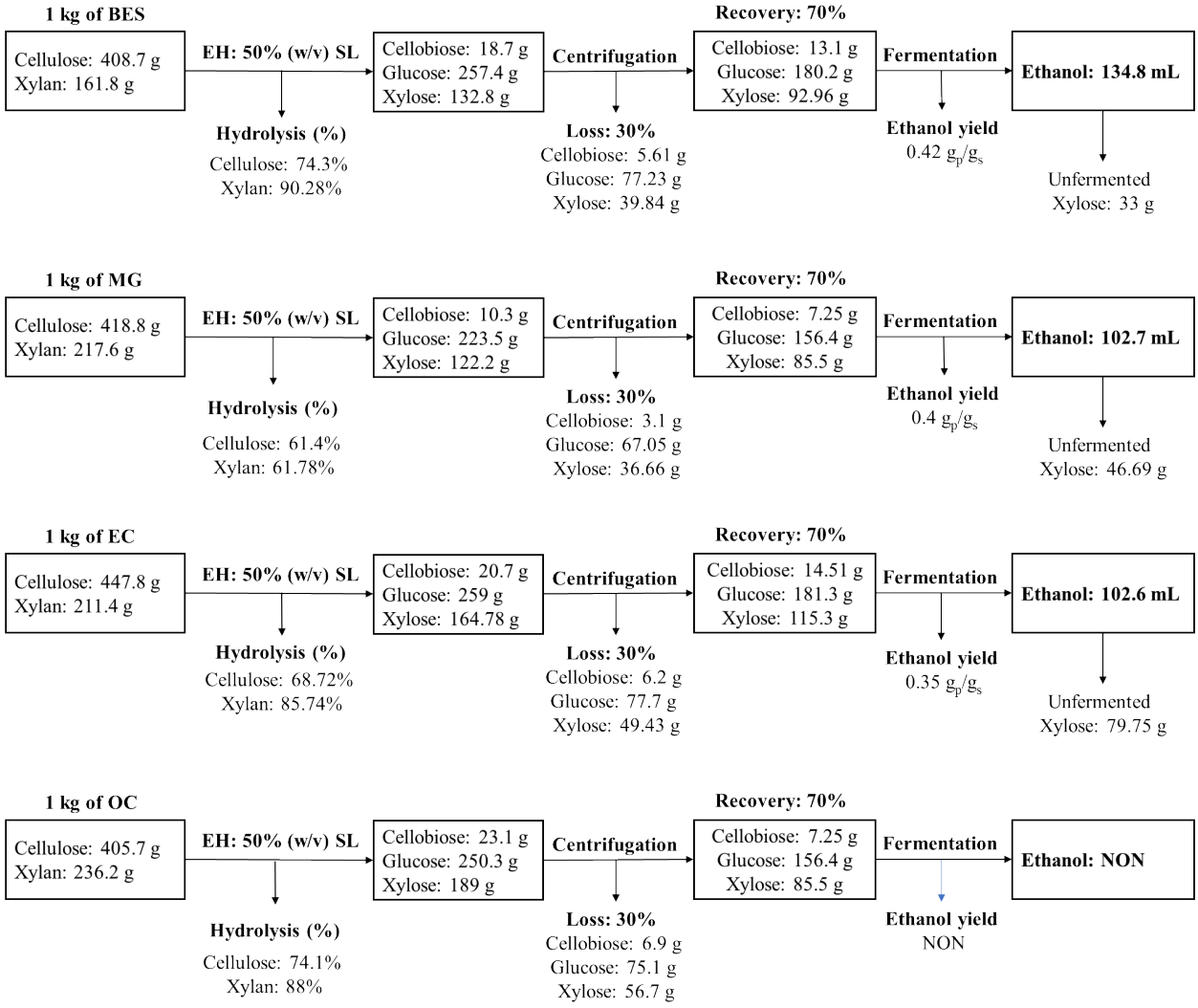


Fig. 6 Scale-up of semi-integrated bioprocess in a bioreactor for bioethanol production from hydrothermal pretreated bioenergy feedstocks a) BES, b) MG, c) EC, and d) OC

(a)


1 kg of BES
Cellulose: 408.7 g
Xylan: 161.8 g
EH: 50% (w/v) SL
Hydrolysis (%)
Cellulose: 74.3%
Xylan: 90.28%
Cellobiose: 18.7 g
Glucose: 257.4 g
Xylose: 132.8 g
Centrifugation
Loss: 30%
Cellobiose: 5.61 g
Glucose: 77.23 g
Xylose: 39.84 g
Recovery: 70%
Cellobiose: 13.1 g
Glucose: 180.2 g
Xylose: 92.96 g
Fermentation
Ethanol yield
0.42 gp/gs
Ethanol: 134.8 mL
Unfermented
Xylose: 33 g
1 kg of MG
Cellulose: 418.8 g
Xylan: 217.6 g
EH: 50% (w/v) SL
Hydrolysis (%)
Cellulose: 61.4%
Xylan: 61.78%
Cellobiose: 10.3 g
Glucose: 223.5 g
Xylose: 122.2 g
Centrifugation
Loss: 30%
Cellobiose: 3.1 g
Glucose: 67.05 g
Xylose: 36.66 g
Recovery: 70%
Cellobiose: 7.25 g
Glucose: 156.4 g
Xylose: 85.5 g
Fermentation
Ethanol yield
0.4 gp/gs
Ethanol: 102.7 mL
Unfermented
Xylose: 46.69 g
1 kg of EC
Cellulose: 447.8 g
Xylan: 211.4 g
EH: 50% (w/v) SL
Hydrolysis (%)
Cellulose: 68.72%
Xylan: 85.74%
Cellobiose: 20.7 g
Glucose: 259 g
Xylose: 164.78 g
Centrifugation
Loss: 30%
Cellobiose: 6.2 g
Glucose: 77.7 g
Xylose: 49.43 g
Recovery: 70%
Cellobiose: 14.51 g
Glucose: 181.3 g
Xylose: 115.3 g
Fermentation
Ethanol yield
0.35 gp/gs
Ethanol: 102.6 mL
Unfermented
Xylose: 79.75 g
1 kg of OC
Cellulose: 405.7 g
Xylan: 236.2 g
EH: 50% (w/v) SL
Hydrolysis (%)
Cellulose: 74.1%
Xylan: 88%
Cellobiose: 23.1 g
Glucose: 250.3 g
Xylose: 189 g
Centrifugation
Loss: 30%
Cellobiose: 6.9 g
Glucose: 75.1 g
Xylose: 56.7 g
Recovery: 70%
Cellobiose: 7.25 g
Glucose: 156.4 g
Xylose: 85.5 g
Fermentation
Ethanol yield
NON
Ethanol: NON

(b)

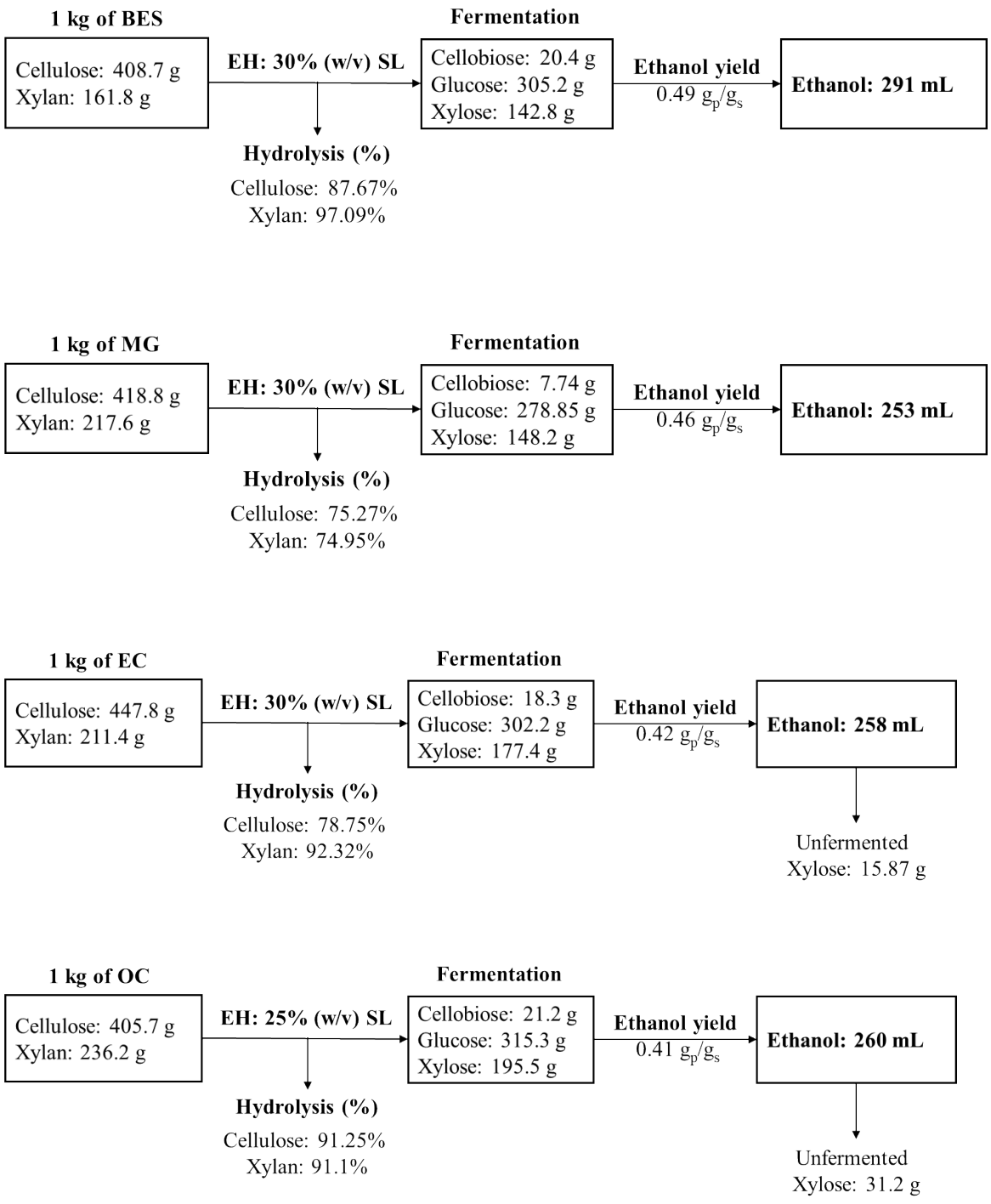


Fig. 7 Mass balance analysis for bioethanol yields for (a) separate hydrolysis and fermentation at 50% (w/v) solid loading and (b) scale-up of semi-integrated bioprocess. *EH, Enzymatic hydrolysis; SL, Solid loading; BES, Bioenergy sorghum; MG, Miscanthus x giganteus; EC, Energy cane; OC, Oilcane.*

Tables

Table 1 Compositional analysis of raw and hydrothermally pretreated feedstocks

Feedstock	Extractives (%)	Cellulose (%)	Xylan (%)	Arabinan (%)	Acetic acid (%)	AIL[†] (%)
Raw biomass						
Bioenergy sorghum	11.82	38.77±0.38	21.76±0.09	NR	NR	14.27±0.23
Miscanthus	14.00	38.30±0.80	20.60±2.20	NR	NR	23.10±0.20
Energy cane	12.64	41.15±3.81	17.35±2.92	1.54±0.22	3.64±0.29	20.21±1.12
Oilcane	20.30	32.03±0.55	17.53±0.93	1.63±0.07	4.37±0.07	16.20±0.51
Hydrothermal pretreatment followed by mechanical disc refining						
Bioenergy sorghum	NA	40.87±0.10	16.18±0.04	0.6±0.01	2.28±0.01	17.55±0.83
Miscanthus	NA	41.88±0.88	21.76±0.17	1.76±0.02	3.92±0.04	16.14±0.03
Energy cane	NA	44.78±0.30	21.14±0.10	1.17±0.01	3.91±0.05	14.71±0.82
Oilcane	NA	40.57±0.87	23.62±0.49	1.17±0.01	4.47±0.05	12.64±0.44

*[†] Acid-insoluble lignin; * Average ± standard deviation; NR, not reported. Raw BES and MG composition were obtained from references.[17,18] Except for miscanthus (170 °C), all other feedstocks were pretreated at 190 °C.*

Table 2. Composition of enzymatic hydrolysates derived from 50% (w/v) solid loading of hydrothermal pretreated bioenergy feedstocks

Biomass	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Furfural (g/L)	5-HMF
BES	9.36±0.80	135.96±0.34	79.09±0.03	NF	5.34±0.04	0.14±0.01	0.04±0.01
MG	20.48±0.18	117.69±0.35	72.96±0.30	NF	8.86±0.03	0.14±0.01	0.04±0.01
EC	10.36±0.04	136.70±0.38	95.08±0.11	0.51±0.06	11.15±0.13	0.14±0.01	0.23±0.03
OCL	11.56±1.93	135.66±5.12	107.27±0.03	1.73±0.01	13.07±0.25	0.55±0.01	0.57±0.02

***** *Average ± standard deviation.*

Table 3 Effect of YP and urea supplementation on ethanol productivity during the fermentation of enzymatic hydrolysates derived from 50% (w/v) solid loading

Time (h)	BES *Ep* (g/L/h)		MG *Ep* (g/L/h)		EC *Ep* (g/L/h)		OC *Ep* (g/L/h)	
	YP	Urea	YP	Urea	YP	Urea	YP	Urea
24	1.82±0.02	0.85±0.03	1.21±0.01	0.16±0.01	0.02±0.01	0.01±0.00	0.01±0.00	0.00±0.00
48	1.53±0.02	1.37±0.01	1.26±0.01	1.05±0.02	0.30±0.13	0.02±0.01	0.01±0.00	0.00±0.00
72	1.09±0.02	0.98±0.01	0.93±0.01	0.86±0.04	0.80±0.08	0.13±0.014	0.00±0.00	0.00±0.00
96	0.83±0.03	0.77±0.01	0.73±0.01	0.66±0.01	0.69±0.04	0.54±0.13	0.00±0.00	0.00±0.00
120	0.65±0.01	0.64±0.01	0.58±0.01	0.56±0.01	0.57±0.03	0.60±0.02	0.38±0.02	0.00±0.00
144	0.56±0.01	0.54±0.01	0.48±0.01	0.57±0.01	0.47±0.02	0.50±0.02	0.41±0.05	0.00±0.00

Ep, Ethanol productivity; * *Average ± standard deviation.*

Table 4. Maximum xylose consumption rates in SHF and SIB using urea as a nitrogen source.

Feedstock	Xylose consumption rate (g/L/h)	
	SHF	**SIB**
BES	0.39±0.03	0.9±0.02
MG	0.39±0.05	0.35±0.03
EC	0.27±0.03	0.66±0.01
OC	NON	0.41±0.05

** Average ± standard deviation; SHF was conducted at 50% (w/v) derived hydrolysates; SIB was calculated in a shake flask at 25% (w/v) solid loading.*

Table 5. Maximum ethanol productivity in SHF and SIB using urea as a nitrogen source.

Feedstock	Ethanol productivities (g/L/h)	
	SHF	**SIB**
BES	1.37±0.01	1.41±0.03
MG	1.05±0.02	1.38±0.01
EC	0.60±0.02	0.97±0.01
OC	0.00±0.00	0.82±0.02

** Average ± standard deviation. SHF was conducted at 50% (w/v) derived hydrolysates; SIB was calculated in a 1 L bioreactor at 30% (w/v) solid loading for BES, MG, EC, and 25% (w/v) solid loading for OC.*

Table 6. Composition of enzymatic hydrolysates derived from 30% (w/v) solid loading of hydrothermal pretreated bioenergy feedstocks

Biomass	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Furfural (g/L)	5-HMF
BES	6.13±0.10	98.82±0.73	55.53±0.03	NF	3.43±0.04	0.09±0.01	NF
MG	9.07±0.13	78.72±0.84	47.09±0.51	NF	5.40±0.03	0.08±0.01	NF
EC	5.50±0.08	97.91±0.25	65.92±0.05	0.30±0.01	7.14±0.02	0.10±0.01	0.06±0.01
OCL	6.36±0.07	101.85±0.58	71.34±0.04	1.02±0.01	8.62±0.025	0.40±0.01	0.10±0.01

Average ± standard deviation.