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# Diverse lignocellulosic feedstocks can achieve high field-scale ethanol yields while providing flexibility for the biorefinery and landscape-level environmental benefits

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## Abstract

Increasing the diversity of lignocellulosic feedstocks accepted by a regional biorefinery has the potential to improve the environmental footprint of the facility; harvest, storage, and transportation logistics; and biorefinery economics. However, feedstocks can vary widely in terms of their biomass yields and quality characteristics (chemical composition, moisture content, etc.). To investigate how the diversity of potential biofuel cropping systems and feedstock supply might affect process and field-scale ethanol yields, we processed and experimentally quantified ethanol production from five different herbaceous feedstocks: two annuals (corn stover and energy sorghum) and three perennials (switchgrass, miscanthus, and mixed prairie). The feedstocks were pretreated using ammonia fiber expansion (AFEX), hydrolyzed at high solid loading (~17%–20% solids, depending on the feedstock), and fermented separately using microbes engineered to utilize xylose: yeast (*Saccharomyces cerevisiae* Y128) or bacteria (*Zymomonas mobilis* 8b). The field-scale ethanol yield from each feedstock was dependent on biomass quality and cropping system productivity; however, biomass yield had a greater influence on the ethanol yield for low-productivity crops, while

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biomass quality was the main driver for ethanol yields from high-yielding crops. The process ethanol yield showed similar variability across years and feedstocks. A low process yield for corn stover was determined to result from inhibition of xylose utilization by unusually elevated levels of hydroxycinnamates (*p*-coumaric and ferulic acids) in the untreated biomass and their acid and amide derivatives in the resulting hydrolyzate. This finding highlights the need to better understand factors that influence process ethanol yield and biomass quality. Ultimately we provide evidence that most feedstocks fall within a similar range of process ethanol yield, particularly for the more resistant strain *Z. mobilis* 8b. This supports the claim that the refinery can successfully diversify its feedstock supply, enabling many social and environmental benefits that can accrue due to landscape diversification.

#### KEYWORDS

biorefinery, ethanol yield, feedstock diversity, fermentation, inhibitors, lignocellulosic hydrolyzate, xylose utilization

## 1 | INTRODUCTION

Cellulosic biofuels generated from vegetative plant biomass are part of a suite of carbon neutral solutions that can meet the world's energy needs (Robertson et al., 2017). Lignocellulosic (second-generation) bioethanol facilities have been largely designed around a process model whereby one, or at most two standardized feedstocks are grown and harvested in nearby fields, then processed, and fermented into ethanol or other bio-based products (POET-DSM, 2014; Raízen, 2014). This approach has perceived advantages with the use of consistent, standardized protocols and equipment for crop production and harvest, biomass transport and preprocessing, and fuel production. It is also consistent with the manner in which first-generation bioethanol facilities currently operate the following: processing a single bioenergy crop specific to their geographical region. For example, ethanol companies in Brazil have optimized to use sugarcane (*Saccharum officinarum* L.), companies in the European Union use sugar beets (*Beta vulgaris* L.) and wheat (*Triticum aestivum* L.), and companies in China and the United States have focused primarily on corn grain (Bonin & Lal, 2012). However, for second-generation ethanol, it may not be feasible or economically advisable to be overly selective in terms of accepted feedstocks, particularly in regions where cropping systems are diversified. As lignocellulosic feedstocks are inherently low-density compared to the sugar- and starch-based crops used by first-generation facilities, less material can be loaded per vehicle, resulting in significantly higher transportation costs (Lin et al., 2016). Without some form of biomass densification in the field, the feedstock collection radius and the subsequent supply for the second-generation biorefinery are economically constrained compared to first-generation facilities.

Depending on the region, there are a large number of potential lignocellulosic feedstocks available for use by a biorefinery, including annual crops such as corn stover (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench); and perennial crops such as switchgrass (*Panicum virgatum* L.), miscanthus (*Miscanthus × giganteus* Greef & Deuter ex Hodkinson & Renvoize), energycane (*Saccharum* spp.), native grass polycultures, and coppiced wood plantations. Additionally, many geographical regions in the United States are capable of producing more than one of these feedstocks at relatively high biomass yields (Lee et al., 2018; Sanford et al., 2016). One criticism of this approach is that feedstock diversity may lead to variability in conversion efficiencies and put an undue burden on biorefineries, resulting in the need to change operating conditions depending on the feedstock. One means to overcome this is through feedstock blending to ensure consistent feedstock properties (Hess, Wright, Kenney, & Searcy, 2009; Shi et al., 2012). However, this may not be necessary as long as the feedstocks are within the same species classification (e.g., grass, hardwood, or softwood). In our previous work, we evaluated the potential to use early successional plant biomass, an inherently unpredictable and diverse set of plant species, as a bioenergy feedstock (Garlock, Bals, Jasrotia, Balan, & Dale, 2012). Based on our findings, although there was a great deal of variability in plant species composition, most of the samples either had the same optimal pretreatment conditions or achieved high yields under a standard set of processing conditions. Additionally, the different materials had consistent hydrolysis yields as long as the feedstocks were mainly comprised of grass species. In another study, we evaluated theoretical ethanol yields from different feedstocks on a field basis and found that ethanol yield was determined more by crop

yield than by feedstock quality (chemical composition) (Sanford et al., 2017). This finding supports the practicable idea that biofuel refineries could process multiple different regional feedstock types without experiencing significant impacts on ethanol production. However, these conclusions were based upon theoretical ethanol yields and not actual fermentation experiments and did not account for specific differences in chemical composition between feedstocks. We have previously shown these can be highly variable and have wide-ranging impacts on microbial conversion of cellulosic sugars to ethanol (Ong et al., 2016).

Here, we extend our previous work on ethanol yield from diverse feedstocks by evaluating the fermentation performance of five different herbaceous bioenergy crops. We evaluated two annuals: corn stover and energy sorghum; and three perennials: switchgrass, miscanthus, and a restored mixed prairie. All feedstocks were grown at the same location (Arlington, WI) and harvested in 2014, pretreated using ammonia fiber expansion (AFEX) and hydrolyzed into fermentable sugars at high solid loadings (~17%–20%, depending on the feedstock). Fermentation studies using engineered ethanologenic microbes (*Saccharomyces cerevisiae* Y128 and *Zymomonas mobilis* 8b) enabled calculation of process and field-scale ethanol yields for all feedstocks. Molecular studies identified significant differences in the chemical compositions of the five feedstocks. This evaluation provides a measure of the relative magnitude of expected feedstock and harvest year variability on process yields.

## 2 | MATERIALS AND METHODS

### 2.1 | Hydrolyzate production

Hydrolyzates were produced from five different AFEX-pretreated feedstocks using enzymatic hydrolysis methods as described previously for AFEX-pretreated corn stover and switchgrass (Serate et al., 2015). Hydrolyzates (~1 L) were generated in a 3 L Applikon ez-control bioreactor system (Applikon Biotechnology, Foster City, CA, USA). Because of the higher glucan and xylan conversions for corn stover, final glucose concentrations were normalized across feedstocks by increasing the solids and enzyme loadings for the other four feedstocks. Solids were loaded at 6% glucan loading for corn stover and 7% glucan loading for the other four feedstocks (~17%–20% solids loading, depending on the feedstock). CTec2 and HTec2 (Novozymes, Franklinton, NC, USA) were used for hydrolysis, at loadings of 32 mg CTec2 protein per g glucan and 9 mg HTec2 protein per g glucan for corn stover, and 48 mg CTec2 protein per g glucan and 13.5 mg HTec2 protein per g glucan for the other four feedstocks, the same as described previously (Serate et al., 2015). The same

solids and enzyme loadings were used for miscanthus, sorghum, and mixed prairie as for switchgrass. Due to the difference in feedstock buffering capacity, different amounts of undiluted HCl were used to neutralize each feedstock prior to hydrolysis: 5.6 ml for corn stover, 6.0 ml for switchgrass and sorghum, 6.5 ml for mixed prairie, and 5 ml for miscanthus. The hydrolysis was carried out at 50°C for 5 days, and the final pH for all hydrolyzates was between 5.0 and 5.5. After the solids were removed by centrifugation at 8,200  $\times$  g and 4°C for 10–12 hr, the supernatant was filter-sterilized sequentially through 0.5- $\mu$ m GVS Maine Glass Prefilters (Thermo Fisher Scientific Inc. Waltham, MA, USA) and 0.2  $\mu$ m 1 L Filter Units (Nalge Nunc International Corporation, Rochester, NY, USA), and stored at 4°C.

### 2.2 | Microbial fermentation of lignocellulosic feedstocks

Engineered xylose-utilizing *S. cerevisiae* Y128 (Parreiras et al., 2014) and *Z. mobilis* 8b (obtained from the American Type Culture Collection, PTA-6976) were used for comparative fermentations. Cultures for inoculation were prepared as described previously (Serate et al., 2015). Fermentations were conducted in 500 ml bioreactors (BIO-STAT Qplus system; Sartorius Stedim Biotech, Bohemia, NY, USA) containing 250 ml of hydrolyzate. Prior to fermentation, the hydrolyzates were adjusted to pH 5 (*S. cerevisiae*) or 5.8 (*Z. mobilis*) using 10 N NaOH or undiluted HCl and filtered through a 0.2- $\mu$ m filter to remove precipitates and to ensure sterility. The seed culture was centrifuged at 14,000 g for 3 min after which the supernatant was discarded and the cell pellets were resuspended into 10 ml of hydrolyzate from the presparged vessels. The starter was then inoculated into each vessel to give an initial OD<sub>600</sub> (optical density at 600 nm) of 0.5 in the bioreactor. Fermentations were conducted at 30°C with continuous stirring (300 rpm) and sparging (150 ml/min; 100% N<sub>2</sub>). During the fermentation, pH was controlled at 5.0 for Y128 and 5.8 for *Z. mobilis* 8b, and samples were periodically removed from the bioreactors for an OD<sub>600</sub> measurement to monitor cell growth and for HPLC-RID analysis of the concentration of glucose, xylose, and the end products as described previously (Serate et al., 2015). To obtain a more accurate final ethanol yield, evaporated ethanol from an off-gas line was trapped in ice water and quantified using HPLC. Growth and substrate uptake rates were calculated as previously described (Sato et al., 2016). Process ethanol yields, expressed as the percentage of maximal theoretical ethanol yield (0.51 g ethanol/g sugar) produced from the total glucose, and xylose present in each hydrolyzate, were calculated from the initial sugar and final ethanol concentrations for each experiment.

To evaluate the inhibition of fermentation by ferulate and *p*-coumarate compounds, additional compounds were supplemented in corn stover (Y2012) hydrolyzate to match the concentrations in corn stover (Y2014) hydrolyzate: ferulic acid (0.027 mM), feruloyl amide (3.262 mM), *p*-coumaric acid (0.858 mM), and *p*-coumaroyl amide (4.441 mM). Feruloyl and coumaroyl amides were synthesized in the laboratory using previously described methods (Keating et al., 2014).

### 2.3 | Chemical genomics

Chemical genomic analysis of the hydrolyzates was performed as described previously, using a collection of ~4,000 yeast deletion mutants (Piotrowski et al., 2017), and ~3,500 *Z. mobilis* transposon insertion mutants in 1578 ORFs (Skerker et al., 2013). Cultures (200  $\mu$ l) of the pooled collection of *S. cerevisiae* deletion mutants or *Z. mobilis* transposon insertion mutants were cultured anaerobically in the different biomass hydrolyzates, rich media (YPD for yeast: 10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose; rich ZRMG for *Z. mobilis*: 20 g/L glucose, 10 g/L yeast extract, and 2 g/L  $\text{KH}_2\text{PO}_4$  (Skotnicki, Tribe, & Rogers, 1980) or synthetic hydrolyzate (SynH) mimic of AFEX-pretreated corn stover hydrolyzate (Keating et al., 2014) without (SynHv2.6) or with inhibitory compounds (SynHv2.7) in triplicate for 48 hr at 30°C. Genomic DNA was extracted from the cells using PureLink™ Pro 96 Genomic DNA Kit (Thermo Fisher Scientific Inc.), and mutant-specific molecular barcodes were amplified using specially designed multiplex primers as described previously (Piotrowski, Simpkins, & Li, 2015). The barcodes were sequenced using an Illumina HiSeq2500 in rapid run mode (Illumina, Inc, San Diego, CA, USA).

Barcode counts were obtained using barseq counter software (Simpkins et al., 2018). Further analysis was performed using R and Bioconductor (Huber et al., 2015; The R Foundation, 2017). The count matrix was filtered to remove failed samples and mutants that did not grow sufficiently well in at least three samples, *that is*, samples with median counts <10 and mutants whose counts did not exceed a set threshold (70 in *S. cerevisiae* and 20 in *Z. mobilis*) in at least three samples. Filtering removed approximately 10% of low-count mutants in each dataset. Gene-level counts used in some analyses were computed by adding up counts from multiple mutants with the same gene deletion. The counts were further processed using Bioconductor limma workflow for RNA-seq (Law, Alhamdoosh, Su, Smyth, & Ritchie, 2016). Specifically, the counts were log-transformed and normalized by the EdgeR TMM method (Robinson, McCarthy, & Smyth, 2010) and observation weights were computed using voom function in limma. Multidimensional scaling (MDS) was performed

using plotMDS function in limma. For purposes of hierarchical clustering, we used gene-level data, which was further filtered to only retain high-variance genes. We kept genes with standard deviations > 1 in *S. cerevisiae* and >0.5 in *Z. mobilis* dataset. Log-count values for each gene were standardized. Average linkage clustering was performed using hclust function in R with distances computed using Pearson correlation for genes and Spearman correlation for samples (Girke, 2018).

### 2.4 | 24-Well assays for yeast growth and xylose consumption

Single colonies of Y330 (a version of Y128 containing a deletion mutation in *FLO8* for reduced flocculation) yeast were inoculated into 5 ml YPD media and grown aerobically at 30°C. After 16–18 hr of growth, cultures were diluted to  $\text{OD}_{600} = 0.3$  and recultured until they reached logarithmic growth phase ( $\text{OD}_{600} = 0.6$ – $0.8$ ). Cells were harvested, pelleted at 3,000  $\times$  g, and washed with sterile water. Cell pellets were resuspended in SynH containing 20 g/L xylose and 60 g/L sorbitol (SynHXS) in lieu of glucose to maintain osmolarity and then inoculated in 1.5 ml SynHXS to a final  $\text{OD}_{600} = 0.1$  in sterile 24-well plates (Greiner Cellstar). Cell growth was monitored by  $\text{OD}_{600}$  measurements in a multimode plate reader (Tecan; Männedorf, Switzerland) every 10 min for 48 hr, at 30°C with continual shaking. Relative growth and xylose consumption were determined by dividing the total cell growth or consumed xylose in the presence of phenolic compounds (ferulic acid, feruloyl amide, *p*-coumaric acid, and/or *p*-coumaroyl amide) by the total cell growth or xylose consumed in the absence of phenolic compounds.

### 2.5 | Statistical analysis of hydrolyzate composition and ethanol yields

Statistical analysis of the hydrolyzate composition was conducted in R-Studio®, version 1.0.143 (Boston, MA, USA). A linear model of each chemical component was developed based on the feedstock, control method, and their interaction, and evaluated using Tukey's HSD test based on 95% confidence intervals (Agricolae package, version 1.2-1) (De Mendiburu, 2009). When a reported value was below the limit of quantitation (LOQ), the value was recalculated as  $\text{LOQ}/\sqrt{2}$  (Croghan & Egeghy, 2003). These recalculated values were used to determine the mean and standard deviation for each sample, and statistical differences between samples. The principal component analysis of hydrolyzate composition was conducted on hydrolyzate batches that had complete suites of data (sugars, aromatic inhibitors, amino acids, and elements). Static and interactive plots were constructed using the ggbiplot (v. 0.55) (Vu, 2011),

ggplot2 (v. 2.2.1) (Wickham, 2009), and plotly packages (v. 4.7.1) (Plotly Technologies Inc, 2015).

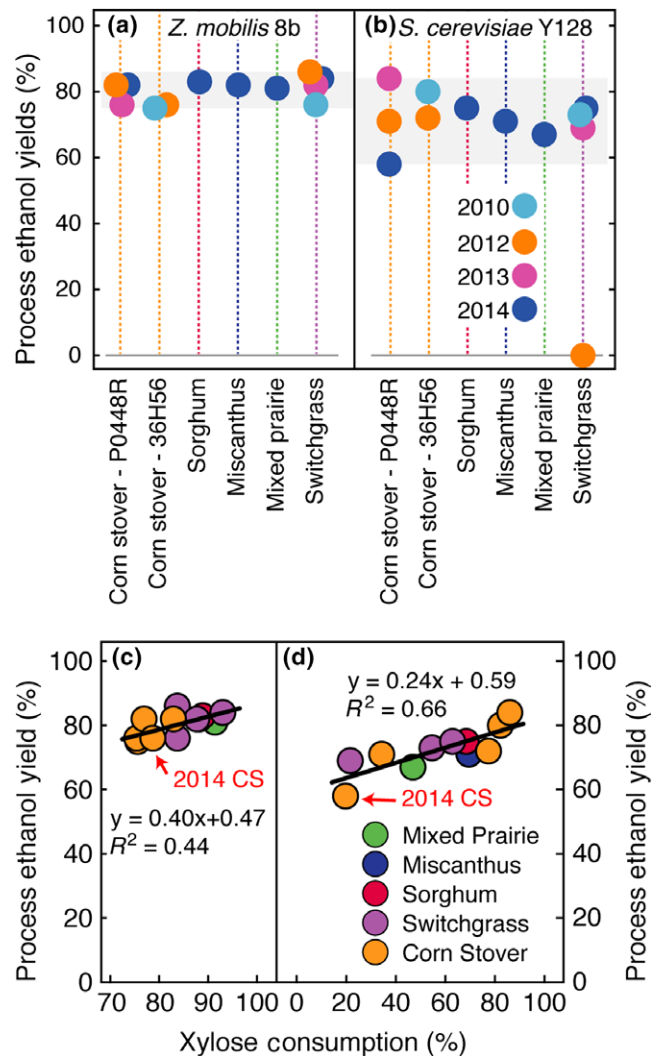
Average ethanol yields per hectare for each feedstock were calculated from the average dry matter yields per hectare (Mg biomass per ha) from the GLBRC biomass cropping system experimental (BSCE) plots and average ethanol yields (L per Mg untreated feedstock) from this study. Corn stover was harvested from other plots, also in Arlington, WI; however, yield data were not collected at harvest, and so the BSCE corn stover yields were used as an estimate. The standard deviations of ethanol yield per hectare were calculated using statistical propagation of uncertainty. Confidence and prediction intervals (95%) were calculated in R (The R Foundation, 2017) based on the linear regression (lm function) of ethanol yields per hectare as a function of the biomass yield per hectare across all feedstocks. The confidence and prediction intervals for the regression equations were calculated and plotted using the ggplot2 package (v.2.2.1) (Wickham, 2009).

Additional methods are included in the supplemental information.

### 3 | RESULTS

#### 3.1 | Impaired xylose fermentation in corn stover hydrolyzate fermentations contributed to lower process ethanol yield compared to other feedstock hydrolyzates

For the 2014 feedstocks, the process ethanol yields from *Z. mobilis* fermentations were relatively similar, ranging from 81% to 84% of the theoretical ethanol yield based on glucose and xylose concentrations in the hydrolyzates (Figure 1a,c and Supporting Information Table S3). This falls within the range of our previously reported values for corn stover and switchgrass (75%–86%) (Figure 1a,c). In contrast, the process ethanol yields from the yeast fermentations showed much greater variability, both for different feedstocks within the same year (2014:58%–75%) and for the same feedstock between years (corn stover: 58%–90%, and switchgrass: 85%–97%) (Figure 1b and Supporting Information Table S3). The lower process yields for corn stover and mixed prairie hydrolyzates (2014 harvest year) seem to be at least partially related to lower extents and/or efficiencies of xylose utilization. For *Z. mobilis* fermentation of the 2014 hydrolyzates, corn stover had a significantly slower rate of xylose consumption (1.5 mM xylose per OD<sub>600</sub> per hour) compared to the other feedstocks (2.4–3.0 mM xylose per OD<sub>600</sub> per hour), (Supporting Information Figure S1f–j and Table S3). With the exception of 2012 switchgrass, which did not support either yeast growth or fermentation, the yeast fermentation process ethanol yields were lowest for the 2014 corn stover



**FIGURE 1** Experimentally determined process ethanol yields are less variable for *Z. mobilis* 8b (a) compared to *S. cerevisiae* Y128 (b) and are positively correlated with xylose consumption for both *Z. mobilis* 8b (c) and *S. cerevisiae* Y128 (d). Anaerobic bioreactor fermentations with hydrolyzates generated from the indicated AFEX-pretreated biomass were performed using *Z. mobilis* 8b (a, c) and *S. cerevisiae* Y128 (b, d) engineered strains. The gray bar (a, b) represents the range in process yields across all feedstocks. Process ethanol yields represent the ethanol produced based on the amount theoretically possible from complete conversion of the glucose and xylose in each hydrolyzate (see Methods). Data for other years and strains of corn stover and switchgrass were published previously (Ong et al., 2016). Xylose consumption is the proportion of xylose consumed in terms of the total xylose available in the hydrolyzate. Average process ethanol yields were calculated from three or four independent biological replicates. To minimize confusion, error bars are not included in this figure; however, standard deviations for the data are included in the supplemental information (Supporting Information Figure S2, Table S3). P0448R and 36H56 are different varieties of corn

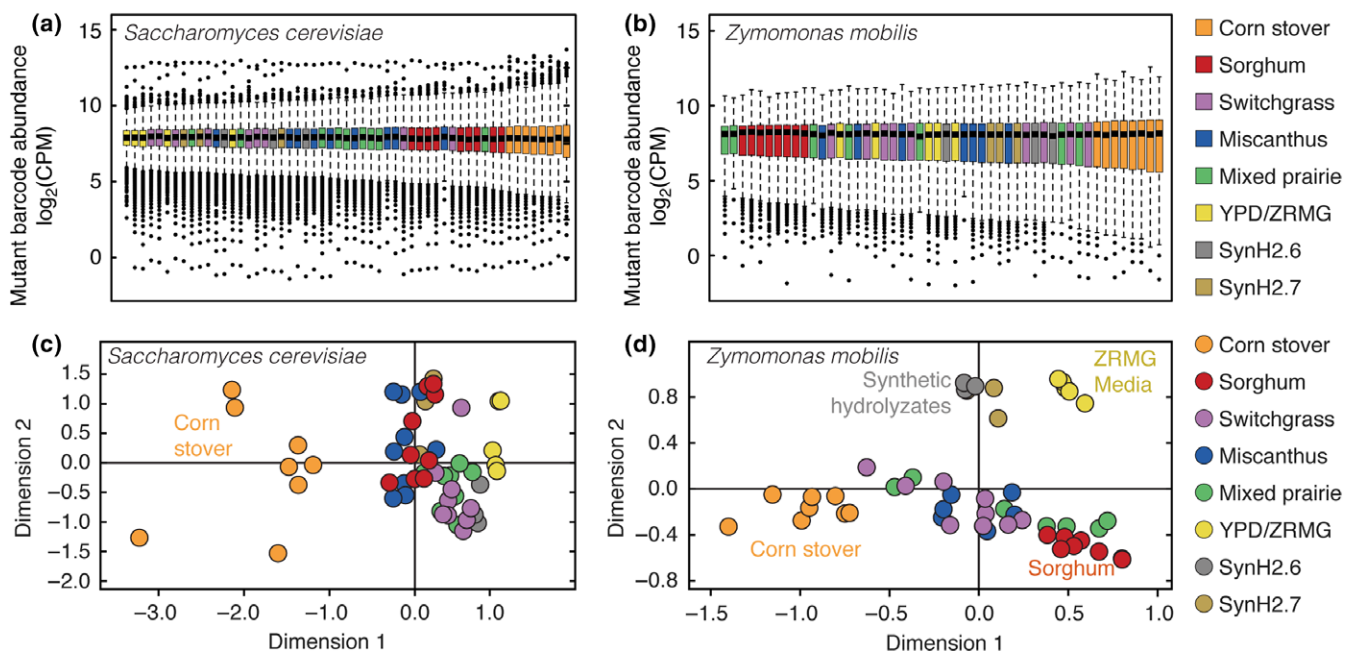
(58%) and the 2014 mixed prairie (67%) hydrolyzates across all feedstocks from all years (Figure 1b, Supporting Information Table S3). Xylose consumption was linearly

correlated with process ethanol yields for yeast fermentations (Figure 1d), and the 2014 corn stover and mixed prairie had the lowest xylose consumption of all the feedstocks evaluated (Figure 1d; Supporting Information Figure S1a,e; and Table S3). Together, these results indicate that yeast xylose fermentation was impaired in the 2014 corn stover and mixed prairie, and bacterial fermentation was also impaired in the 2014 corn stover hydrolyzate. This was one likely reason for lower process ethanol yields from these feedstocks. Notably, although the mixed prairie contained a mixture of herbaceous dicots (forbs) and grasses (Supporting Information Table S4), it was not significantly lower quality, and achieved similar ethanol yields compared to the other feedstocks investigated, particularly when *Z. mobilis* was used as the ethanologen.

### 3.2 | Yeast and bacterial fitness were significantly altered in corn stover hydrolyzate

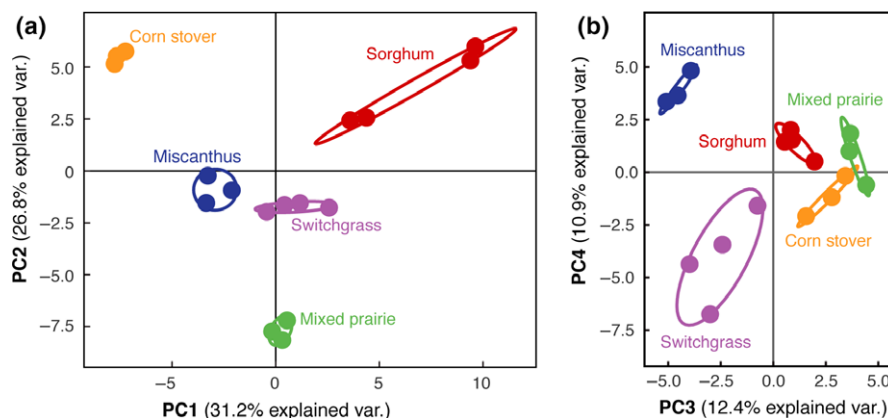
The impaired xylose utilization by both yeast and bacteria suggests that the quality of the 2014 corn stover hydrolyzate, in terms of its fermentability, may be lower compared to the four other feedstocks. Previously, we found that the Y128 yeast strain fermented xylose from hydrolyzates generated from AFEX-pretreated corn stover harvested in

multiple years (Figure 1d) (Ong et al., 2016; Parreiras et al., 2014; Serate et al., 2015), suggesting that this effect is specific to 2014 corn stover. Chemical genomic profiles were compiled for all five hydrolyzates to evaluate their relative fitness for microbial growth. Each showed a distinctive chemical genomic profile, with corn stover hydrolyzates clustered together in both the *S. cerevisiae* and *Z. mobilis* profiles (Supporting Information Figure S3). There was also a greater range in the relative fitness of *S. cerevisiae* and *Z. mobilis* mutants cultured in corn stover hydrolyzates compared to the other four hydrolyzates (Figure 2a,b). This indicates that, in general, any single gene deletion will have a greater negative or positive effect on a mutant's fitness in the corn stover hydrolyzate compared to the other four. This unique effect of the corn stover hydrolyzate on *S. cerevisiae* and *Z. mobilis* was also visualized using multidimensional scaling (MDS) plots generated from the chemical genomic data (Figure 2c,d). These show that the corn stover hydrolyzates segregated strongly from the other feedstocks by first and second dimension for *S. cerevisiae* and by first dimension for *Z. mobilis*. Together, these results support our fermentation results that the hydrolyzate from 2014 corn stover was a significantly different environment for microbial growth compared to the hydrolyzates generated from the four other feedstocks.



**FIGURE 2** Yeast and bacteria mutant fitness are more strongly affected in corn stover hydrolyzates compared to other 2014 feedstocks. Boxplots of mutant fitness ordered by length of the interquartile region reveal that corn stover hydrolyzates segregate with the greatest interquartile range for both *S. cerevisiae* (a) and *Z. mobilis* (b) mutants. Corn stover also segregates within multidimensional scaling (MDS) plots on fitness of both *S. cerevisiae* (c) and *Z. mobilis* (d) mutants. All experiments were cultured anaerobically. Dimensions in the MDS plot are based on the leading log<sub>2</sub>(FC). CPM = counts per million





**FIGURE 3** Principal component analysis of hydrolyzate composition shows each feedstock fully segregates. Miscanthus and switchgrass hydrolyzates have more similar compositions compared to the other feedstocks (a) and are only differentiated by components 3 and 4 (b)

### 3.3 | The 2014 corn stover and hydrolyzates contained elevated levels of hydroxycinnamic acids and their derivatives

The fermentation and chemical genomic studies indicated that the 2014 corn stover was of lower quality relative to the other feedstocks harvested that year, which may have been due to compositional differences in the hydrolyzates that affected xylose fermentation. Principal component analysis of these data showed that all five feedstocks had distinctive compositional signatures (Figure 3). However, the 2014 corn stover hydrolyzate was the most different of the five, as this segregated clearly through the first principal component (31% of the variance in the data). The second principal component (27% of variance) further segregated sorghum and mixed prairie, and the third and fourth principal components (12% and 11% of the variance, respectively) fully differentiated the miscanthus and switchgrass hydrolyzates, which appeared to be the most similar of the five based on their analyzed compositions.

A number of compounds contributed toward the segregation of corn stover from the other feedstocks, including four hydroxycinnamates that were present at comparatively high concentrations in the 2014 corn stover hydrolyzate compared to the others: ferulic acid, feruloyl amide, *p*-coumaric acid, and *p*-coumaroyl amide (Supporting Information Table S5, Files S1 and S2). The concentrations of the acids and amides in the untreated feedstocks and hydrolyzates were strongly correlated (Figure 4), which indicates that untreated feedstocks that have higher concentrations of hydroxycinnamates were more likely to release higher concentrations of hydroxycinnamates during enzymatic hydrolysis. For all hydrolyzates, a higher and consistent proportion of the amide form was present compared with the acid form: feruloyl amide (97%–99%) and *p*-coumaroyl amide (79%–88%). Untreated corn stover tended to have higher levels of hydroxycinnamates and correspondingly

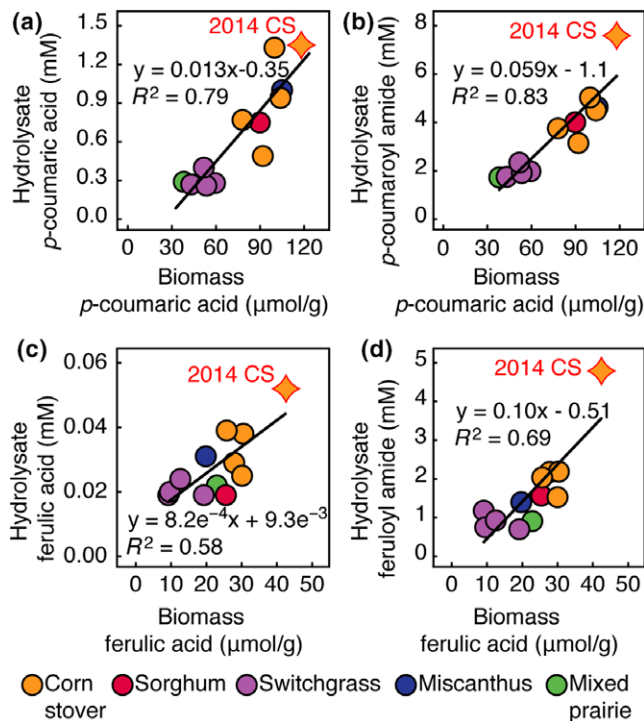
higher levels of the derivatives in the hydrolyzates compared to switchgrass. However, of all the feedstocks including other years of corn stover, the 2014 corn stover had the highest concentrations of ferulic and *p*-coumaric acid in the untreated biomass, and likewise the hydrolyzate contained the highest concentrations of ferulic and *p*-coumaric acids and amides (Figure 4). In particular, the ferulic acid levels in the untreated 2014 corn stover were more than 40% higher than the other feedstocks. These results indicate that, in terms of the sample set, the 2014 corn stover was unique in terms of the biomass and hydrolyzate hydroxycinnamate contents.

### 3.4 | Elevated hydroxycinnamates in 2014 corn stover hydrolyzate impair xylose fermentation by yeast

Specific amounts of ferulic and *p*-coumaric acids, and chemically synthesized feruloyl and *p*-coumaroyl amides, were added to noninhibitory 2012 corn stover hydrolyzate, elevating their concentrations equal to that found in the inhibitory 2014 corn stover hydrolyzate. The subsequent anaerobic yeast fermentations with 2012 corn stover hydrolyzate with and without supplementation of the phenolic compounds are shown in Figure 5b,c. In the unmodified 2012 corn stover hydrolyzate, the Y128 yeast strain fermented xylose at a rate similar to that observed previously (Ong et al., 2016). In 2012 corn stover hydrolyzate containing additional phenolic acids and amides, xylose fermentation by the Y128 strain was substantially reduced, similar to what was seen with 2014 corn stover hydrolyzate (Figure 5a).

When Y330 yeast (a less flocculant version of Y128) was cultured in SynHXS media (a synthetic hydrolyzate with only xylose as a carbon source) and supplemented with the individual phenolic inhibitors (0 to 13.8 mM), inhibition of xylose consumption was observed to be

mostly additive, with all four compounds contributing to the inhibition (Figure 6). The ferulates were more inhibitory than *p*-coumarates, particularly at low concentrations.

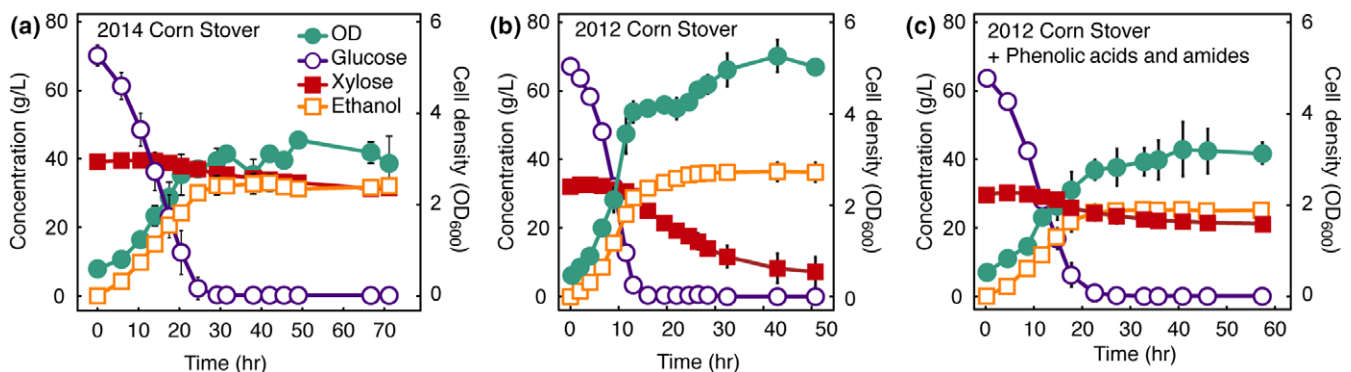


**FIGURE 4** Elevated hydroxycinnamates in the 2014 corn stover hydrolysate trace back to the untreated feedstock. The indicated phenolic compounds were quantified in hydrolysates from AFEX-pretreated biomass hydrolysates and the corresponding untreated biomass. Values reported are averages and standard deviations from technical triplicates. Hydrolysate values for feedstocks harvested in 2010, 2012, and 2013 were reported previously (Ong et al., 2016). Data for corn stover (36H56 and P0448R) and switchgrass (Shawnee and Cave-In-Rock (CIR)) included two different varieties. The data points for the 2014 corn stover are labeled as a red-outlined star

Ferulic acid was the most inhibitory compound tested, with ~20% inhibition of xylose consumption at the lowest concentration tested (1.9 mM) (Figure 6). Although less inhibitory than the acids, the amides were present in the hydrolysates at concentrations shown to inhibit xylose consumption (7.6 and 4.8 mM, for *p*-coumaroyl amide and feruloyl amide, respectively, vs. 1.3 and 0.05 mM for *p*-coumaric acid and ferulic acid, respectively) (Figure 6). The acid amide mix at 13.8 mM was significantly inhibitory toward xylose utilization, achieving only ~20% of the xylose consumption observed in the synthetic media. This is similar to the relative difference in % xylose consumption between the 2012 and 2014 corn stover, with the 2014 corn stover xylose consumption only 25% of the 2012 corn stover xylose consumption. Combined, these results indicate that the uncommonly elevated ferulic acid and *p*-coumaric acid levels in 2014 corn stover resulted in higher concentrations of hydroxycinnamates in the hydrolysates, which negatively affected yeast xylose utilization and ethanol production.

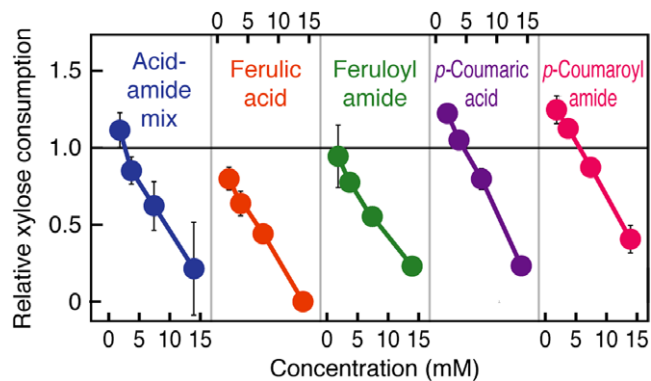
### 3.5 | Ethanol yields per hectare are largely determined by biomass productivity rather than biomass quality

Field-scale ethanol yields (L/ha) were calculated based on the amount of dry biomass harvested per hectare and the experimental data from yeast and *Z. mobilis* fermentations (Figure 7 and Table 1). Ethanol yields for yeast fermentations ranged from 740 to 3,618 liters of ethanol per hectare, with the exception of drought-stressed 2012 switchgrass, which was too inhibitory to sustain yeast growth. Ethanol yields for *Z. mobilis* fermentations were slightly higher than those obtained for *S. cerevisiae* and ranged from 802 to 4,073 L ethanol/ha. Ethanol yield per



**FIGURE 5** Elevated hydroxycinnamates impair anaerobic xylose fermentation by yeast. The Y128 yeast strain was cultured anaerobically in bioreactors containing hydrolysate from 2014 corn stover (a), and 2012 corn stover without (b) or with the addition of ferulic and *p*-coumaric acids and feruloyl and coumaroyl amides to equal the final concentrations in 2014 corn stover hydrolysate (c). Graphs display average cell densities ( $OD_{600}$ , teal circles), and extracellular glucose (purple circles), xylose (red diamonds), and ethanol (orange diamonds) with standard deviations from three independent biological replicates

hectare from 2014 corn stover was higher than the median (1,642 L/ha) for *Z. mobilis* fermentations and at the median (1,603 L/ha) for *S. cerevisiae* fermentations, and were within the range expected based on linear regression of the data (Figure 7). With the exception of drought-year (2012) switchgrass, which was completely unfermentable for reasons previously identified (Ong et al., 2016), field ethanol yields were highly correlated with biomass yields based on experimental data for both *S. cerevisiae* ( $R^2 = 0.95$ ) and *Z. mobilis* ( $R^2 = 0.95$ ) fermentations (Figure 7).

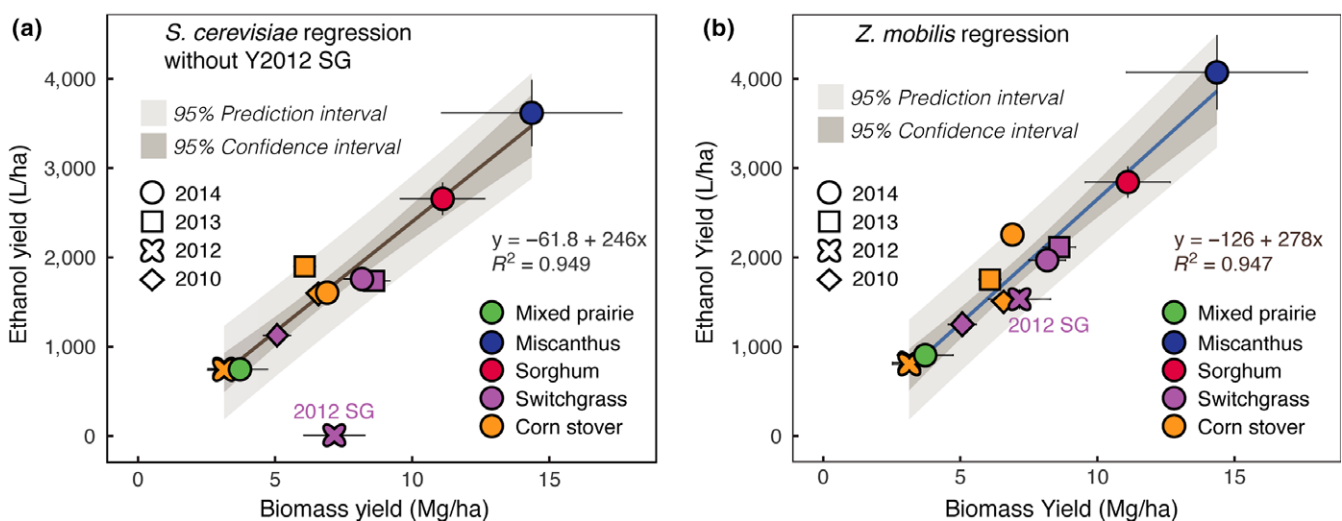


**FIGURE 6** Hydroxycinnamates inhibit yeast xylose consumption. A nonflocculant derivative of the Y128 strain was cultured anaerobically in 24-well plates containing synthetic SynHXS media (see Methods) and the indicated concentration of phenolic compounds. Each compound or compound mix was added at 1.9, 3.8, 7.5, and 13.8 mM. For the acid amide mix, the ratios for each compound were identical to the ratio of concentrations in 2014 corn stover hydrolyzate (0.4% ferulic acid, 9.8% *p*-coumaric acid, 34.8% feruloyl amide, and 55.1% *p*-coumaroyl amide). Xylose consumption was determined by the differences in initial and final extracellular xylose concentrations for each condition relative to SynHXS without the addition of phenolic compounds

The ethanol yield (L/ha) resulting from an increase in biomass productivity of 1 Mg/ha for each feedstock was compared to the calculated increase in biomass ethanol yield (L/Mg of dry biomass) that would be necessary to achieve this same field-scale ethanol yield without increasing biomass productivity. The calculated biomass ethanol yield increase plotted against the actual biomass yield showed that the two parameters were highly correlated via an inverse power law function (Figure 8). This result indicates that biomass quality and yield improvements have different effects on field-scale ethanol yields, depending on whether the biomass has low or high productivity. At low biomass productivities, biomass yield increases have a greater impact on field-scale ethanol yields compared with improvements in biomass quality or conversion efficiencies, whereas the opposite is true at high biomass productivities. For example, increasing the biomass yield of the 2014 mixed prairie from 3.7 to 4.7 Mg/ha would increase the ethanol yield (L/ha) by an amount that is comparable to increasing the biomass ethanol yield by 54 or 65 L/Mg (depending on the microorganism). In contrast, miscanthus, which already has a high productivity of 14.4 Mg/ha, would only require an increase in biomass ethanol yield of 18–20 L/Mg to equal a biomass yield increase of 1 Mg/ha.

## 4 | DISCUSSION

Recently, there has been interest in generating renewable biofuels in an economical, as well as an environmentally and socially sustainable manner (Robertson et al., 2017). Industrial biofuel processing facilities have generally focused on using one type of feedstock that is of high quality and is also highly productive. However, there are a number of potential bioenergy feedstocks that can achieve



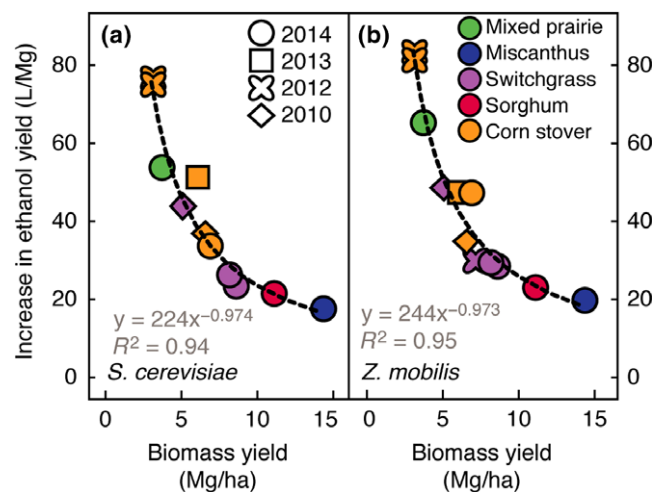
**FIGURE 7** Experimentally determined ethanol yields per hectare correlate with biomass yield. (a) *S. cerevisiae* linear regression with 2012 SG excluded from the regression analysis, and (b) *Z. mobilis* linear regression

**TABLE 1** Summary of ethanol yield data for *S. cerevisiae* Y128 and *Z. mobilis* 8b fermentations of five feedstocks. Data are averages  $\pm$  standard deviation. Ethanol yield standard deviations were calculated using propagation of error. Data on feedstocks from years other than 2014 are from (Ong et al., 2016). Dry matter yields for the actual corn stover plots were unavailable and instead were estimated based on the values for the GLBRC BCSE plots. Both were planted at the same site in Arlington, Wisconsin

Feedstock	Harvest year	Dry matter yield (Mg/ha)	<i>Saccharomyces cerevisiae</i>		<i>Zymomonas mobilis</i>	
			Ethanol yield (L/Mg untreated feedstock)	Ethanol yield (L/ha)	Ethanol yield (L/Mg untreated feedstock)	Ethanol yield (L/ha)
Corn Stover 36H56	2010	6.58 $\pm$ 0.45	243 $\pm$ 6	1,600 $\pm$ 52	230 $\pm$ 4	1511 $\pm$ 47
Corn Stover 36H56	2012	3.14 $\pm$ 0.61	241 $\pm$ 19	756 $\pm$ 71	262 $\pm$ 2	822 $\pm$ 72
Corn Stover P0448R	2012	3.14 $\pm$ 0.61	236 $\pm$ 7	740 $\pm$ 65	255 $\pm$ 5	802 $\pm$ 70
Corn Stover P0448R	2013	6.08 $\pm$ 0.40	312 $\pm$ 2	1,897 $\pm$ 56	288 $\pm$ 6	1752 $\pm$ 54
Corn Stover P0448R	2014	6.90 $\pm$ 0.36	233 $\pm$ 9	1,605 $\pm$ 47	327 $\pm$ 22	2,255 $\pm$ 85
Switchgrass	2010	5.07 $\pm$ 0.51	223 $\pm$ 3	1,128 $\pm$ 51	246 $\pm$ 7	1,249 $\pm$ 58
Switchgrass	2012	7.16 $\pm$ 1.13	1.1 $\pm$ 0.1	8 $\pm$ 1	214 $\pm$ 5	1532 $\pm$ 109
Switchgrass	2013	8.61 $\pm$ 0.59	202 $\pm$ 3	1,737 $\pm$ 54	245 $\pm$ 3	2,114 $\pm$ 65
Switchgrass	2014	8.17 $\pm$ 0.67	215 $\pm$ 12	1,757 $\pm$ 79	241 $\pm$ 2	1968 $\pm$ 73
Sorghum	2014	11.11 $\pm$ 1.55	239 $\pm$ 15	2,656 $\pm$ 182	256 $\pm$ 2	2,843 $\pm$ 177
Miscanthus	2014	14.36 $\pm$ 3.30	252 $\pm$ 2	3,618 $\pm$ 373	284 $\pm$ 2	4,073 $\pm$ 419
Mixed prairie	2014	3.72 $\pm$ 1.02	200 $\pm$ 10	747 $\pm$ 93	243 $\pm$ 20	905 $\pm$ 116

high biomass yields in the eastern United States (Lee et al., 2018; Sanford et al., 2016). If a lignocellulosic biorefinery could accommodate multiple feedstocks, there would be many potential benefits for both the biorefinery and the surrounding landscape. Logistical benefits include reductions in the area required for biomass procurement with resulting reductions in transportation costs and emissions (Maung et al., 2013) and the possibility of staging harvest timing to reduce biomass storage requirements (Rentizelas, Tolis, & Tatsiopoulos, 2009). Having the capability to process a variety of materials also provides resiliency against abnormally low biomass yields, allowing the refinery to switch feedstocks if some are in short supply. Environmental benefits from implementing landscape feedstock diversity can include soil carbon sequestration, climate stabilization, water quality (Oates, Duncan, Gelfand, et al., 2016; Robertson et al., 2008; Robertson, Hamilton, Grosso, & Parton, 2011), as well as reduced N<sub>2</sub>O emissions (Oates, Duncan, Gelfand, et al., 2016), and nitrate losses (Donner & Kucharik, 2008; Duran, Duncan, Oates, Kucharik, & Jackson, 2016) that occur with a reduction in fertilizer requirements and greater nutrient use efficiency (Ruan, Bhardwaj, Hamilton, & Robertson, 2016). Greater crop diversity in the landscape, particularly in the form of an increased proportion of perennials, provides increased wildlife and insect habitat and accrues associated biodiversity

benefits such as pest suppression and pollination services (Bennett & Isaacs, 2014; Werling et al., 2014; Werling, Meehan, Robertson, Gratton, & Landis, 2011) and plant-associated soil microbes (Oates, Duncan, Sanford, Liang, & Jackson, 2016). Adding perennial crops can also increase belowground C inputs and the attendant reduction in C debt (Robertson et al., 2017). These benefits may improve long-term yield stability and allow the use of marginal lands to increase yield potential (Gelfand et al., 2013). Perennials can also be strategically added to sensitive or low-productivity regions within the landscape, such as marginal field sites, riparian buffers, and in-field erosion strips (Bonner, Muth, Koch, & Karlen, 2014; Gopalakrishnan, Cristina Negri, & Salas, 2012; Ha & Wu, 2015; Ssegane, Negri, Quinn, & Urgun-Demirtas, 2015). This incorporation of perennials into the landscape would avoid competition with food production while maintaining economic productivity and providing most of the ecosystem services that would be gained by completely replacing annual crops with perennials (Asbjornsen et al., 2014; Lautala et al., 2015). Feedstock diversity can also improve harvest logistics and reduce overall labor and equipment requirements using the same equipment to harvest multiple feedstocks at different times during the growing season. By moving from a single feedstock to three feedstocks with different harvest windows, the refinery could save an expected 25% of the



**FIGURE 8** Improvements in biomass productivity are more important for increasing field-scale ethanol yields at low biomass productivity, whereas biomass quality improvements have a greater impact at high biomass productivity. The graphs show the increase in biomass ethanol yield required to match the field-scale ethanol yield resulting from a 1 Mg/ha increase in productivity for each feedstock for *S. cerevisiae* (a) and *Z. mobilis* (b) fermentations. Power law regressions were fit to the data points. The *S. cerevisiae* figure does not include 2012 switchgrass, which was unfermentable

total logistics costs (Correll, Suzuki, & Martens, 2014). A broad range of cropping systems would also provide greater economic opportunities for rural communities and may increase food security through better integration of lignocellulosic biomass and animal feed production (Dale et al., 2014).

To evaluate whether there are any major differences in fermentation efficiency and ethanol yields from key herbaceous lignocellulosic feedstocks, we investigated the fermentability of five feedstocks that had been processed using AFEX pretreatment and high solid enzymatic hydrolysis. These feedstocks were all harvested at the same location in 2014 and included both annual crops and residues (corn stover and energy sorghum) and perennial mono- and polyculture feedstocks (switchgrass, miscanthus, and mixed prairie). Each feedstock was separately fermented using a yeast, *S. cerevisiae* Y128, or bacteria, *Z. mobilis* 8b, both of which have been engineered to utilize xylose. Additional information on the cropping systems used in these experiments can be found in a number of papers that evaluate crop yields (Sanford et al., 2016), biomass characteristics and predicted ethanol yields (Sanford et al., 2017), potential farm-level economic returns (Skevas, Swinton, Tanner, Sanford, & Thelen, 2016), and environmental impacts of the cropping systems including nitrous oxide emissions during establishment (Oates, Duncan, Gelfand, et al., 2016), and differences in soil microbial communities (Oates, Duncan, Sanford, et al., 2016).

Our findings ultimately depended on the ethanol yield metric that was used to compare the feedstocks. Process ethanol yields represent the efficiency of the microbes to convert sugars to ethanol during fermentation. The process ethanol yields are affected by the resiliency of the microorganism, initial sugar concentrations in the hydrolyzate, and any inhibitors that may also be present. These latter two parameters are in turn dictated by the interaction between the untreated feedstock composition and the pretreatment method used to increase access to the sugars. In contrast, field-scale ethanol yield represents the ethanol production potential per land area, often in L/ha. This metric encompasses both biomass yield and feedstock quality in terms of the ability to achieve a high process ethanol yield. We have previously reported that field-scale ethanol yield was more dependent on biomass productivity (Mg/ha) than biomass quality (digestibility) for a variety of feedstocks (Sanford et al., 2017). However, this finding was based on constant defined process ethanol yields (i.e., metabolic yields) for each feedstock (0.931 and 0.897 g ethanol per g sugar consumed for corn stover, and all perennials, respectively). These field-scale ethanol yields therefore included experimental variability due to enzymatic hydrolysis but not fermentation. However, our experimental fermentation data also revealed that biomass yield is a key driver of field-scale ethanol yield, as indicated by the strong linear correlation between the two values across feedstocks. The relative influence of biomass quality (ability to generate high ethanol yield from a given feedstock) and biomass productivity also varied in a consistent manner across all of the feedstocks. When the total biomass produced is low, increasing biomass productivity, as opposed to increasing the biomass ethanol yield (L/Mg), has a larger effect on the field-scale ethanol yield. As biomass productivity increases the opposite holds true; each incremental increase in productivity has less of an impact compared to potential improvements in biomass ethanol yields (L/Mg). From a grower's or breeder's perspective, although both productivity and quality influence the results and should ideally be improved simultaneously, in the event this is not possible, it would be more efficient to focus on strategies to improve the ethanol yield for specific crops. For crops that are already highly productive, such as miscanthus, instead of targeting improvements in crop yield, it may instead be more efficient to increase the ethanol yield by improving biomass quality and/or conversion efficiency. In contrast, for feedstocks that are low yielding, it may be more efficient to increase crop yield rather than targeting improvements in biomass quality or conversion. That said, biomass yield is still critically important for the farmer and perennial crops are inherently risky, with extremely high breakeven yields to achieve the same economics compared to corn (20–100 Mg/ha) (Skevas et al., 2016).

Although field-scale ethanol yield is important for growers and breeders of lignocellulosic crops, process ethanol yield is more important for the biorefinery as it is a key factor affecting the minimum ethanol selling price (MESP), the main metric of biorefinery economic sustainability (Vicari et al., 2012). From our results, the range and variability in the process ethanol yield were highly dependent on the microbe used for fermentation. *S. cerevisiae* fermentations tended to have lower process ethanol yields and greater variability (58%–75%) compared to *Z. mobilis* (81%–84%) (Figure 1). In our previous study, we found that for these particular strains, *Z. mobilis* is significantly more resistant to inhibitors and generates higher process ethanol yields compared to *S. cerevisiae* Y128 (Ong et al., 2016). Process yields also showed similar levels of variability whether compared between feedstocks or between years. Evaluation of the 2014 feedstocks showed that corn stover from that year had significant inhibition of xylose consumption that resulted in process yield losses for that feedstock. In another study, process ethanol yields from glucose and xylose contributed ~25% of the uncertainty in MESP calculations (Vicari et al., 2012). Efficient microbial utilization of both glucose and xylose will be important to achieve high process ethanol yields and low MESP. There are a number of ways to increase biomass ethanol yield including: increasing the biomass polymeric sugar content, increasing the sugar released during enzymatic hydrolysis, or increasing the process ethanol yields during fermentation. These changes can be attained either through biomass or process modifications. Identifying biomass characteristics that contribute to inefficient conversion is critical to designing feedstocks or modifying processes to overcome these limitations and achieve high yields. We were able to utilize a tiered approach to confirm the uniqueness of the 2014 corn stover compared to the other hydrolyzates and identify the inhibitors responsible for the poor xylose utilization. During this analysis, we discovered that untreated corn stover harvested in 2014 contained higher than average concentrations of the hydroxycinnamates, ferulic acid, and *p*-coumaric acid. During AFEX pretreatment, these were largely converted to their amide forms (feruloyl amide and *p*-coumaroyl amide). Further experiments revealed that the ferulates and the acid forms of the hydroxycinnamates were more inhibitory of *S. cerevisiae* xylose utilization compared to the *p*-coumarate and amide forms, respectively. This may indicate that although the AFEX-pretreated biomass generated amides that inhibited xylose fermentation, it may be less inhibition than would be caused by an acidic pretreatment that leaves these compounds predominantly in the acid form (Chundawat et al., 2010). A previous study found a similar effect on yeast xylose utilization for the different hydroxycinnamate derivatives, with ferulic acid having the strongest negative

effect (Tang et al., 2015), though the mechanism of inhibition in yeast has yet to be determined. In *E. coli*, feruloyl amide has been shown to impair xylose metabolism by inhibiting glutamine PRPP amidotransferase (PurF), the first step in de novo purine biosynthesis (Pisithkul, Jacobson, O'Brien, Stevenson, & Amador-Noguez, 2015). This may also be the mechanism for the inhibition of *Z. mobilis* xylose utilization in the 2014 corn stover hydrolyzate.

It is unclear as to the reason for the elevated hydroxycinnamate levels in the 2014 corn stover compared to the other years investigated for the same hybrid (2012 and 2013). Maize pest herbivory has been shown to cause an increase in hydroxycinnamate content (Santiago et al., 2017), and it may be that pest infestation of the maize grown in 2014 was higher compared with other years, although we have no data on pest infestation to support or disprove this hypothesis. Another possibility is that environmental conditions, such as temperature and precipitation, affected the quality of the corn biomass during that growing season. This is similar to what we found for switchgrass grown and harvested during 2012, a major drought year in the Midwest. The switchgrass from 2012 built up high concentrations of soluble sugars due to drought stress that were converted by AFEX pretreatment to pyrazines and imidazoles, which completely blocked yeast, but not *Z. mobilis*, growth, and glucose fermentation (Ong et al., 2016).

Together, these results suggest that cellulosic biofuel producers can assess for high concentrations of hydroxycinnamates or soluble sugars in their untreated biomass and subsequently make decisions on how to ferment their feedstock. Although the inability of yeast to ferment xylose from 2014 corn stover had minimal impact on ethanol yield per hectare, this limitation still affected the process ethanol yield. Thus, additional measures can be made to maximize ethanol production. Based on our results, it may be desirable to improve biomass quality by reducing the biomass hydroxycinnamate content, which can be accomplished by repressing expression of certain BAHD acyltransferase genes (de Souza et al., 2018; Molinari, Pellny, Freeman, Shewry, & Mitchell, 2013). This would be expected to have a positive effect on fermentation performance by reducing the concentration of hydroxycinnamates in the biomass and resulting hydrolyzate, but may come at the expense of feedstock viability. Higher hydroxycinnamate contents have been shown to increase plant resistance to herbivory and pathogen infestation, and reductions in these levels can lead to losses in crop yields (Barros-Rios, Santiago, Jung, & Malvar, 2015; Buanafina & Fescemyer, 2012; Reem et al., 2016; Santiago, Barros-Rios, Alvarez, & Malvar, 2016). Alternatively, bioethanol producers could utilize *Z. mobilis* or other tolerant microbial ethanologens, or reengineer yeast with greater tolerance to hydroxycinnamates and other lignocellulose derived inhibitors, such as has been done with *E. coli*

(Sariaslani, 2007). This would likely require minimal modifications to processing but may require more significant research investment to identify key genes related to improved resistance and engineer tolerant strains. Alternatively, cellulosic plant operators may opt to perform additional hydrolyzate conditioning to remove high concentrations of ferulic acid or other phenolic inhibitors prior to fermentation (Tomek et al., 2015). One option is to use an ammonia extraction step that is able to remove the majority of hydroxycinnamates (da Costa Sousa, Foston, et al., 2016; Mittal et al., 2017). The benefit of this approach is twofold. First, the resulting biomass is significantly more digestible (da Costa Sousa, Jin, et al., 2016), and second, the hydroxycinnamates could be sold or biologically upgraded into value-added products (Linger et al., 2014).

## 5 | CONCLUSIONS

We have provided evidence supporting the idea that multiple plant types with a range in feedstock quality can be used without a major impact on field-scale ethanol yields. Instead of being overly concerned about biomass quality, as long as biomass yields are low, feedstock producers can focus on increasing productivity. In contrast, for feedstocks that are already high-yielding, it may be more efficient to focus on improving feedstock quality, which can have a major impact on the biorefinery by increasing process ethanol yields and lowering the MESP. In our study, with a few notable exceptions, most feedstocks showed very similar process ethanol yields across multiple harvest years. Although most of the feedstocks were grasses, they all have very different characteristics (morphology, chemical composition, etc.), so the similarity in their yields is encouraging. It seems likely that bioethanol producers can be somewhat feedstock agnostic in terms of the materials they are able to take in and process. Together, this data provide evidence that the biofuel industry could successfully produce and process multiple sources of lignocellulosic feedstocks, which could achieve key social, economic, and environmental goals by increasing supply while offering numerous ecosystem services. As an added benefit, the industry can accomplish these goals while avoiding the use of food/feed crops such as corn grain, without significant impacts on net bioethanol production.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## AUTHOR CONTRIBUTIONS

YZ, DC, DE, LGO, JSP, JJC, JR, RGO, GRS, and TKS designed the project and experiments. YZ, YB, MKY, AH, LGO, TKS, and RGO wrote the manuscript with input from all authors. DE, GRS, YZ, JS, DX, EP, JP, MKY, AH, and SDK performed experiments. YB and RGO performed computational data analysis.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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