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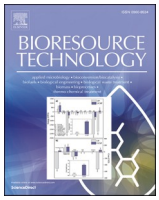

pH adjustment increases biofuel production from inhibitory switchgrass hydrolysates

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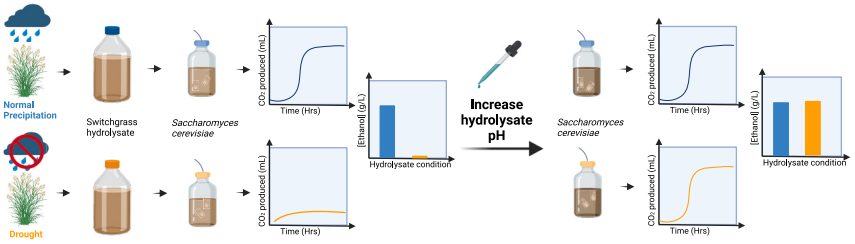
HIGHLIGHTS

- Interventions were tested to improve biofuel production from inhibitory feedstocks.
- A bench scale Soaking in Aqueous Ammonia (SAA) pretreatment method was developed.
- SAA and AFEX pretreatments generate hydrolysates with similar sugar titers.
- SAA pretreatment leads to more acids, while AFEX leads to more amides in hydrolysates.
- Higher fermentation pH increases biofuel titers from inhibitory hydrolysates.

GRAPHICAL ABSTRACT



ABSTRACT

Biofuels derived from renewable and sustainable lignocellulosic biomass, such as switchgrass, offer a promising means to limit greenhouse gas emissions. However, switchgrass grown under drought conditions contains high levels of chemical compounds that inhibit microbial conversion to biofuels. Fermentation of drought switchgrass hydrolysates by engineered *Saccharomyces cerevisiae* and *Zymomonas mobilis* results in lower ethanol production than does fermentation of hydrolyzed switchgrass from a typical rainfall year. Here, it is demonstrated that this inhibitory effect can be alleviated by altering the pH of drought switchgrass hydrolysates produced by two different pretreatment methods: Ammonia Fiber Expansion (AFEX) and Soaking in Aqueous Ammonia (SAA). Fermentation rates and biofuel production by *Saccharomyces cerevisiae* and *Zymomonas mobilis* were higher at pH 5.8 than at pH 5.0 from all feedstock years and following both pretreatment methods. SAA pretreatment of drought switchgrass furthermore enabled increased fermentation rates and biofuel titers compared to AFEX pretreatment. A synthetic mimic of switchgrass hydrolysate was developed and identified relief from pH-dependent inhibition by lignocellulose-derived inhibitors as the cause of increased biofuel production above a pH of 5.0. These results demonstrate that SAA pretreatment and pH adjustment can significantly improve fermentation and biofuel production from inhibitory feedstocks by industrial microorganisms.

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1. Introduction

Lignocellulosic-derived biofuels are being investigated as a sustainable and renewable solution for combatting climate change and reducing greenhouse gas emissions. Although electrification has provided an alternative carbon–neutral energy source to replace traditional liquid automobile fuels, electrification is less feasible for the aviation industry. Sustainable aviation fuels (SAF) generated from alcohol-to-jet (ATJ) processes are one of many proposed approaches to decarbonize the aviation sector (Geleynse et al., 2018; Holladay et al., 2020; Tiwari et al., 2023). These alcohols include ethanol, which can be catalytically converted into longer chain ethers, olefins, and paraffins (Xie et al., 2024), as well as isobutanol, which can be converted to branched alkenes and alkanes, and cyclic molecules (Geleynse et al., 2018; Holladay et al., 2020). Both ethanol and isobutanol can be produced through microbial fermentation of renewable lignocellulosic feedstocks. Potential plant feedstocks include switchgrass, a drought- and heat-tolerant grass species with resistance to insects and disease that can grow on agricultural land unsuitable for food production (Rinehart, 2006; Keshwani & Cheng, 2009; Larnaudie et al., 2022). Moreover, switchgrass creates over 500 % more energy than is used to produce it (Schmer et al., 2008), making switchgrass a promising feedstock for ATJ biofuels.

Like all living organisms, switchgrass responds to both abiotic and biotic stressors, which induce physiological responses that affect their biomass compositions. For example, others have studied the influence of nitrogen application rate, harvest year, and field location on switchgrass composition (Hoover et al., 2022). Other stressors, including extreme temperatures, floods, and drought also have downstream consequences on biofuel production by inhibiting conversion microbes such as the yeast *Saccharomyces cerevisiae* and bacterium *Zymomonas mobilis*. In 2012, a severe drought in the Midwest (Jin et al., 2019; Mallya et al., 2013) impacted the composition of switchgrass grown in Wisconsin (Ong et al., 2016; Chipkar et al., 2022). When deconstructed with Ammonia Fiber Expansion (AFEX) pretreatment and enzymatic hydrolysis, the 2012 switchgrass proved highly inhibitory to fermentation by *S. cerevisiae* but not by *Z. mobilis* (Ong et al., 2016; Chandrasekar et al., 2021; Chipkar et al., 2022). This effect was not observed in fermentations of deconstructed switchgrass grown in 2010 and 2016, which experienced higher seasonal rainfall. Chemical analysis of the deconstructed 2012 switchgrass contained higher levels of inhibitory compounds including pyrazines, imidazoles, and saponins (Ong et al., 2016; Chipkar et al., 2022). This result suggests that one or more compounds present at higher concentrations in 2012 switchgrass hydrolysates causes these inhibitory effects on yeast fermentation in a potentially additive fashion.

In addition to these inhibitory compounds, deconstructed switchgrass hydrolysates contain numerous other chemical compounds that have varying effects on microbial fermentation and biofuel production. Some of these inhibitory compounds are directly produced by the plants (Orczyk et al., 2020; Chipkar et al., 2022) whereas others, such as phenolic compounds, are generated during the pretreatment process (van der Pol et al., 2014). The mechanisms by which these compounds inhibit biofuel-producing yeast and bacteria are only somewhat understood (Díaz et al., 2001; Cunha et al., 2018; Diderich et al., 2018). Some known inhibitory compounds, such as weak acids (acetic acid), furans (furfural), and phenolic acids (ferulic and *p*-coumaric acids), can damage membranes, decrease cellular pH, and induce depletion of cellular ATP (Piotrowski et al., 2014; Vanmarcke et al., 2021). Phenolic compounds are known to have various inhibitory effects on cellular growth and ethanol yield (Adeboye et al., 2014). To overcome some of these inhibitors, others have shown that increasing the pH of some lignocellulosic hydrolysates with the addition of potassium, can alleviate inhibitory effects of furfural and acetic acid, resulting in increased ethanol production (Lam et al., 2021).

Here, two potential interventions were explored to alleviate the inhibitory effect of drought switchgrass hydrolysates on downstream microbial fermentation: an alternative Soaking in Aqueous Ammonia (SAA) pretreatment method, and pH adjustment of hydrolysates. We hypothesized that increasing the pH of hydrolysate to at or near the pK_a of some key acidic inhibitors would have beneficial effects on microbial fermentation. Switchgrass from drought (2012) and normal precipitation years (2010 and 2016) were pretreated using both AFEX and SAA methods followed by enzymatic hydrolysis. The pH of hydrolysates produced using both pretreatment methods was adjusted to 5.0 and 5.8, based on previous research (Serate et al., 2015), and these feedstocks were fermented by both *S. cerevisiae* and *Z. mobilis*. It was determined that pH adjustment enabled substantially higher biofuel production rates from both hydrolysates, as well as from two formulations of synthetic switchgrass hydrolysate. Additionally, a clear distinction between the compositions of the hydrolysates from the two different pretreatment processes was found, while still yielding similar sugar titers.

2. Materials and methods

2.1. Feedstocks

Switchgrass grown at the Arlington Research Station, Arlington WI, in 2010, 2012, and 2016 between May to October were harvested, dried, and milled to 5 mm particle size. Growing conditions, cultivation, and milling were as previously described (Serate et al., 2015; Ong et al., 2016; Zhang et al., 2020). The drought in 2012 occurred between June to September, which was also when the highest 30-year averages for precipitation occurred during the growing season (Ong et al., 2016). Glucan contents (%) for the 2010, 2012, and 2016 switchgrass were previously determined to be 34.8 %, 30.5 %, and 34.5 %, respectively (Ong et al., 2016; Zhang et al., 2020).

2.2. Biomass pretreatment

AFEX pretreatment of switchgrass was described previously for the 2010 and 2012 harvests (Serate et al., 2015) and for the 2016 harvest (Zhang et al., 2020). The Soaking in Aqueous Ammonia (SAA) pretreatment method was developed based on a previously published patent (Dunson et al., 2007) and is described in Fig. 1. 15 g of milled switchgrass were transferred into 120 mL glass pressure tubes (Chemglass, Cat# CG 1880–06). 60 mL of 18 % diluted ammonium hydroxide (28.0 to 30.0 % (w/w) purity, Fisher Chemical, Cat# A669-212) was slowly added to each tube, with mild agitation if needed, and the tubes were then sealed tightly. Sealed tubes were left upside down for 1–2 min and then mixed by inversion to ensure biomass saturation. Tubes were then placed in a rotator set at 19 RPM within a hybridization oven (ProBlot) set at 75 °C. After 1.5 hr incubation, tubes were removed and mixed, then returned to the 75 °C oven for an additional 2 hr of incubation (3.5 hr total incubation time at 75 °C). After incubation at 75 °C for 3.5 hr, tubes were placed inside of a fume hood for 30 min to cool. After cooling, the caps were carefully unscrewed to release the pressure.

SAA-pretreated samples were dried in two stages. First, pressurized drying with compressed air was performed using clear silicone tubing that connects house air to a rotary meter set to ~ 20 L/min. A metal spatula was used to make a gap in the center of the biomass to the bottom of the glass tube. An end of silicone tubing was placed into the gap ~ 0.25 in. from the bottom of the glass tube to blow forced air into the sample. After 48 hr of pressurized drying, the pretreated biomass was transferred to weigh boats and further dried in a fume hood for 5 days. Dried SAA-pretreated biomass was then prepared for enzymatic hydrolysis or transferred to a glass bottle for storage. A minimum of 0.5 g of dried SAA-pretreated biomass was used for moisture content analysis (Ohaus MB35 Moisture Analyzer).

2.3. Enzymatic hydrolysis of pretreated switchgrass

Enzymatic hydrolysis was carried out as described in Fig. 1. AFEX-

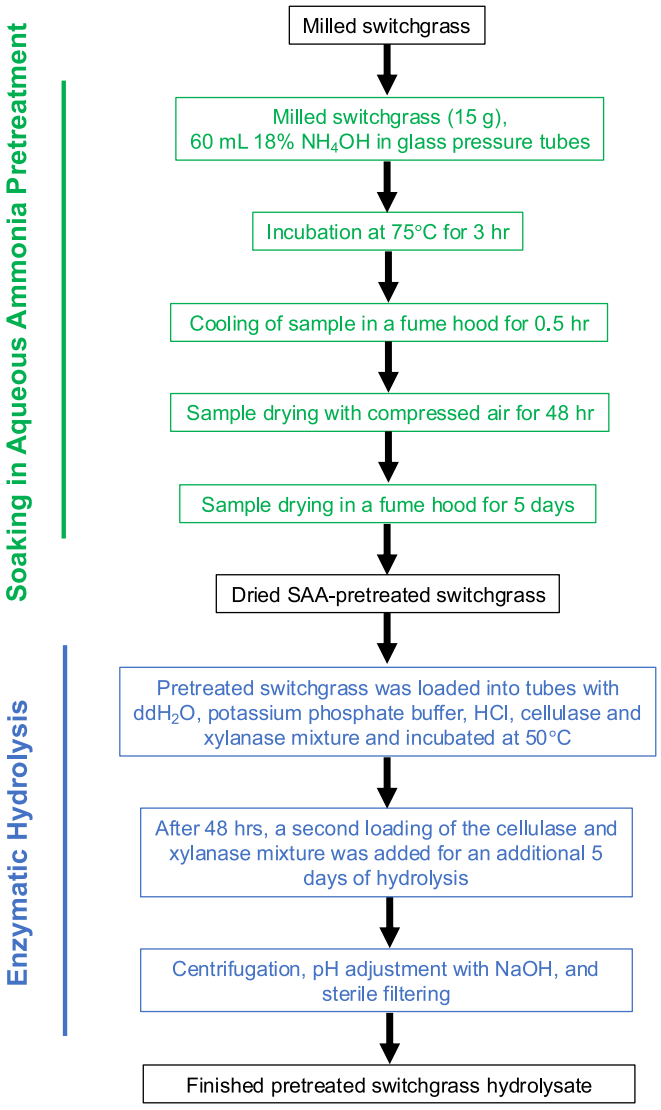

Fig. 1. Soaking in Aqueous Ammonia (SAA) pretreatment and enzymatic hydrolysis can be customized for small-scale hydrolysate production. A flowchart provides detailed methods used for SAA (in green) and enzymatic hydrolysis (in blue) of switchgrass used in this study.

and SAA-pretreated switchgrass samples were loaded into 85 mL Oak Ridge tubes (Thermo Scientific, Nalgene, Cat# 3118–0085) for 7 % glucan loading in 50 mL total volume. Pretreated biomass was brought to 32.7 mL with double-deionized water (ddH_2O) and mixed with 5 mL 1 M potassium phosphate buffer to stabilize the pH, and then autoclaved. After the biomass was cooled, 150 µL of HCl (~37–38 % w/v) was added and mixed, and then 5.8 mL of a sterile-filtered enzyme mixture (1 mL cellulase cocktail, Novozymes NS 22257; 0.17 mL xylanase cocktail, Novozymes NS 22244; 4.7 mL ddH_2O) was added. Sealed tubes were mixed and incubated on a rotator at 50 °C. After 2 days of hydrolysis, a second loading of the 5.8 mL enzyme mixture, composed of the same enzymes as described above, was added to the SAA-pretreated biomass, whereas the AFEX-pretreated material received 5.8 mL of ddH_2O. After 5 additional days of hydrolysis at 50 °C, the hydrolysate was centrifuged at 15,000 x *g*, pH adjusted to 5.0 with 10 N NaOH or 37 % HCl, and then sterile filtered with a 0.2-µm filter unit (Nalgene). When needed, half of the volumes of each hydrolysate at pH 5.0 were further adjusted to pH 5.8 with NaOH.

2.4. Analysis of hydrolysate chemical compositions

Detection and quantification of chemical compounds in hydrolysates were determined by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) as described previously (Zhang et al., 2020). In brief, hydrolysate samples were diluted ten-fold with chilled 2.5 µM vanillin-$^{13}C_6$ in water, mixed, centrifuged at 14,000 x g for 2 min at 4 °C, and then the supernatant was transferred to an amber glass autosampler vial with glass vial insert for LC-MS/MS analysis. Standard calibration curves for quantitation of 84 chemical compounds were generated with twelve total standard concentration levels ranging from 0.024 µM to 50 µM. Each standard level was transferred to a low volume amber autosampler vial with insert and then placed into an autosampler at 4 °C for injection.

LC-MS/MS analyses were performed with a 5 µL injection volume by Vanquish Split Sampler HT autosampler (Thermo Scientific). Sample components were separated using an ACQUITY UPLC HSS T3 reversed phase column (2.1 x 150 mm, 1.8 µm particle diameter, Waters Corporation) held at 40 °C, and a Vanquish Binary pump (150 µL/min flow rate; Thermo Scientific). Mobile phase A consisted of 0.1 % formic acid in water. The solvent mobile phase B consisted of 100 % purity acetonitrile, which is used to with mobile phase A to separate and elute analytes from the column. Mobile phase B was increased from 7.5 % to 30 % over 20 min. Mobile phase B was further increased to 100 % over 14.5 min and then held at 100 % for 1.5 min. The column was re-equilibrated with mobile phase B at 7.5 % for 2 min before the next injection.

The LC system was coupled to a Q Exactive Orbitrap mass spectrometer through a heated electrospray ionization (HESI II) source (Thermo Scientific). Source conditions were as follows: HESI II and capillary temperature at 275 °C, sheath gas flow rate at 30 units, auxiliary gas flow rate at 6 units, sweep gas flow rate at 0 units, spray voltage at |4.0 k| for positive mode and |4.5 k| for negative mode, and Slens RF at 60.0 units. To promote fragmentation, in-source collision-induced dissociation energy was set to 25.0 eV. The MS was operated in a polarity switching mode within the same injection. Acquisition parameters for full MS scans in both modes were 35,000 resolution, 1 x 105 automatic gain control (AGC) target, 50 ms ion accumulation time (max IT), and 50-360 *m/z* scan range. Targeted MS-MS scans in both modes were then performed at 17,500 resolution, 1 x 105 AGC target, 100 ms max IT, 1.0 *m/z* isolation window, stepped normalization collision energy at 20, 30, 40, and a 5.0 s dynamic exclusion. The resulting LC-MS/MS data was processed using a custom TraceFinder 4.1 (Thermo Scientific) method using a mass precision of 4 ppm and mass tolerance of 10 ppm. The prepared standard solution was used to identify appropriate peaks for peak area analysis.

2.5. Media and microbial strains

The xylose-fermenting *Saccharomyces cerevisiae* strain GLBRCY1455 was derived from the strain GLBRCY1327 (Lee et al., 2021) with an additional deletion mutation in *FLO8* to limit flocculation. *FLO8* was deleted by homologous recombination and marker rescuing with the *hphMX* cassette (Güldener et al., 1996). The generation of the isobutanol/ethanol co-producing hybrid *S. cerevisiae* strain yHRW253 was described previously (Pastore de Lima et al., 2023). Both *S. cerevisiae* strains were cultured in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L > 97 % purity dextrose). Xylose-fermenting *Zymomonas mobilis* 2032 (Yang et al., 2018), obtained from the American Type Culture Collection (PTA-6977), was cultured in *Zymomonas* Rich Defined Medium (ZRDM; (Enright et al., 2023)) with tetracycline (Tc) and chloramphenicol (*Cm*). Synthetic hydrolysate versions 4.0 and 4.1 (SynHv4.0 and SynHv4.1) mimic the composition of 7 % glucan loading AFEX-pretreated switchgrass as previously determined (Serate et al., 2015; Zhang et al., 2020). SynHv4.1 contains specific lignocellulose-derived inhibitors (*e.g.*, ferulic and *p*-coumaric acids),

whereas SynHv4.0 lacks these inhibitors. Chemical compositions of SynHv4.0 and SynHv4.1 can be found in the supplementary material. Tween-80 and ergosterol were made by dissolving ergosterol (75 % purity, E6510-10G, Sigma) and Polysorbate 80 (>96 % purity, Tween 80, 59924–1 KG-F, Fluka) in 100 % ethanol. The pH of all media and hydrolysates were adjusted with HCl and NaOH to pH values of 3–7 as needed.

2.6. Hydrolysate fermentations

Fermentations of hydrolysates by yeast and bacteria were performed as described in (Chandrasekar et al., 2021) with modifications. *S. cerevisiae* and *Z. mobilis* cultures were grown overnight in YPD and ZRDM + Tc + *Cm* respectively. The next day, cultures were diluted to an optical density at 600 nm wavelength (OD_{600}) = 0.3 with fresh media and grown for approximately 3–5 h until cultures reach log phase at OD_{600} = 0.6–0.8. Cultures were then harvested and used to inoculate 4 mL of hydrolysate in sterile serum bottles (Fisher: 06406H) at an OD_{600} = 0.2. Inoculated bottles were sealed with 20 mm butyl rubber stoppers (ChemglassLife Sciences) and rendered anaerobic by three alternating vacuum and 100 % N_2 gas-sparging cycles with syringe needles and tubing. Anaerobic cultures were connected to the Challenge Technology (Springdale, AR, USA) AER-800 respirometer system by piercing the rubber stoppers with syringe needles connected to tubing. Inoculated cultures connected to the respirometer were shaken at 150 RPM at 30 °C for 48 h. At the end of the experiment, cultures were harvested to measure the final cell density and centrifuged to collect the supernatant for metabolomic analysis. Supernatant glucose, xylose, and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) and refractive-index detection (RID) as previously described (Schwalbach et al., 2012). Isobutanol concentrations were determined by gas chromatography as previously described (Gambacorta et al., 2022). Visualization and statistical analyses of growth metrics, metabolites, and hydrolysate composition were performed in R v4.4.1.

3. Results

3.1. Soaking in Aqueous Ammonia and AFEX pretreatments yield switchgrass hydrolysates with similar sugar titers but different molecular compositions

The first step was to test if the inhibitory effect of the 2012 switchgrass hydrolysate would persist in a pretreatment different from AFEX, which is known to generate higher levels of phenolic amides (Keating et al., 2014; Zhang et al., 2020). Soaking in Aqueous Ammonia (SAA) pretreatment (Zhao et al., 2020) has been used to effectively deconstruct corn stover (Kim & Lee, 2007), rice straw (Ko et al., 2009), and switchgrass (Pryor et al., 2011), and it has been patented for industrial processes (Dunson et al., 2007). This method was customized to pretreat small amounts (10–15 g) of biomass in batches of 12–24 samples at a time. For this SAA-deconstruction pipeline (Fig. 2**A**), milled switchgrass samples harvested in 2010, 2012, and 2016 were incubated with mixing in 18 % (wt/vol) NH_4OH for 3.5 hr at 75 °C in 120 mL glass pressure

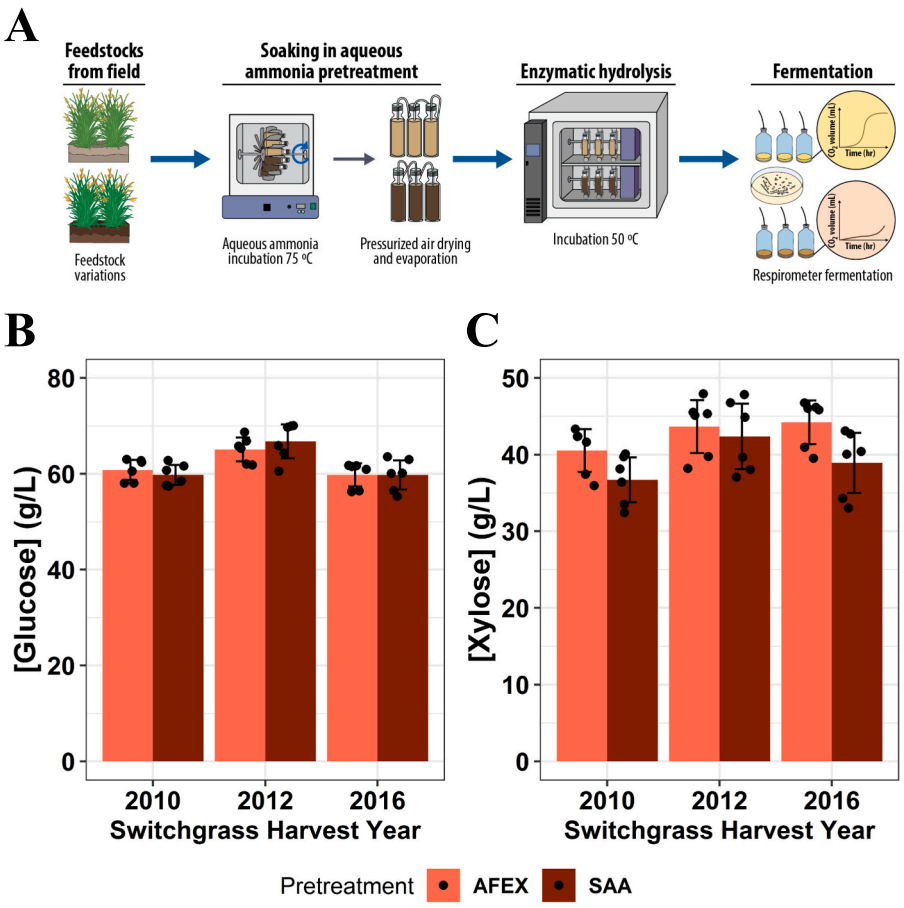

Fig. 2. A small-scale Soaking in Aqueous Ammonia pretreatment pipeline generates switchgrass hydrolysates with sugar titers comparable to AFEX pretreatment. (**A**) A schematic diagram of the SAA deconstruction pipeline that incorporates enzymatic hydrolysis and microbial fermentation. Comparison of glucose (**B**) and xylose (**C**) titers in hydrolysates generated by AFEX and SAA pretreatment and enzymatic hydrolysis. Columns and bars show the mean and standard deviation (SD) of six independent hydrolysate batches.

tubes, then cooled and dried with compressed air in a fume hood (see Materials and Methods section). Dried SAA-pretreated switchgrass samples at 7 %-glucan loading were enzymatically hydrolyzed to produce 20–27 mL of filtered hydrolysate. To facilitate direct comparisons, AFEX-pretreated switchgrass samples from the same harvest years were hydrolyzed in parallel. Our SAA pretreatment conditions differed from previous AFEX pretreatment conditions used to deconstruct 2010, 2012, and 2016 switchgrass in a number of ways. These included different reactor vessel scales, ammonia–ammonium loadings, temperatures, pressures, and pretreatment times (Table 1). It was expected that the differences in pretreatment conditions would result in hydrolysates with distinct compositions.

After hydrolysis of the pretreated biomass, the resulting sugar titers were analyzed to compare the hydrolysis efficiencies between the two different pretreatments. Glucose titers from SAA- and AFEX-deconstructed switchgrass were virtually indistinguishable within harvest years (Fig. 2**B**). Xylose titers were, on average, 3.5 g/L lower (min $p = 0.065$, two-sided Wilcoxon tests) across harvest years in SAA-pretreated hydrolysates compared to the paired AFEX-pretreated switchgrass samples (Fig. 2**C**). A previous study achieved lower sugar titers in SAA-pretreated switchgrass compared AFEX pretreated (Tao et al., 2011); the differences in sugar titers from our study were likely due to the different versions of commercial enzymes used for hydrolysis. The study by Tao et al., 2011 utilized older Genencor Spezyme CP and ß-glucosidase enzyme cocktails, whereas this study utilized the latest publically available Novozymes cellulase (NS 22257) and xylanase (NS 22244) cocktails. These results indicated that both SAA and AFEX pretreatment methods were similarly efficacious when coupled with these effective cellulase and xylanase mixtures to produce hydrolysates with similar sugar titers.

To investigate whether different pretreatment methods yielded hydrolysates with distinct inhibitor compositions, targeted quantification was performed for 48 analytes by mass spectrometry (MS) from both SAA- and AFEX-pretreated switchgrass hydrolysates adjusted to pH 5.0 and pH 5.8, except for 2016 at pH 5.0 (see supplementary material). Hydrolysates from each harvest year and pretreatment clustered tightly together in a principal component analysis (PCA) of their chemical compositions, with clear separation between these groups (Fig. 3**A**). The most variance was described by pretreatment method (principal component 1), whereas principal component 2 captured separation by harvest year and precipitation. The next focus was on the effect of pretreatment on the concentrations of several characterized microbial inhibitors (Fig. 3**B-C**). As observed previously (Zhang et al., 2020), AFEX-pretreated switchgrass hydrolysates contained high levels of phenolic amides, such as feruloyl amide and courmaroyl amide. The concentrations of feruloyl amide (1122 ± 394 μM) and coumaroyl amide (2277 ± 533 μM) in AFEX pretreated switchgrass hydrolysates made by the small-scale hydrolysis method (see supplementary material) were higher than in hydrolysates made from unmilled switchgrass with a large bioreactor (feruloyl amide, 701 ± 15 μM; coumaroyl amide, 1100 ± 20 μM; (Zhang et al., 2020)). In contrast, the levels of feruloyl amide (2189 ± 471 μM) and coumaroyl amide (938 ± 159 μM) were approximately 1.4- to 1.8-fold lower in the SAA-deconstructed samples, while the levels of ferulic acid and *p*-coumaric acid were approximately 2.9- to 3.8-fold higher ($p < 0.001$, two-sided Wilcoxon tests). There was a strong inverse correlation between the concentrations of coumaric acid and coumaroyl amide (Fig. 3**B**; Spearman's rho: −0.7, $p = 1.7e\text{-}7$), as well as ferulic acid and feruloyl amide (Fig. 3**B**; Spearman's rho: −0.51, $p = 5.3e\text{-}4$). This result suggests that ammonolysis reactions that convert carboxylic acid groups to amides during AFEX pretreatment (Chundawat et al., 2010) occurred at a higher rate than with our SAA pretreatment method. The higher levels of ammonolysis reactions with AFEX pretreatment may be due to the higher temperatures and pressures used compared to SAA pretreatment (Table 1). Overall, SAA pretreatment yields hydrolysates with similar sugar content, but they have distinct chemical profiles compared to AFEX pretreatment.

Table 1
Summary of conditions used for pretreatment 2010, 2012 and 2016 switchgrass.

Pretreatment Condition	Large-scale AFEX[a]	Small-scale AFEX[b]	Small-scale SAA
Reactor size	19 L Parr reactor	0.2 L custom reactor	0.12 L glass tubes
Biomass amount	600–800 g	25 g	15 g
NH_3/NH_4OH loading	2 g NH_3/g dry biomass	2 g NH_3/g dry biomass	60 mL 18 % NH_4OH
Reaction temperature	100 °C	120 °C	75 °C
Reaction time	30 min	30 min	3 h
Pressure	60 psi	60 psi	Not determined[c]

[a] Ong et al., 2016.
[b] Chandrasekar et al., 2021.
[c] No exogenous pressure was introduced.

3.2. Soaking in aqueous ammonia pretreatment modestly relieves drought hydrolysate inhibition

Next, it was tested whether the differing chemical compositions of SAA- and AFEX-pretreated hydrolysates translated into different effects on microbial fermentation at pH of 5.0, particularly for the inhibitory 2012 feedstock. After enzymatic hydrolysis of the pretreated biomass the resulting hydrolysate has pH of approximately 5.0, which was the rationale for selecting this pH value in a previous study (Serate et al., 2015). SAA- and AFEX-pretreated hydrolysates at pH 5.0 were fermented by the engineered, xylose-fermenting *Saccharomyces cerevisiae* strain GLBRCY1455 (Y1455, see Materials and Methods), as well as by *Zymomonas mobilis* strain Zm2032 (Yang et al., 2018) (Fig. 4**A-B**). Final CO_2 production, lag time (length of delay in minutes for the start of CO_2 production), glucose and xylose consumption, final cell density (OD_{600}), and ethanol titers were all measured after 48 h.

Fermentations at pH 5.0 of SAA-pretreated drought hydrolysates by Y1455 were found to be highly variable between hydrolysate batches, with ethanol titers after 48 h ranging from 0.11 to 49.55 g/L (see supplementary material). These results nonetheless represented an approximately 6-fold increase in median ethanol titers compared to paired AFEX-pretreated hydrolysates, although this difference was not statistically significant ($p = 0.31$, two-sided Wilcoxon test). By contrast, no meaningful difference in ethanol production was observed for Y1455 in fermentations of AFEX- and SAA-pretreated hydrolysates from normal precipitation years (see supplementary material). Similar trends were observed for Zm2032, with SAA-pretreated drought hydrolysates yielding 2.5-fold higher median ethanol titers than AFEX-pretreated hydrolysates ($p = 0.49$, two-sided Wilcoxon test; see supplementary material). Thus, the differing makeup of hydrolysates generated from SAA and AFEX pretreatment did not translate into a full alleviation of the inhibitory effects of drought-grown feedstocks at the pH of 5.0. Moreover, SAA-pretreated drought hydrolysates yielded unpredictable fermentation performances that were hindered by strong batch effects, although still improving ethanol production by Y1455 and Zm2032 on average.

3.3. Increasing hydrolysate pH from 5.0 to 5.8 results in increased ethanol production by S. cerevisiae and Z. mobilis

Next, the effect of altering hydrolysate pH on fermentation by Y1455 and Zm2023 was tested (Fig. 4). Previous research has suggested an optimal pH of 4.0–6.0 for *S. cerevisiae* (Narendranath & Power, 2005), and pH of 4.0–7.5 for *Z. mobilis* (Lawford et al., 1988; Panesar et al., 2006). Due to these inherent differences in pH preferences, previous comparisons between these microbes have often been made using different pH values that were tailored to the microbe's preference under optimal non-hydrolysate conditions (Serate et al., 2015; Ong et al.,

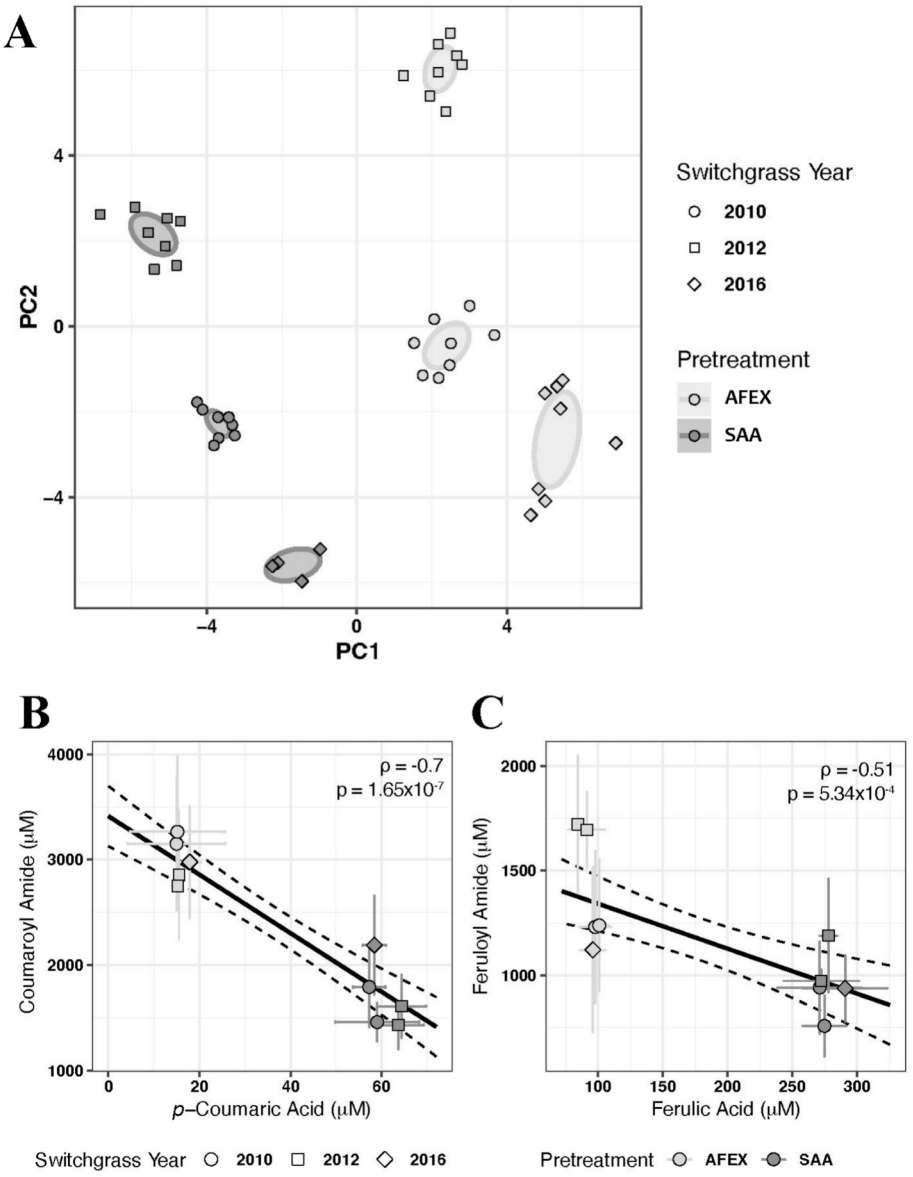

Fig. 3. Switchgrass hydrolysates from SAA and AFEX pretreatments are chemically distinct. Principal component analysis (PCA) plot (**A**) displays all the analyzed hydrolysate samples in principal component space for principal components 1 and 2. Ellipses show confidence intervals for each specified group. Scatterplots display the mean and standard deviation of coumaroyl amide and *p*-coumaric acid (**B**), as well as feruloyl amide and ferulic acid (**C**) concentrations from four independent batches of the indicated hydrolysates. Black lines show linear regressions and 95% confidence intervals.

2016; Zhang et al., 2018). Across both pretreatment methods and all feedstock years, pH adjustment to 5.8 increased median ethanol production by Y1455 by a minimum of 12.2 % ($p = 0.002$, two-sided Wilcoxon test). The greatest increase in 48-hour ethanol production by Y1455 was observed in AFEX-pretreated drought switchgrass hydrolysate, where median ethanol titers leapt approximately 46-fold, from 0.88 g/L at pH 5.0 to 40.39 g/L at pH 5.8 (Fig. 4**A**). A similar trend was observed for fermentations with Zm2032, where median ethanol production at pH 5.8 was an average of 4.26-fold higher than in matched hydrolysates at pH 5.0 (Fig. 4**B**). Here, as well, the greatest increase of ~ 9-fold was observed for AFEX-pretreated hydrolysates from drought switchgrass.

Similar trends were observed for CO_2 production, lag phase, cell growth, and glucose and xylose consumption (see supplementary material). Y1455 produced significantly more CO_2 at pH 5.8 than at pH 5.0 within pretreatment types for all three harvest year hydrolysates (all $p \leq 0.005$, two-sided Wilcoxon tests; see supplementary material). Similarly, Zm2032 produced significantly lower final CO_2 at pH 5.0 than at pH 5.8 for the hydrolysates from each pretreatment method (see supplementary material), with the exception of the 2016 SAA-pretreated hydrolysates ($p = 0.34$, two-sided Wilcoxon test). Lag time was generally shorter for pH 5.8 fermentations by Y1455, but it could not be accurately estimated for fermentations that experienced severe inhibition, including all pH 5.0 fermentations by Zm2032 (see supplementary material). Final cell densities (OD_{600}) for both Y1455 and Zm2032 were universally higher in pH 5.8 fermentations (see supplementary material), although these differences were not statistically significant for some *Z. mobilis* fermentations.

Finally, these trends of increased cell growth and fermentation at higher pH were recapitulated in sugar consumption profiles (see supplementary material). Y1455 nearly fermented all glucose present in hydrolysates from non-drought years, regardless of pH or pretreatment. However, pH adjustment rescued glucose consumption by Y1455 in drought hydrolysates, increasing the median percent consumed by ~ 82-fold and ~ 5.8-fold in AFEX- and SAA-pretreated hydrolysates, respectively (see supplementary material). Y1455 consumed an average of 1.7-

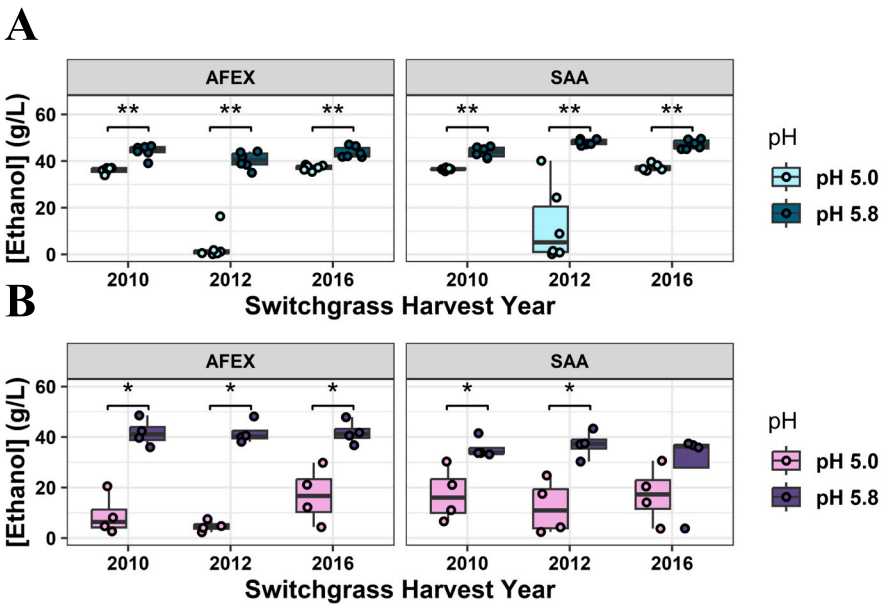

Fig. 4. Increasing pH improves yeast and bacterial fermentation in both SAA- and AFEX-pretreated switchgrass hydrolysates. Boxplots display the final ethanol titers from fermentations of switchgrass hydrolysates by the *S. cerevisiae* strain Y1455 (**A**) and the *Z. mobilis* strain Zm2032 (**B**). Brackets denote significance as determined by two-sided Wilcoxon tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

fold more xylose from AFEX-pretreated hydrolysates and 2.5-fold more xylose from SAA-pretreated hydrolysates at pH 5.8 compared to pH 5.0 (all $p \leq 0.004$, two-sided Wilcoxon tests; see supplementary material). Zm2032 also nearly completely fermented all glucose in pH 5.8 hydrolysates from all years, regardless of pretreatment, (all $p \leq 0.03$ except 2016 SAA, two-sided Wilcoxon tests; see supplementary material). Zm2032 consumed ~ 1.9-fold more xylose from AFEX-pretreated hydrolysates and ~ 1.3-fold more from SAA-pretreated hydrolysates at pH 5.8 (all $p = 0.03$ except 2016 SAA, two-sided Wilcoxon tests; see supplementary material**).** Therefore, pH adjustment afforded substantial improvements to ethanol production and fermentation by *S. cerevisiae* and *Z. mobilis* across hydrolysates with varying chemical compositions and inhibitory effects.

3.4. Ethanol production by S. cerevisiae and Z. mobilis is greater at higher pH

Our results suggested that pH adjustment might represent a generalizable intervention to alleviate the effects of lignocellulose-derived (LCD) inhibitors in diverse hydrolysates. To test this hypothesis, synthetic mimics of AFEX-pretreated switchgrass hydrolysate were developed (Synthetic Hydrolysate or "SynH"). Previously, versions of SynH reconstructed from pure compounds that were identified and quantified from AFEX-pretreated corn stover hydrolysate were described (Keating et al., 2014). For this study, compositional data was utilized from chemical analysis of AFEX-pretreated switchgrass hydrolysate (Zhang et al., 2020) to formulate a synthetic version of switchgrass hydrolysate. Furthermore, because SynH is reconstituted with pure, off-the-shelf chemical compounds, SynH was generated without LCD inhibitors ("SynHv4.0") and with many, but not all, of the LCD inhibitors ("SynHv4.1"), including ferulic acid and *p*-coumaric acid, as well as feruloyl amide and coumaroyl amide.

To test the interaction between pH and LCD inhibitory compounds on yeast and bacterial fermentations, each engineered *S. cerevisiae* and *Z. mobilis* strain was cultured in SynHv4.0 or SynHv4.1 media at pH 3, 4, 5, 6, and 7, and final ethanol titers after 48 h were quantified. Clear dose–response kinetics were observed between pH and ethanol production (Fig. 4), with notable differences between microorganisms and SynH formulations. Ethanol production was universally inhibited at pH 3, and in SynHv4.1 at pH 4. Both microbes produced substantially more ethanol from the LCD inhibitor-less SynHv4.0 than SynHv4.1 at pH 4, indicating a key interaction between pH and the action of LCD inhibitors. Supporting this notion, there were no statistically significant differences in ethanol production from SynHv4.0 and SynHv4.1 by either strain between pH 5 and 7. The lack of difference at pH 5 compared to higher pH levels is likely due to missing inhibitors in the synthetic hydrolysates that are found in switchgrass hydrolysate. Increasing pH between 4 and 7 generally led to increasing ethanol titers by both strains and in both SynH formulations, with notable differences in the inflection points of non-linear trends. For example, Y1455 produced only ~ 2.8 % more ethanol from SynHv4.0 at pH 5 compared to pH 4, whereas Zm2032 produced ~ 3.1-fold more (Fig. 5). These differences likely reflect each organism's preferred pH range (Lawford et al., 1988; Narendranath & Power, 2005; Panesar et al., 2006), in the absence of perturbations by LCD inhibitors. Between pH 4 and pH 5 in SynHv4.1, Y1455 ethanol titers increased ~ 12-fold, and Zm2032 ethanol titers increased ~ 17-fold. Between pH 5 and pH 7 in both SynH types, substantial linear increases in ethanol production by both microbes, with median titers ~ 24 % higher for Y1455 (adjusted R^2: 0.72, $p = 1e^{-7}$, linear regression) and ~ 9 % higher for Zm2023 (adjusted R^2: 0.09, $p = 0.12$, linear regression) were further observed. Finally, the effect of pH on ethanol production was recapitulated in overall fermentative growth as measured by CO_2 production, cell density, and sugar consumption (see supplementary material). Thus, increasing pH to the range of 5–7 greatly improved both growth and metabolism by Y1455 and Zm2032, especially in the presence of LCD inhibitors.

3.5. Increasing hydrolysate pH increases isobutanol titers by a hybrid S. cerevisiae ethanol and isobutanol co-producing strain

Isobutanol (IBA) is a potential substrate for the formation of alkanes and alkenes used for aviation fuel production, making it a key second-generation biofuel (Geleynse et al., 2018; Holladay et al., 2020). The engineered hybrid *S. cerevisiae* strain yHRW253 (Pastore de Lima et al., 2023), which can simultaneously ferment glucose from hydrolysate into ethanol and isobutanol, also experiences inhibition by drought hydrolysates. Therefore, this strain was tested for whether isobutanol production could also be improved by increasing the hydrolysate pH.

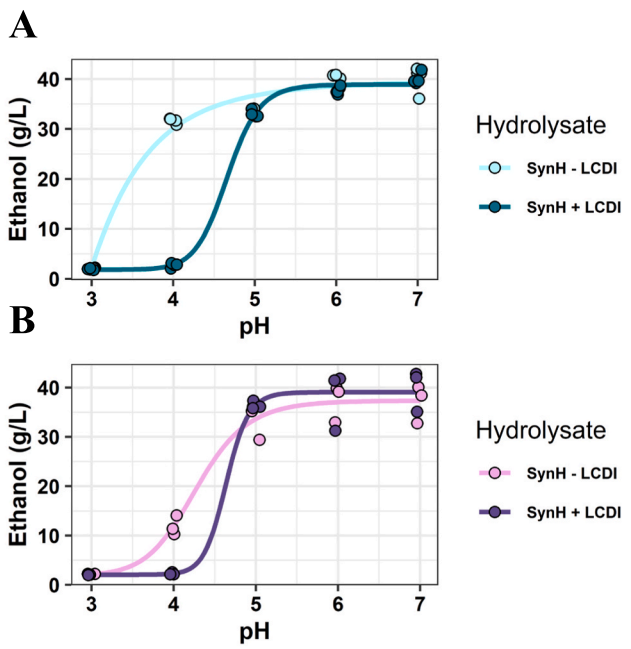

Fig. 5. Increasing pH of synthetic hydrolysates increases ethanol production by *S. cerevisiae* and *Z. mobilis*. Dose-response curves display final ethanol titers from fermentations of synthetic hydrolysate without (SynH − LCDI, SynHv4.0) or with lignocellulose-derived inhibitors (SynH + LCDI, SynHv4.1) at pH 3–7 by *S. cerevisiae* Y1455 (**A**) and *Z. mobilis* Zm2032 (**B**).

Fermentation experiments were performed with yHRW253 in both AFEX- and SAA-pretreated switchgrass hydrolysates at pH 5.0 and 5.8.

Neither pretreatment nor pH had a significant effect on lag phase or total cell growth for yHRW253 (see supplementary material). CO_2 production was only significantly different, lower in this case, in SAA-pretreated 2016 hydrolysate ($p = 0.03$; see supplementary material). Glucose was nearly completely consumed in all hydrolysates from normal precipitation years at pH 5.0 and 5.8 (see supplementary material). Increasing the pH of the drought year switchgrass hydrolysates recapitulated the near total consumption of glucose seen in normal year switchgrass hydrolysates, but not in a statistically significant manner ($p \geq 0.3$, two-sided Wilcoxon tests).

Median ethanol production was modestly lower (~19 % on average) at pH 5.8 than pH 5.0 across all feedstock years and deconstruction methods (Fig. 6**A**), although this difference was only statistically significant in the 2010 SAA-pretreated hydrolysate ($p = 0.03$, two-sided Wilcoxon test). Interestingly, comparisons between pretreatment methods of the same harvest year switchgrass at pH 5.0 and at 5.8 did not have a significant effect on ethanol production (min $p = 0.06$, two-sided Wilcoxon tests, see supplementary material). Crucially, almost no isobutanol was produced from 2012 hydrolysates at pH 5.0 (Fig. 6**B**). In 2012 AFEX- and SAA-pretreated hydrolysates, and in 2010 SAA-pretreated hydrolysate, there were statistically significant increases in isobutanol production at pH 5.8 compared to 5.0 (max $p = 0.03$, two-sided Wilcoxon tests) (Fig. 6**B**). Notably, fermentation of SAA- and AFEX-pretreated hydrolysates at pH 5.8 yielded ~ 5.9-fold and ~ 1.8-fold more isobutanol, respectively, than fermentations of the same hydrolysates at pH 5.0 in non-drought years (Fig. 6**B**). By contrast, for the inhibitory 2012 hydrolysates, median isobutanol production increased in SAA- and AFEX-pretreated hydrolysates at pH 5.8 by ~ 30-fold and ~ 56-fold respectively. Overall, the quantitative effect of increased pH suggests that pH adjustment is a promising strategy to improve isobutanol production from lignocellulosic hydrolysates by engineered *S. cerevisiae*.

4. Discussion

To identify ways to overcome the impaired fermentation of drought switchgrass by engineered *S. cerevisiae* and *Z. mobilis*, fermentations of drought and non-drought switchgrass hydrolysates were compared at two different pH values, and from two different pretreatment methods. First, it was tested whether the inhibitory effect of hydrolysates from drought year feedstocks could be alleviated by using the SAA pretreatment method, which produced similar yields of glucose and xylose compared to AFEX pretreatment (Fig. 2**B-C**). The overall compositions of the SAA-pretreated and AFEX-pretreated hydrolysates differed

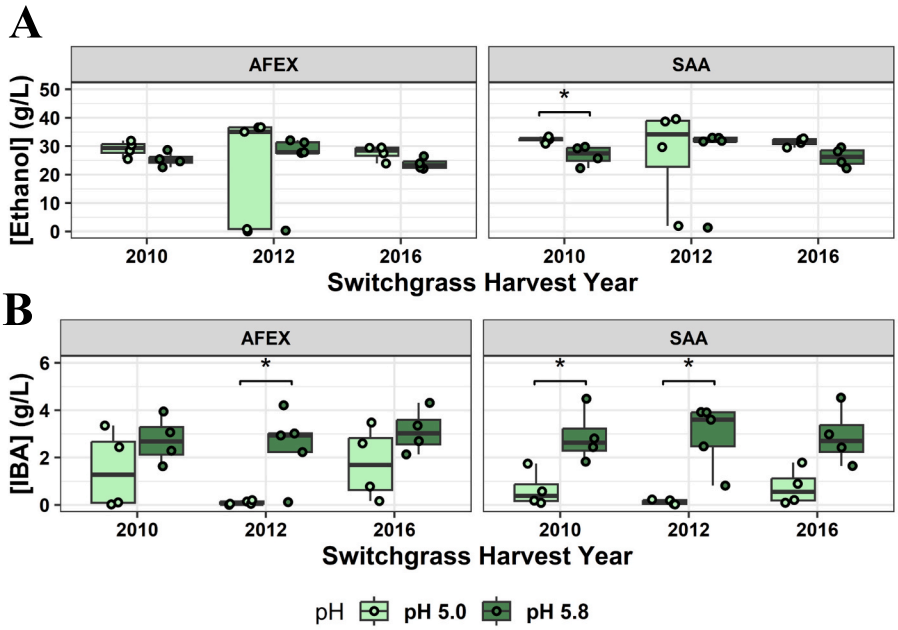

Fig. 6. pH adjustment improves isobutanol (IBA) production from hydrolysates. Boxplots display final ethanol (**A**) and isobutanol (**B**) titers from SAA- and AFEX-pretreated hydrolysates from three harvest years, fermented at pH 5.0 and 5.8 by *S. cerevisiae* yHRW253 hybrid. Brackets denote significance level as determined by two-sided Wilcoxon tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

greatly, including increased concentrations of inhibitory amides in AFEX-pretreated hydrolysates compared to higher concentrations of the conjugate acids in SAA-pretreated hydrolysates (Fig. 3). Differences in the concentrations of these compounds likely occurred because of different conditions for each pretreatment, such as reaction temperatures and pressures (Table 1). Phenolic acids such as ferulic acid, *p*-coumaric acid, and other compounds can inhibit *S. cerevisiae* fermentation (Klinke et al., 2004; Mussatto & Roberto, 2004; Adeboye et al., 2014), as can high levels of acetic acid (Palmqvist et al., 1999; Guaragnella & Bettiga, 2021). *Z. mobilis* also experiences inhibitory effects from phenolic compounds and other inhibitors found in lignocellulosic-derived hydrolysates (Franden et al., 2013; Shabbir et al., 2023). It was found that ferulic acid and *p*-coumaric acid were at higher concentrations in SAA-pretreated hydrolysate, whereas feruloyl amide and coumaroyl amide were at higher concentrations in AFEX-pretreated hydrolysate, producing an inverse relationship between pretreatment and acid vs. amide formation (Fig. 3**B-C**). This difference was likely due to the SAA pretreatment leading to lower conversion of the acids to their amide forms than AFEX pretreatment from ammonolysis reactions (Chundawat et al., 2010). A further benefit of the SAA pretreatment is its lower requirements for temperature, pressure, and biomass, which could create a lower energy cost for the process (Table 1). SAA pretreatment should also have lower capital costs, since the AFEX pretreatment needs specialized reactors to safely accommodate the high pressures used with ammonia gas. This could lead to a more resilient and economical process. Therefore, the SAA pretreatment method is comparable to AFEX pretreatment for the deconstruction of switchgrass and potentially more cost effective. Although it has been the subject of some investigation (Tao et al., 2011; Singh et al., 2021), a thorough technoeconomic and environmental analysis of these pretreatment methods at scale remains to be undertaken.

As SAA-pretreatment alone did not relieve the inhibitory effects of drought-grown switchgrass hydrolysates on microbial fermentation, the effect of manipulating hydrolysate pH was tested next. Increased pH has been previously shown to increase ethanol production by yeast (Narendranath & Power, 2005) through mechanisms that include the neutralization of weak acids (Lam et al., 2021), and increased pH has also been shown to increase ethanol production by *Z. mobilis* (Lawford et al., 1988; Panesar et al., 2006). The higher level of acids may render SAA-pretreated hydrolysates more amenable to intervention because pH adjustment from 5.0 to 5.8 can shift more of the weak acids into their deprotonated forms (ferulic acid $pK_a = 4.58$; *p*-coumaric acid $pK_a = 4.64$), preventing them from acidifying the cytosol after passively diffusing into the cell, and thereby mitigating their inhibitory effects (Carmelo et al., 1996; Johnston et al., 2020). In contrast, the pH adjustment from 5.0 to 5.8 has little effect on the basic amides within this pH range. This led us to believe that the microbes in the SAA-pretreated hydrolysate at pH 5.8 grow to higher cell density and produce more ethanol or isobutanol (Fig. 4, Fig. 6, see supplementary material) because the neutralization of acids in the media created a less inhibitory environment for fermentation and growth. This result does not explain overall increases in ethanol or isobutanol yields at higher pH in AFEX-pretreated hydrolysates, and further study into the tradeoffs between producing these competing alcohol products is needed. The increased pH could still have neutralization effects on the lower concentrations of acids within AFEX-pretreated hydrolysates by creating a less inhibitory environment. A hydrolysate pH of 5.8 could also be nearer to the optimal pH value for these microbes to grow in the presence of LCD inhibitors (Fig. 4). Overall, it was found that the most promising method to overcome the inhibitory effect of hydrolysates from drought-affected switchgrass on downstream microbial fermentation was to increase the pH, regardless of the pretreatment method. This intervention was shown to be sufficient to substantially increase production of biofuels by strains of *S. cerevisiae* and *Z. mobilis* from both real (Fig. 4, Fig. 6) and synthetic hydrolysates (Fig. 5) with diverse chemical compositions. While only the effects of pH adjustment on fermentations of a limited number of hydrolysates were tested, the consistent improvements observed across these feedstocks with distinct chemical compositions, along with work by others (Lam et al., 2021), suggest that pH adjustment may be a viable and generalizable strategy for improving biofuel yields from lignocellulosic biomass. Future research may investigate the technoeconomic impacts of pH adjustment and alternative deconstruction methods for diverse feedstocks implemented at industrially relevant scales.

5. Conclusion

Adjustments to hydrolysate composition were tested in an effort to develop sustainable and economical methods that create lignocellulosic-derived biofuels from switchgrass, while acknowledging the influence on microbial fermentation of uncontrollable factors during plant growth. Extreme weather events, such as drought, are predicted to increase in frequency with climate change. Droughts, such as in Wisconsin in 2012, can affect the composition of biofuel crops like switchgrass, which may in turn impact microbial biofuel production by lignocellulosic biorefineries. Previous work has shown the inhibitory effect of AFEX-pretreated drought year switchgrass hydrolysate on *S. cerevisiae* fermentation and others have shown that increasing the pH of other inhibitory hydrolysates can improve fermentation. To determine if increasing the pH of inhibitory drought switchgrass hydrolysates was beneficial for multiple pretreatment approaches, a lab-scale SAA pretreatment method was developed and used to determine that increasing the pH of SAA and AFEX pretreated drought switchgrass could overcome the fermentation inhibition of 2012 year drought switchgrass hydrolysate. This work shows that the selection of pretreatment methods and the pH of deconstructed sugar streams are important factors in producing sustainable biofuels from switchgrass grown under different environmental conditions. Our results imply that the SAA pretreatment methods and pH adjustment are likely to represent generalizable interventions that can boost the production of valuable biofuels from diverse lignocellulosic feedstocks.

CRediT authorship contribution statement

Lillian M. Barten: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Johnathan G. Crandall:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Dan Xie:** Writing – review & editing, Methodology, Investigation. **Jose Serate:** Writing – review & editing, Methodology, Investigation. **Evan Handowski:** Writing – review & editing, Methodology, Investigation. **Annie Jen:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Katherine A. Overmyer:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Joshua J. Coon:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Chris Todd Hittinger:** Writing – review & editing, Supervision, Resources, Investigation, Formal analysis, Conceptualization. **Robert Landick:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Yaoping Zhang:** Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Trey K. Sato:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132651.

Data availability

Data will be made available on request.

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