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Gluconic Acid Pretreatment of Wheat Straw and Fermentation to Ethanol

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Gluconic Acid Pretreatment of Wheat Straw and Fermentation to Ethanol

By

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THESIS

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Abstract

Gluconic acid was evaluated to be a promising pretreatment agent on wheat straw. The pretreatment was tested at different gluconic acid concentrations (0.125-1M), temperatures (160-190°C), and reaction times (30-120 minutes). After each pretreatment, the slurries were separated into hemicellulose hydrolysate and pretreated solid residue. The sugar yields in stage 1 (hemicellulose hydrolysate) and stage 2 (enzymatic hydrolysis of pretreated solid residue) were reported. The highest overall xylose achieved 89.6±3.4% yield that was observed at the pretreatment condition of 0.125M gluconic acid concentration, 170°C pretreatment temperature, and 30-minutes reaction time. The highest overall glucose achieved 91.5±2.0% yield at the pretreatment condition of 0.5M gluconic acid concentration, 190°C pretreatment temperature, and 30-minutes reaction time. The overall sugar (glucose and xylose combined) yield of 83.5±2.4% was achieved at the pretreatment condition of 0.5M gluconic acid concentration, 170°C pretreatment temperature, and 30-minutes reaction time. Preference of utilizing lower gluconic acid concentration was also evaluated. The overall sugar yield of 81.1±0.2% was achieved at the pretreatment condition of 0.125M gluconic acid concentration, 170°C pretreatment temperature, and 60-minutes reaction time.

The evaluation for ethanol production utilized the hemicellulose hydrolysate and pretreated solid residue generated from the pretreatment condition of 0.125M gluconic acid concentration, 170°C pretreatment temperature, and 60-minutes reaction time because of lower gluconic acid concentration consumption and high overall sugar yield.

Roughly 4.2±0.14% of the gluconic acid was lost during the pretreatment process and a portion may be lost from being stuck to pretreated solid residue. The remaining 75.6±0.59% of gluconate in the detoxified hemicellulose hydrolysate was fermented to ethanol along with other hemicellulose sugars (glucose, xylose, and arabinose). The engineered microorganisms such as *Escherichia coli* AH003 and *E. coli* SL100 were evaluated for ethanol production in the detoxified hemicellulose hydrolysate. The theoretical maximum yield obtained from the detoxicated hemicellulose hydrolysate fermentation was 107.5±1.2% from *E. coli* AH003 using Luria-Bertani (LB) media and 90.4±1.8% from *E. coli* SL100 using low-salt (AM1) media. Additionally, *E. coli* AH003 and *Saccharomyces cerevisiae* D5A were used as the ethanologens to convert the pretreated solid residue to ethanol via simultaneous saccharification and fermentation (SSF) with cellulase loading of 20 FPU and β-glucosidase loading of 20IU/g cellulose. The ethanol yield obtained from *E. coli* AH003 in LB media from SSF was unclear since glucose, xylose, and a small amount of gluconate could be hydrolyzed from the pretreated solid residue. Meanwhile, *S. cerevisiae* D5A exclusively consumed glucose in lean media and the ethanol yield was 92.8±2.0% with cellulose conversion of 70.8±0.8%. Overall, evaluation of fermentation suggests that casamino acids in LB media could promote high cell growth and ethanol yield.

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1. Introduction

1.1. Background and motivation

The increase of global energy demands and reliance on the limited fossil fuels attracted further interest in biofuel production. Cellulosic biomass is a valued raw material for biofuel production because of its low cost, plentiful supply, and non-interference with food crops (1,2,3). Furthermore, interests in cellulosic biomass to biofuel production continued to increase with the desires to reduce greenhouse gas emissions, decrease reliance of imported finite fossil fuel reserves, and reduce agricultural waste accumulation, further benefiting rural communities economically (3,4). Despite the numerous advantages of cellulosic biofuels, the widespread commercialization of cellulosic bioprocessing is hindered by the lack of low-cost processing technologies (5). As shown in **Figure 1**, the conventional process of cellulosic biomass conversion into fuel and chemical products requires the main steps: pretreatment, cellulase production, enzymatic hydrolysis, fermentation, and product recovery. The preliminary steps of sugar production (pretreatment, cellulase production, and enzymatic hydrolysis) has a negative impact on cost-competitive biofuel production due to high cost of pretreatment and enzyme utilization efficiency (6,7,8,9). However, these steps are crucial for the reduction of the recalcitrance of cellulosic biomass by making it more amenable to biofuel production. Cellulosic biomass is a complex and rigid structure composed of three major components; cellulose, hemicellulose, and

lignin. The cellulose component exists as both crystalline[1] and amorphous[2] forms that are packed tightly into microfibrils, protected by the hemicellulose and lignin components (10,11). The dominant cellulose crystallinity form, along with the presence of hemicellulose and lignin add major difficulties for effective enzyme digestibility (12,13,14). Therefore, the importance of pretreatment can further remove these barriers for more efficient enzyme digestion (12,14,15,16). In turn, the capabilities of effective and optimized pretreatment technologies could decrease the downstream cost of enzymes (usage, production, etc.) and overall biofuel production.

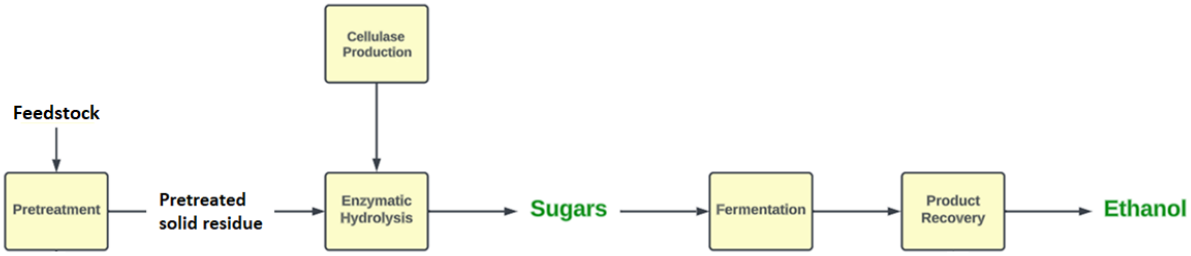


Figure 1: Conventional cellulosic biomass into biofuel production.

1.2. The conventional pretreatment strategies

Pretreatment technologies are a quintessential part to unlocking the potential of cellulosic biofuel production (17). These technologies are primarily used to enhance the chemical, enzyme, or biological hydrolysis rates. However, further research in the endeavors of effective pretreatment technology require the following goals to be met:

A. Improving sugar yields by enzyme digestibility

B. Reduce sugar loss

C. Reduce the formation of sugar degradation products

[1] Linearly organized chains of cellulose that are rigid and difficult to hydrolyze.
[2] Unorganized chains of cellulose that are susceptible to hydrolysis.

D. Maintain cost effectiveness

The diversity in pretreatment methods is vast and continually expanding to include technologies such as ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid, flowthrough (FT), lime, and sulfur dioxide were evaluated (12,18). Additionally, mechanical comminution, pyrolysis, steam explosion, hot liquid water, CO_2 explosion, ozonolysis, alkaline hydrolysis, oxidative delignification (wet oxidation), organosolv process, biological pretreatment, and ionic liquids were also studied (10,14,16,19,21). Furthermore, the listed pretreatment processes could be improved upon, for example microwave-assisted alkali pretreatment was able to improve enzymatic digestibility compared to conventional-heating alkali methods (20). Of these technologies; AFEX, ARP, controlled pH, dilute sulfuric acid, lime, and flowthrough are leading pretreatment methods (18). The focus of this thesis will be evaluating the interests in the continually growing and promising dilute acid pretreatment technology used for industrial applications.

1.3. The formation and inhibition of sugar degradation products

Sugar degradation products can be formed during dilute acid pretreatment (DAP) and appear in the aqueous component (hemicellulose hydrolysate). During pretreatment, a portion of hemicellulose-derived sugars such as pentoses and hexoses could degrade into furfural and 5-hydroxymethylfurfural (HMF) respectively, along with acetate. Depending on pretreatment severity (acid concentrations, pretreatment temperatures, and reaction times), furfural could further yield formate or polymerize,

while HMF could be converted into formate and levulinic acid (40). Non-sugar compounds such as solubilized lignin fragments can also be degraded into phenolic compounds (16,37). These degradation products are inhibitory in the downstream enzymatic hydrolysis and fermentation processes (16). Of these, furfural and HMF are considered to be the most potent inhibitors due to their destruction of cell growth and DNA, reduction of enzymatic and biological activities, and inhibition of protein and RNA synthesis (38). Additionally, phenolic compounds could incorporate further inhibitory effects in the hemicellulose hydrolysate (39). Thus, adequate strategies to reduce inhibitory compounds and improve the tolerance in microorganisms must be achieved for a viable cellulosic biofuel process.

1.4. The mechanism and effects of dilute acid pretreatment on cellulosic structure

DAP is a widely used and tested approach which can include different hydrolyzing agents such as sulfuric, nitric, hydrochloric, and phosphoric acids (22,23). DAP reduces recalcitrance of cellulosic biomass by disrupting lignocellulosic composite linkages, breaking weak[3] and covalent bonds (21). This process hydrolyzes hemicellulose into sugars, exposing the cellulose structure for further biodegradability, and disrupt lignin; however lignin is not dissolved and is re-adsorbed in a modified form (24). Specifically, sulfuric acid is preferred as an pretreatment agent for DAP amongst other acids since it is inexpensive and effective (22). The DAP of cellulosic biomass can be performed at different temperatures, solid loadings, acid concentrations, and

[3] Van der Waal & hydrogen bonds.

pretreatment reaction times (19,22). After the pretreatment, slurry mixture of acid and pretreated solid residue can be separated into pretreated solid residue and hemicellulose hydrolysate. Analysis of the pretreatment shows that hemicellulose is predominant in the hydrolysate while cellulose is predominant in pretreated solid residue (37). The pretreated solid residue could further undergo a water-washing method, which can help promote glucose and xylose release in the downstream enzymatic hydrolysis (18). The hemicellulose hydrolysate can be processed for product recovery or neutralization (16,19,23,27). The factors of temperature, acid concentration, and pretreatment time have major effects on cellulose hydrolysis and xylose conversion yields. Therefore, the implementation of the combined severity factor (CSF) can be used to compare and facilitate control of DAP conditions. In literature, DAP experiments observed that higher CSF could promote higher xylan removal and cellulose conversions in enzyme hydrolysis but would also affect xylose yield from xylan due to formation of degradation products (25,26). Therefore, the high yield of xylose conversion in the hemicellulose hydrolysate and high cellulose conversion from pretreated solid residue are desirable for a cost-effective pretreatment (16,19).

DAP is an effective technology for cellulosic biofuel production but it is not perfect. Firstly, DAP promotes sugar loss and the formation of sugar degradation products that are inhibitors to many fermentation processes (24). Secondly, neutralization of the acid is required for downstream enzymatic hydrolysis or fermentation processes, which form gypsum and present disposal challenges (19,27). Lastly, acid recovery is an essential part to obtaining an economically viable process, as it would be expensive otherwise (23).

1.5. Dilute organic acid pretreatment

The growing interest in DAP studies has sparked consideration for the use of organic acid as a possible alternative to the traditionally employed sulfuric acid, especially dicarboxylic organic acids. The advantage of the dicarboxylic acid group allows for selective hydrolysis of β-(1,4)-glycolic bonds, decreasing glucose degradation compared to sulfuric acid (28,30). Additionally, dicarboxylic organic acids are more efficient in hydrolysis of cellulosic biomass over a range of temperature and pH values (29).

Dilute organic acid pretreatment has been studied vastly in literature. The pretreatment process comparing maleic and fumaric acids showed that the organic acid pretreatment formed lower sugar degradation products compared to sulfuric acid pretreatment (31). Other studies compared oxalic, citric, tartaric, and acetic acids against sulfuric acid; reporting high yields of xylooligomers and effective cellulose hydrolysis (32). Overall, studies indicated that organic acids are effective pretreatment catalysts of cellulosic biomass compared to sulfuric acid (31,32,33,34,35). While the benefits of organic acid pretreatment are acknowledged, the challenges related to DAP persist without complete resolution. The economic viability of employing dilute organic acid pretreatment hinges on adopting smaller acid concentrations and implementing an organic acid recycling system, as the organic acid expenses involved are notably higher compared to sulfuric acid (31,36).

1.6. Fermentation strategies from DAP

The fermentation process involves the conversion of sugars or organic compounds into biofuels using bacteria or yeasts. As shown in **Figure 1**, sugars derived from pretreated solid residue are able to be utilized in fermentation for the conventional pretreatment process. Additionally, the sugars produced hemicellulose hydrolysate could also be used in fermentation. Thus, an economical approach to fermentation strategy following DAP involves the use of both product streams in fermentation, as represented in **Figure 2**. Furthermore, addressing economic considerations for industrial-scale fermentation might prove impractical unless the utilization of high cost nutrients such as yeast extract and tryptone are minimized or avoided (41,42,43,59).

Fermentation can be performed in batch, fed-batch, or continuous modes. The continuous fermentation is much lower in cost and higher in ethanolic productivity compared to batch fermentation (39). Additionally, bioprocessing strategies of fermentation includes; separate hydrolysis and fermentation (SHF), separate hydrolysis and co-fermentation (SHCF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (34,35,44). Of these, SSF is extensively studied and effective in many biofuel production research (44). During SSF, both the enzymatic hydrolysis and fermentation processes are combined into one process that can positively contribute to the capital cost. In one process, microorganisms will instantly consume hydrolyzed sugars from pretreated solids which reduces the sugar accumulation and inhibition of enzymes (44). However, challenges arise when using the fermentation of hemicellulose hydrolysate from DAP. The hydrolysate cannot be directly used in fermentation due to

the high concentration of inhibitory compounds. Therefore, detoxification methods are applied for effective fermentation, these include; physical, adsorbent, solvent extraction, membrane separation, chemical, alkaline, reducing agent, persulfate, biological, enzymatic, microbial, compound detoxification methods (39,45). Of these, the adsorbent method such as activated carbon is effective and low cost (45,47,48,49). The activated carbon combines with inhibitors and the removal of inhibitors can be vacuum filtered to separate spent activated carbon from supernatant where supernatant can undergo pH readjustment for fermentation (45). Additionally, the alkaline (overliming) method may also be economically viable if it can result in higher productivity during the fermentation process since the used alkali cannot be recovered (46,47). The overliming method can be cost-effective by using inexpensive $Ca(OH)_2$ inorder to increase pH to 9-10. This method removes inhibitors by precipitation. Further readjustment of pH is necessary for the fermentation process (39,47).

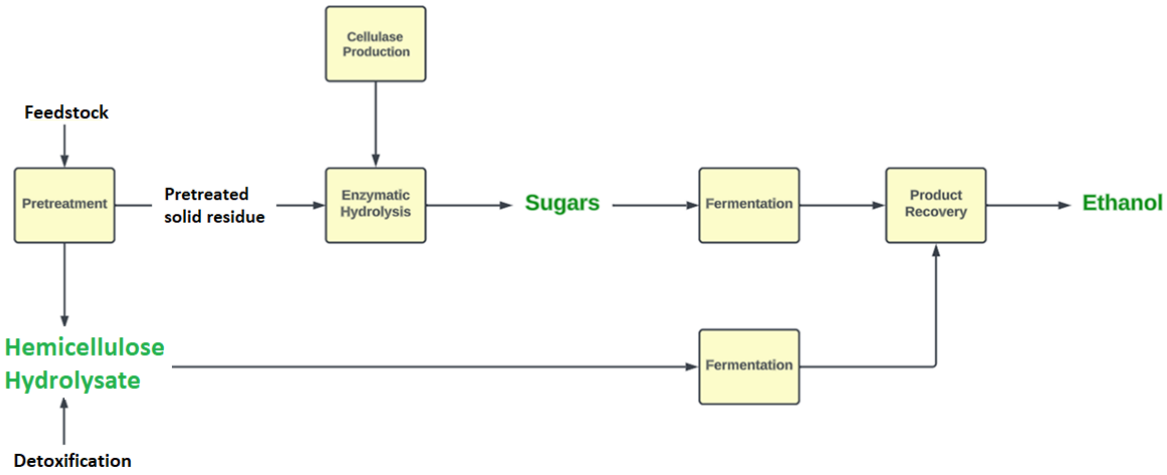
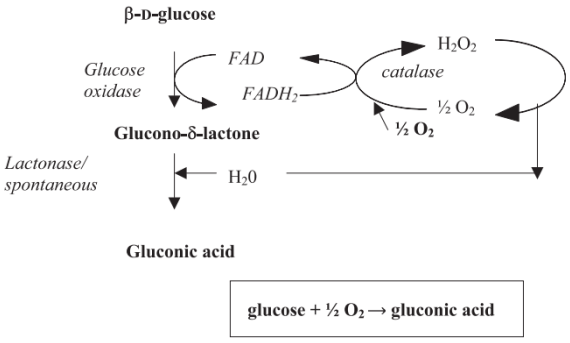


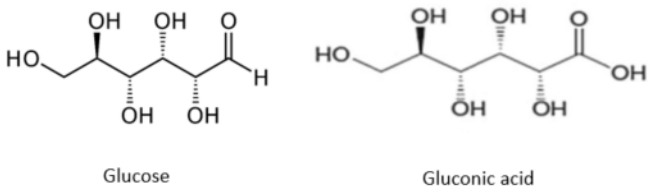
Figure 2: Conventional cellulosic biomass into biofuel production modified for DAP economic feasibility.

1.7. Gluconic acid utilization & pretreatment

Gluconic acid is a nontoxic, low corrosive, nonvolatile, and mild organic acid with capabilities of forming water-soluble complexes with divalent and trivalent metal ions (50,51,52). These traits allow for gluconic acid, containing forms of □-lactone and gluconates (sodium gluconate, potassium gluconate, etc.), to be widely used in industries; construction (45%), food (35%), pharmaceutical (10%), and others (50,51,52,53). As shown in **Figure 3a**, gluconic acid can be produced from glucose via oxidation using *Aspergillus niger* (51).



a



b

Figure 3: Oxidation pathway of glucose into gluconic acid from *Aspergillus niger*. Reproduced from Ramachandran, S., Fontanille, P., Pandey, A., & Larroche, C. (2006). Gluconic Acid: Properties, Applications and Microbial Production. *Food Technology and Biotechnology, 44*, 185-195 (a) Chemical structure of glucose and gluconic acid (b).

In **Figure 3b**, glucose and gluconic acid only differ in their carbonyl group. That is gluconic acid exhibits a monocarboxylic group that has a pKa of ~3.86, which can provide the necessary hydronium ions to catalyze hydrolysis of hemicellulose bonds in cellulosic biomass as compared to other dilute acid pretreatment studies (32,35,54). Research in gluconic acid pretreatment focused heavily on the production of xylooligosaccharides (XOS) and glucose for various industrial applications. Pretreatment condition study, done by Zhou et al., on sugarcane bagasse at 150°C, pretreatment reaction time of 60 minutes, and using 5% gluconic acid concentration that generated XOS yield of 53.2% and glucose yield of 86.2% (55). In comparison to their prior pretreatment experiment at the same temperature, pretreatment reaction time of 45 minutes, and using 10% acetic acid that generated a lower XOS yield of 39.1% and slightly higher glucose yield of 88.6% (54,55). In addition, Han et al. studied gluconic acid pretreatment on corncob generating XOS yield of 56.2% and cellulose conversion of 86.3% using 154°C, pretreatment reaction time of 47 minutes, and 0.6M of gluconic acid (56). Dai et al. experimented using sorghum straw, achieving XOS yield of 50.3% and high enzymatic hydrolysis yield of 90.8% at 168°C, pretreatment reaction time of 35 minutes, and 7.5% gluconic acid concentration (57). A study from Gu et al., solely focused on the production of XOS (57.73% yield) using 167°C, pretreatment reaction time of 28 minutes, and 4.6% gluconic acid concentration (58).

Additionally, what makes gluconic acid an attractive agent for cellulosic biomass pretreatment is that gluconate, the salt form of gluconic acid, can be utilized as a carbon source by microorganisms. Therefore, acid recovery of the pretreatment process can be avoided. Studies have shown that the Entner-Doudoroff pathway in *E. coli* is able to

catabolize gluconate and gluconate metabolism varies with each study on cell growth (78,79,80,81,82,83,84). Novel experimental gluconate fermentation reported that *Escherichia coli* KO11 was able to metabolize gluconate faster than glucose, producing ethanol and acetate as the main products (41). Additionally, the same study reported that gluconate and glucose are able to be co-utilized in fermentation. Furthermore, Hildebrand et al. engineered the strain *E. coli* AH003, a derivative from *E. coli* KO11 with the pyruvate formate lyase activity gene (*pflA*) and lactate dehydrogenase gene (*ldh*) knocked out, improving the yield of ethanol from gluconate fermentation from 87.5% to 97.5% of the theoretical maximum (60). A related study by Fan et al., suggests that co-fermentation of gluconate and glucose could decrease nutrient requirements in media for fermentation of strains lacking *pfl* gene (59). Additionally, Tao et al. investigated the co-fermentation of gluconate and glycerol for ethanol production via a fed batch fermentation process. The ethanol yield that was achieved from this experiment was about 97.2% of the theoretical maximum using an engineered *Klebsiella oxytoca* WT26, with deleted *pta, frd, ldh, pflA, a*nd *pduC* genes from *K. oxytoca* BW21 (61).

The advantage of gluconic acid pretreatment incorporates an effective catalyst and a downstream substrate in its gluconate form. The potential to mitigate acid recovery and removal is extremely valuable for the fermentation process, particularly for microorganisms that are able to utilize sugars and gluconate simultaneously. This can potentially offset acid cost, which can positively affect the overall cellulosic biofuel production.

1.8. Research Objectives

A process for gluconic acid pretreatment of cellulosic biomass has been studied. Gluconic acid pretreatment of wheat straw will be conducted and analyzed for an effective pretreatment process. Subsequently, the hemicellulose hydrolysate and pretreated solid residue of the effective pretreatment process will be used in fermentation. These goals will be met from the following objectives:

1. Investigate gluconate loss from different pretreatment temperature conditions.
2. Investigate sugar and inhibitor yields from different pretreatment conditions.
3. Analyze the ethanol production in detoxified hemicellulose hydrolysate fermentation and SSF.

2. Material and Methods

2.1. Materials and microbial strain

Wheat straw samples were collected from Idaho National Laboratory in Jefferson, Idaho. The wheat straw were harvested on August 15, 2014, and were sieved to 1 inch and stored at room temperature. Wheat straw dry weight was around 93%, dried in an oven (Fisher Scientific Isotemp Oven) set to 105°C. The composition of the wheat straw contains 33.8% glucan, 17.87% xylan, and 3.17% arabinan (Idaho National Laboratory reference material).

The wheat straw samples used in the pretreatment process were milled to the size of 0.45-1 mm. The gluconic acid solutions were created by dissolving

D-Glucono-1,5-lactone (ThermoFisher Scientific, West Sacramento, California, USA) in deionized (DI) water. The engineered biological strains; *E. coli* SL100 was kindly provided by University of Florida, *S. cerevisiae* D5A was purchased from ATCC, and *E. coli* AH003 was provided by Fan lab and engineered by Hildebrand et al. (60). The Cellulase enzyme blend SAE0020-50mL and Glucosidase from almonds (source BCCJ1434) were purchased from Sigma-Aldrich Co., 3050 Spruce Street, St. Louis, MO 63103 USA.

2.2. Gluconic acid degradation studies

The degradation studies of gluconic acid were conducted using sealed stainless steel, 14-mL tubular reactors (designed by BAE Shop and Fan lab). The gluconic acid solutions were placed into the tubes. The tubes were heated in an initial oil bath (HH oil bath) set to 200°C to bring the acid solutions to the desirable temperature quickly. Immediately, as the desired temperature is reached, the tubes were transferred to a second oil bath (Memmert oil bath one) set at the designated temperature to be maintained at a steady condition where the pretreatment reaction time will initiate. Temperatures were calibrated and recorded using the PARR temperature recorder (PARR 4843). Once pretreatment is completed, the tubes were immersed in an ice water bath to cool the contents below 50°C quickly. The resulting solutions were analyzed for the concentrations of gluconic acid using HPLC.

2.3. Gluconic acid pretreatment

The pretreatment was performed using sealed stainless steel, 14-mL tubular reactors (designed by BAE Shop and Fan lab). The wheat straw was presoaked in the gluconic acid solution overnight. The solid loading used was 10% (w/w). After presoaking, the pretreatment experiments were carried out in silicon oil baths. Temperatures were calibrated and recorded using the PARR temperature recorder (PARR 4843). The tubes were heated in an initial oil bath (HH oil bath) set to 200°C to bring the acid-solid mixed samples to the desirable temperature quickly. Immediately, as the desired temperature is reached, the tubes were transferred to a second oil bath (Memmert oil bath one) set at the designated temperature to be maintained at a steady condition where the pretreatment reaction time will initiate. Once the pretreatment reaction time is reached, the pretreatment was stopped by cooling the tubes to below 50°C using an ice water bath.

After the tubes are cooled and safe to handle, the mixed acid-solid samples (slurry form) were filtered to separate the hemicellulose hydrolysate from the pretreated solid residue. Hemicellulose hydrolysate and pretreated solid residue were collected using glass microfiber filters (Whatman GF/F diameter 21-mm) and ceramic crucibles. Additionally, larger slurry forms were collected using large glass fiber filters (Whatman circles diameters of 125-mm and 15-cm) and large ceramic crucibles.

The densities (units of [g/mL]) of the hemicellulose hydrolysate were recorded by using a pipette (eppendorf) to collect 1-mL and measured on a weighing scale (Ohaus explorer scale). A parallel experiment for pipette accuracy was conducted to calibrate

for measured densities by using a similar method with DI water. Afterwards, the hemicellulose hydrolysates were analyzed for inhibitory compounds. A fraction of the hydrolysate was neutralized using $CaCO_3$ to pH of 5.0-7.0 for monomer analysis. A post hydrolysis took place as the remaining neutralized supernatant was added to a final concentration of 4% sulfuric acid and autoclaved at 121°C for 60-minutes to hydrolyze remaining oligomers into monomers. After autoclaving and cooling to room temperature, samples were collected and neutralized for oligomer analysis. Experimental procedures for monomers, oligomers, and inhibitors were analyzed using Laboratory Analytical Procedure (LAP) from National Renewable Energy Laboratory (NREL) (62). Additionally, parallel pretreatment experiments were conducted for the analysis of pH of gluconic acid after pretreatment.

The pretreated solid residues were washed with large quantities of DI water to remove remaining hemicellulose hydrolysate sugar and metabolite residues. Afterwards, the solids were left to air-dry at room temperature for subsequent compositional analysis using LAP from NREL (63). The compositional analysis of the pretreated solid residue was analyzed by weighing out ~0.2 grams and adding 2-mL of 72% H_2SO_4 in pressure tubes (Ace glass incorporated). The samples were incubated at 30°C for 60-minutes in a DI-water bath while shaking for well-mixing. After the incubation period, samples were added with DI-water to convert the total volume to have 4% H_2SO_4. A control sample containing glucose and xylose in 4% H_2SO and samples were autoclaved at 121°C for 60-minutes for sugar hydrolysis and calibration.

In addition, an optimized gluconic acid pretreatment was conducted in a 200-mL PARR reactor (PARR Instrument Co. Moline, IL, USA T316 012214) to generate a large

working volume of hemicellulose hydrolysate and pretreated solid residue for the fermentation process. Temperature was recorded using the PARR temperature recorder (PARR 4843). The pretreatment was conducted at a desired temperature for a period of pretreatment reaction time and once completed, reaction was stopped by submerging the reactor into an ice water bath for fast cooling.

2.4. Enzymatic hydrolysis

Preliminary cellulase activity was measured prior to the enzymatic hydrolysis process using LAP from NREL (64). The enzymatic hydrolysis of the pretreated solid residue was conducted using 1% (w/w) solid concentration in 0.05M citrate buffer solution with pH of 4.8. The hydrolysis was incubated in a rotary shaker running at 150 rpm, 50°C for 72-hours following the LAP from NREL with some modifications (65). In addition, sodium azide was added at a final concentration of 0.1% to prohibit potential microbial contamination during the hydrolysis process. A cellulase loading of 60 FPU/g cellulose was used. The samples were collected at 72-hours and samples were boiled for 5-minutes to deactivate enzymes immediately and spun down using centrifuge (Beckman Coulter microfuge 16). The remaining supernatants were collected for sugar analysis.

2.5. Fermentation seed culture

The ethanologenic strains of *E. coli* was used as the ethanologen for fermentation. A culture tube containing 3-mL of LB media (10-g/L of casein peptone, 10-g/L of sodium chloride, and 5-g/L of yeast extract) was inoculated with a single

colony of *E. coli* and incubated in a rotary shaker (Thermo Scientific MaxQ6000) for 8-hours at 37°C and 200 rpm. From the pre-culture, 1-mL was transferred to a 200-mL seed serum bottle containing 100-mL of LB with 2% glucose. The seed culture was incubated for 12-16-hours at 30°C and 200 rpm. The seed cultures are then stored in 4°C for further use to make more seed bottles prior to fermentation processes.

The ethanologenic strain *S. cerevisiae* D5A was also used. A Yeast Peptone (YP) medium contains the concentration of 20-g/L of peptone and 10-g/L of yeast extract was used to inoculate the strain. A culture tube containing 3-mL of the YP medium was inoculated with a single colony and incubated in a rotary shaker for 8-hours at 35°C and 200 rpm. From the pre-culture, 1-mL was transferred to a 200-mL seed serum bottle containing 100-mL of YP with 2% glucose. The seed culture was incubated for 12-16-hours at 35°C and 200 rpm. The seed cultures are then stored in 4°C for further use to make more seed bottles prior to fermentation processes.

The fermentation experiments were initiated by inoculating the fermentation-ready serum bottles with mid-log phase strains at an initial OD of ~0.1. Multiple trials of fermentation were analyzed in serum bottles on a rotary shaker at 37°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of substrates and metabolites.

2.6. Simultaneous saccharification and fermentation (SSF)

2.6.1. Enzyme activity

Prior to running SSF, enzyme assays were conducted to determine the enzyme activity. The cellulase activity was measured using LAP from NREL (64). The □-glucosidase activity was measured following the IUPAC protocol (66).

2.6.2. Preparation of SSF using *E. coli* AH003

Pretreated wheat straw solids were added to serum bottles along with LB medium at an equivalent glucose content of 20-g/L. The pH was adjusted to 6.5-7.0 and airspace was purged with nitrogen gas before autoclaving at 121°C. After autoclave and cooling to room temperature, enzymes were added through filter sterilization (cellulase loading of 20 FPU/g cellulose and □-glucosidase loading of 20 IU/g cellulose). Mid-log phase *E. coli* AH003 were inoculated into the serum bottles and incubated for fermentation analysis. The batch experiments were initiated by inoculating the serum bottles with mid-log phase *E. coli* AH003 strain at an initial OD of ~0.1. The serum bottles were cultured in a rotary shaker (Thermo Scientific MaxQ6000) at 37°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of substrates and metabolites.

2.6.3. Preparation of SSF using *S. cerevisiae* D5A

Pretreated wheat straw solids were added to serum bottles along with lean medium at an equivalent glucose content of 20 g/L. The lean medium contained 5mM

MgSO4 and 0.3% (v/v) corn steep liquor (CPC International, Summit-Argo, IL) (67). The pH of the medium was adjusted to 4.5-5.0 and airspace was purged with nitrogen before autoclaving at 121°C. After autoclaving and cooling to room temperature, enzymes were added through filter sterilization (cellulase loading of 20 FPU/g cellulose and □-glucosidase loading of 20 IU/g cellulose). The batch experiments were initiated by inoculating the fermentation serum bottles with mid-log phase *S. cerevisiae* D5A strain at an initial OD of ~0.1. The serum bottles were cultured in a rotary shaker (Thermo Scientific MaxQ6000) at 37°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of substrates and metabolites.

2.7. Detoxified hemicellulose hydrolysate and fermentation

2.7.1. Detoxification of hemicellulose hydrolysate

The hemicellulose hydrolysate was detoxified using a modified method based on López-Linares and Preechakun et al. (47,48). Initially, the hydrolysate was over-limed to pH 10 using $Ca(OH)_2$. The over-limed hydrolysate was incubated at 50°C at 200 rpm in a rotary shaker for 30 min. The product was pH adjusted back to 2.0-2.5 by adding concentrated 72% H_2SO_4. After the pH adjustment, the treated hydrolysate was filtered through a glass fiber filter to separate liquid and sediments. The filtrate (over-limed hydrolysate) was added with activated carbon at 4.5% (w/v) and incubated at 45°C for 1-hour. The activated carbon treatment was filtered to separate solids from liquid, where the liquid (combined hydrolysate) remained at pH of 2.0-2.5. Subsequently, the

combined hydrolysate would be neutralized to pH 6.5-7.0 using KOH for the fermentation process.

The composition of the original, over-limed, and combined hydrolysates were analyzed for inhibitor, gluconic acid, and sugar concentrations. The phenol contents were analyzed using gallic acid as the standard based on the Folin-Ciocalteu Micro Method (68).

2.7.2. Preparation of detoxified hemicellulose hydrolysate fermentation using *E. coli* AH003

The pH of the detoxified hemicellulose hydrolysate was adjusted to 6.5-7.0 using KOH. The 10x concentrated LB medium was added at a volume of 3.5 mL into 100-mL serum bottles, purged with nitrogen gas, and autoclaved. After cooling, the pH adjusted hydrolysate at a volume of 30.5-mLwas then filtered sterilized to the 10x concentrated LB medium. The fermentation was initiated by inoculating the serum bottles with mid-log phase *E. coli* AH003 at an initial OD of ~0.1. Serum bottles were cultured in a rotary shaker (Thermo Scientific MaxQ6000) at 37°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of substrates and metabolites.

2.7.3. Preparation of detoxified hemicellulose hydrolysate fermentation using *E. coli* SL100

The strain *E. coli* SL100 was used in previous studies when conducting hemicellulose hydrolysate fermentation in low salt media (73,74). The detoxified hemicellulose hydrolysate was pH adjusted to 6.5-7.0 using KOH. The fermentation

media used was low alkali metals and total salts (AM1 media) where the final concentrations was achieved: alkali metals (mmol/L): $(NH_4)_2HPO_4$, 19.92; $NH_4H_2PO_4$, 7.56; KCl, 2.00; $MgSO_4 \cdot 7H_2O$, 1.50; Betaine · KCl, 1.00; and total salts: (µmol/L): $FeCl_3 \cdot 6H_2O$, 8.88; $CoCl_2 \cdot 6H_2O$, 1.26; $CuCl_2 \cdot 2H_2O$, 0.88; $ZnCl_2$, 2.20; $Na_2MoO_4 \cdot 2H_2O$, 1.24; H_3BO_3, 1.21; $MnCl_2 \cdot 4H_2O_2$, 2.50 (69). Concentrated 10x AM1 medium was added at a volume of 3.5-mL into 100-mL serum bottles, purged with nitrogen gas, and autoclaved at 121°C. After cooling to room temperature, the hydrolysate was adjusted to pH of 6.5 using KOH and filtered sterilized (30.5-mL) to the concentrated AM1 medium. The fermentation was initiated by inoculating the serum bottles with mid-log phase *E. coli* SL100 at an initial OD of ~0.1. Serum bottles were cultured in a rotary shaker (Thermo Scientific MaxQ6000) at 37°C and 200 rpm. Samples were taken at various time intervals to analyze the concentrations of substrates and metabolites.

2.8. Analytical methods

The sugars, gluconate, and inhibitors samples were analyzed using a Shimadzu High Performance Liquid Chromatography (HPLC) equipped with a refraction index detector, and a photodiode array detector. The IC Sep ICE-ION-300 and Carbo Sep Coregel-87C and Coregel-87P columns (Transgenomic, San Jose, CA, USA) were used. 5 mM of H2SO4 was used as the mobile phase to run the IC Sep ICE-ION-300 column at 60°C for the analysis of inhibitors, milli-Q water was used as the mobile phase to run the Carbo Sep Coregel-87P column at 80°C for the analysis of sugars, and 4mM of CaCl2 was used as the mobile phase to run the Carbo Sep Coregel-87C

column at 80°C for the analysis of gluconate. The flow rate of the mobile phase was controlled at 0.5 mL/min.

3. Results and Discussion

3.1. The degradation of gluconic acid during pretreatment

Gluconic acid is a sugar acid that can potentially degrade at high temperatures. Gluconic acid solutions of concentrations of 0.125M (pH 2.37) and 1M (pH 1.8) were subjected to different temperature conditions of 170-190°C and different reaction times of 0.5-2.5-hours. Shown in **Figure 4**, when the pretreatment temperature was 170°C, roughly 10.5% of 1M gluconic acid was degraded at 2.5-hours. At the same pretreatment reaction time, when temperature was increased to 180°C, roughly 17% of 1M gluconic acid was degraded. About 24.5% of 1M gluconic acid was lost at 190°C. Gluconic acid concentration at 0.125M degraded slightly slower compared to the 1M gluconic acid concentration at the same temperatures. About 4.7% of gluconic acid was degraded at the most severe pretreatment condition (1M gluconic acid, 30-minutes, and 190°C). Furthermore, the specific degradation products of gluconic acid have not been identified. Assuming that gluconic acid degradation followed first-order kinetics, the calculated first-order reaction rate constants for gluconic acid degradation at different temperatures was shown in **Table 1**.

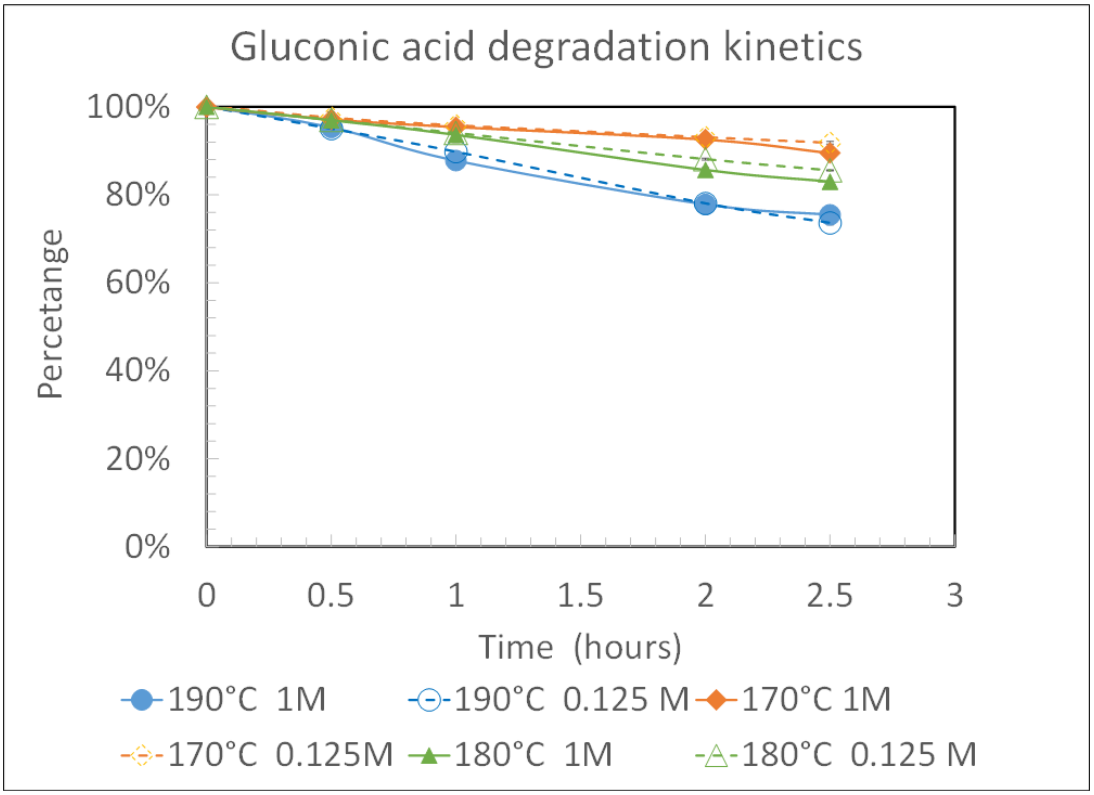


Figure 4: Profiles glucose acid decomposition at various concentrations and reaction temperatures.

Table 1. The calculated first order reaction rate constant for gluconic acid decomposition.

	0.125M (hour^{-1})	1M (hour^{-1})
170°C	0.030±0.002	0.039±0.007
180°C	0.064±0.002	0.083±0.007
190°C	0.122±0.004	0.123±0.008

3.2. Gluconic acid pretreatment of wheat straw

3.2.1. Stage 1 sugar yields

The pretreatment experiments were conducted at different temperatures (160°C & 170°C), different reaction times, and a fixed 0.125M gluconic acid concentration is shown in **Figure 5a-b**. Additionally, the latter pretreatment experiment is conducted on different gluconic acid concentrations (0.125M-1M), different temperature conditions (170-190°C), and at a fixed reaction time of 30-minutes (**Figure 5c-d**).

As shown in **Figure 5**, the glucose monomer (GM), glucose oligomer (GO), xylose monomer (XM), and xylose oligomer (XO) were found in the hemicellulose hydrolysate. The yield of xylose is much greater than glucose. In **Figure 5a**-**b**, the trend showed that the rate of sugar monomers yields tend to increase with the increase of reaction time and temperature. However, the sugar oligomers yields decreased with the increase of temperature and reaction time. The case exists for both glucose and xylose yields. When comparing different gluconic acid concentrations and temperatures at 30-minute reaction time, as shown in **Figure 5c**, the GO yield decreases as pretreatment temperature increases. In contrast, the yields of GM increased with the increase of acid concentration. However, the increase in GM yield is limited to the increase of reaction temperatures from 170°C to 180°C. At the lowest gluconic acid concentration (0.125M), GM yield was highest at 190°C but the rate of GM yield increased and exceeds at 180°C as gluconic acid concentration increased. The temperature of 190°C is the most severe temperature tested, thus higher sugar liberation and degradation products could be more apparent as the hemicellulose

structure is degraded during pretreatment (40). In **Figure 5d**, the XM yields increased with increasing acid concentration limited to temperatures 170°C to 180°C. The temperature of 190°C can cause XM yields to decrease with the increase of gluconic acid concentration. As such, the most severe temperature condition along with increasing acid concentration has further promoted XM loss. The XO yields decreased with the increase of acid concentrations and pretreatment temperature, where yields were lowest at 190°C. The highest XM yield (39.3%) was achieved at 180°C, 1M of gluconic acid concentration, and 30-minutes reaction time. The highest XO yield (58.6%) was achieved at 170°C, 0.125M of gluconic acid concentration, and 30-minutes reaction time.

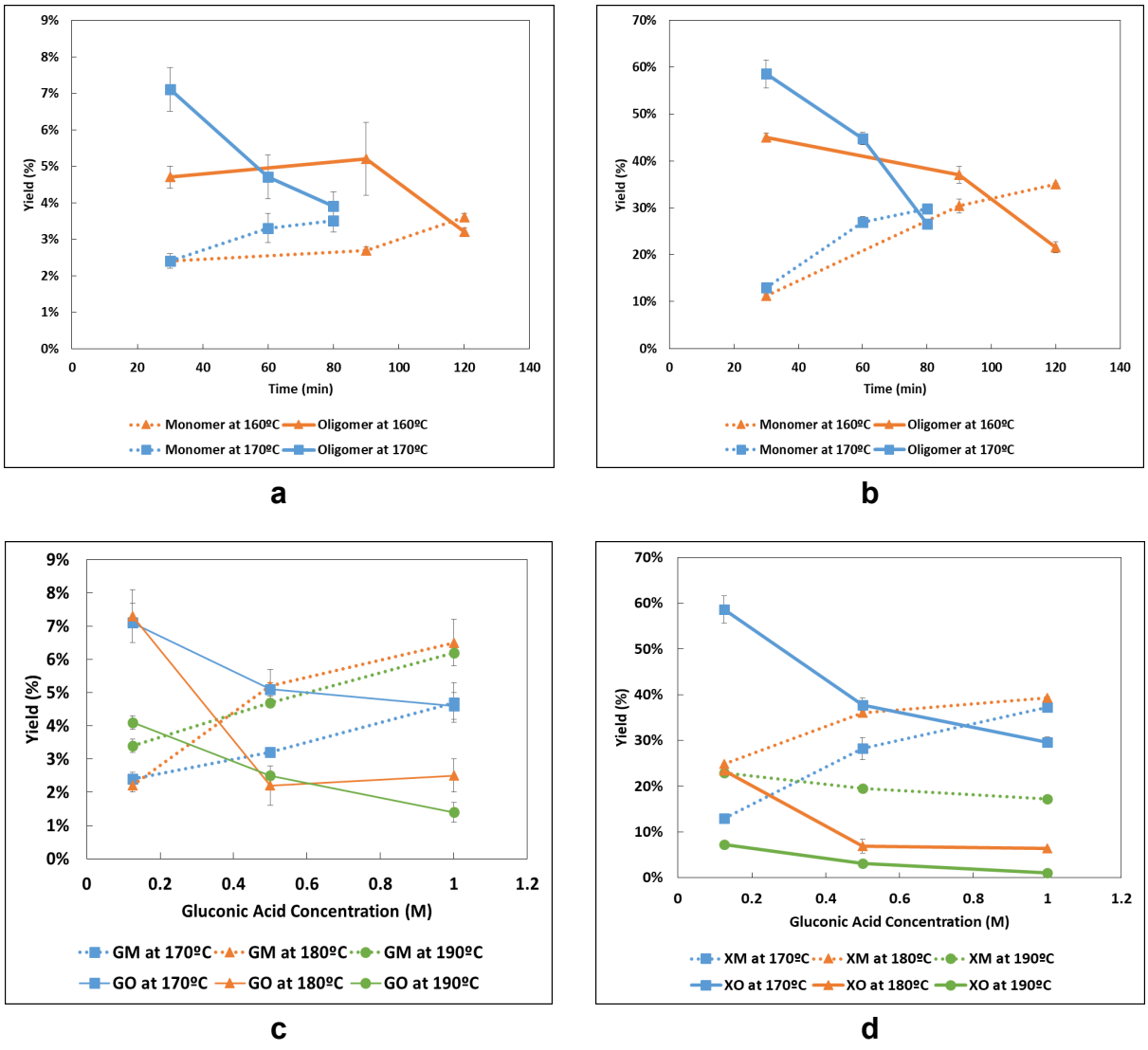
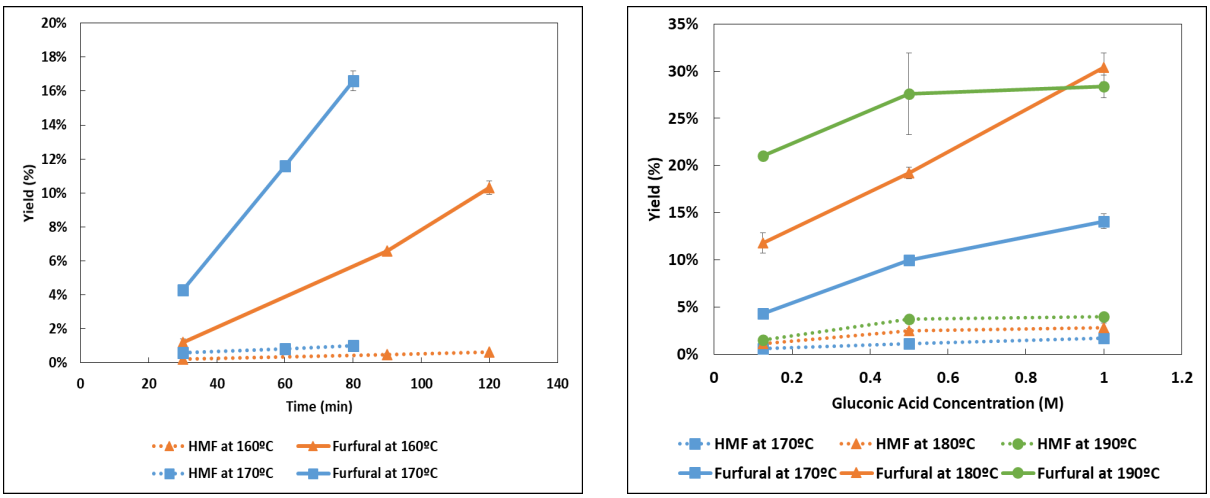


Figure 5: Overall sugar yields of stage 1; (a) glucose yield at constant 0.125M gluconic acid concentration (b) xylose yield at constant 0.125M gluconic acid concentration (b) glucose yield at constant 30-minutes reaction time (d) xylose yield at constant 30-minutes reaction time. Error bars denote the standard deviations from triplicates.

3.2.2. Stage 1 sugar degradation products

Increasing the severity of pretreatment conditions; including elevated concentrations, longer duration, and higher temperatures, has shown to lead to higher generation of degradation products in the hemicellulose hydrolysate. Furfural concentrations were higher than HMF concentration, which aligns with higher xylose concentration compared to glucose concentration. These trends align with the pretreatment using other organic acids (31, 32, 33). In **Figure 6,** the increase in pretreatment temperature shows an upward trend of HMF and furfural yields. Furthermore, **Figure 6a** showed that increasing pretreatment reaction time and temperature will cause HMF and furfural yields to increase. In addition, **Figure 6b** shows an increase in both HMF and Furfural as acid concentration becomes more severe with the increase of temperature. However, at the highest acid concentration (1M), furfural yield is much less at 190°C compared to 180°C. This may be due to the degradation of HMF and furfural to form levulinic acid and formate as discussed in other studies (32,40).

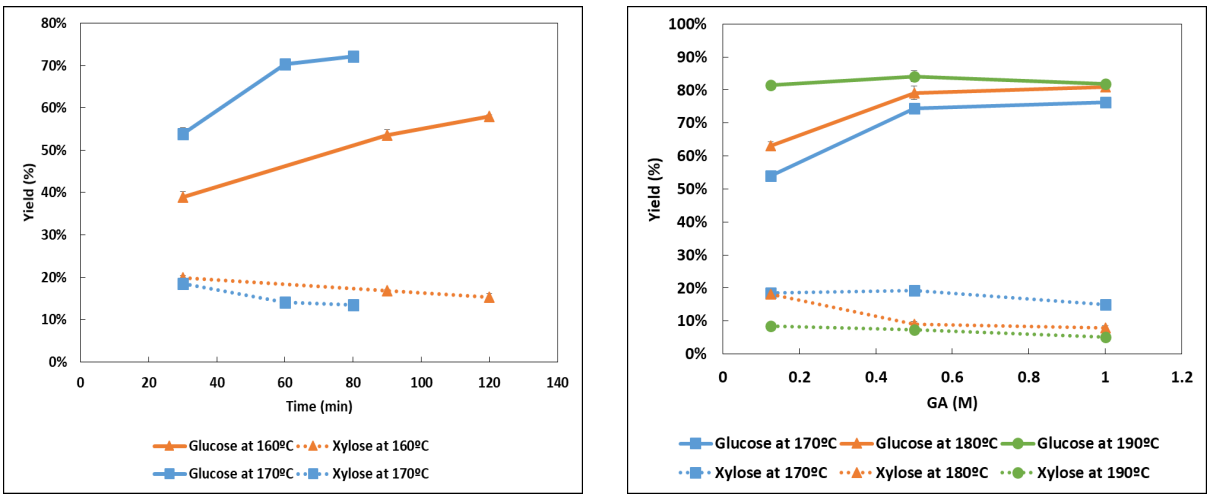


a **b**

Figure 6: Overall sugar degradation products of stage 1; (a) degradation products at fixed 0.125M gluconic acid concentration (b) degradation products at fixed pretreatment reaction time of 30-minutes. Error bars denote the standard deviations from sample triplicate runs.

3.2.3. Enzymatic digestion of gluconic acid pretreated solid residue

The enzymatic hydrolysis of the pretreated solid residue was carried out at an enzyme loading of 60FPU/g of glucan for three days. From the pretreated solids, the glucose yield was highest with the increase of temperatures, acid concentrations, and pretreatment times. However, **Figure 7b** shows that the glucose yield at 190°C peaked at the acid concentration of 0.5M. At this temperature, the further increase of the acid concentration led to a decrease in the glucose yield. The stage 2 xylose yield decreased with the increase of the temperatures, pretreatment reaction times, and gluconic acid concentrations. The highest xylose yield in stage 2 was achieved at a pretreatment temperature of 160°C, 0.125M gluconic acid concentration, and pretreatment reaction time of 30-minutes.



a **b**

Figure 7: Overall sugar degradation products of stage 2; (a) Sugar yield products at fixed 0.125M gluconic acid concentration (b) Sugar yield products at fixed pretreatment reaction time of 30-minutes. Error bars denote the standard deviations from sample triplicate runs.

3.2.4. Combined glucose and xylose yields from pretreatment and enzymatic hydrolysis and the combined severity factor analysis

The combined severity parameter analysis allows the comparison of data under different pretreatment conditions (**APPENDIX EQUATION 11**). **Figure 8** plots the overall glucose yield, overall xylose yield, and the combined glucose and xylose yield (overall sugar yield) vs. the combined severity factor of all the pretreatment conditions measured. As shown in **Figure 8a**, the overall glucose yield increased with the increase of the combined severity factor. The highest glucose yield was achieved at the log CS of about 1.21, corresponding to the pretreatment of 190°C, 30-minutes reaction time, and 0.5M gluconic acid concentration. **Figure 8b** shows the highest overall xylose yield was

achieved at the log CS of about 0.22, corresponding to the pretreatment conditions of 170°C, 30-minutes reaction time, and 0.125M gluconic acid concentration. Lastly, **Figure 8c** shows that the overall sugar yield was insensitive to the combined severity factor.

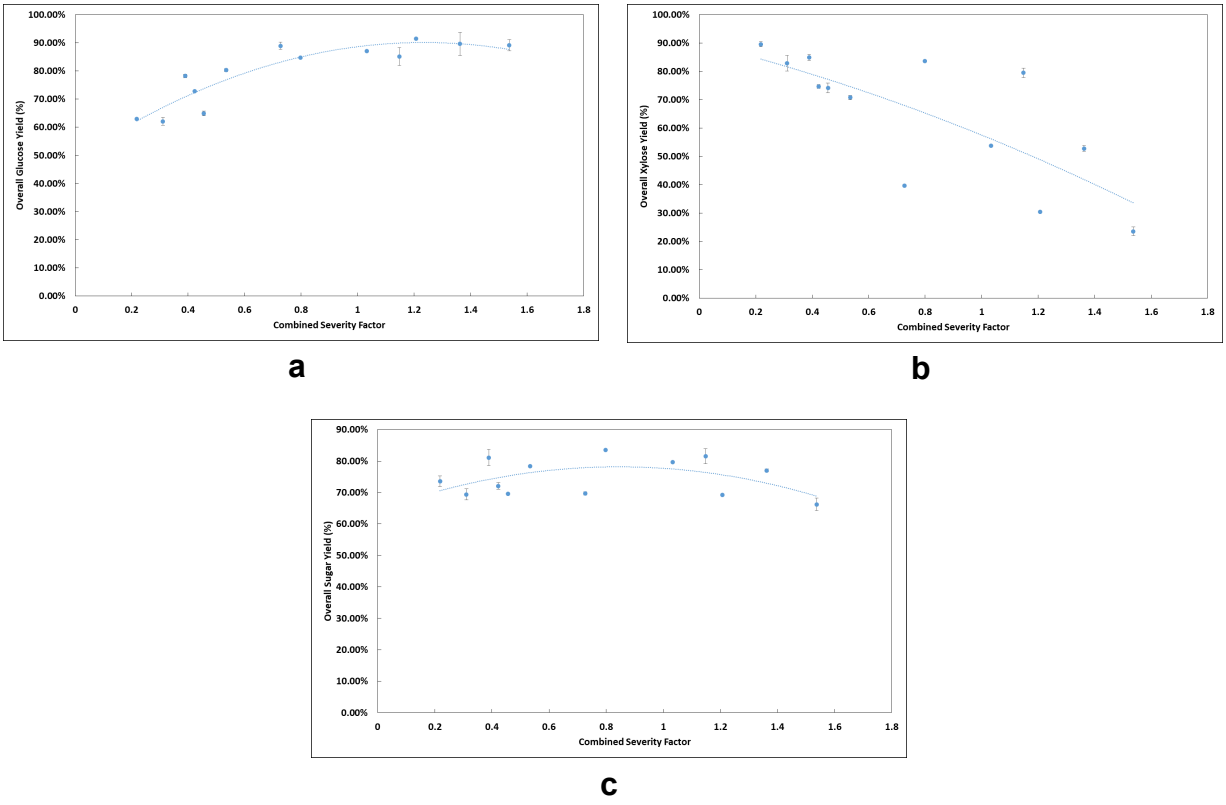


Figure 8: Overall sugar yields of stages 1 and 2 combined; (a) glucose vs severity factor (b) xylose vs severity factor (c) combined glucose and xylose vs severity factor. Error bars denote the standard deviations from sample triplicate runs.

4. Fermentation Process

4.1. Characterization of hemicellulose hydrolysate generated using the PARR reactor

The pretreatment condition of 0.125M gluconic acid, pretreatment time of 60-minutes, and temperature of 170°C was used to generate hemicellulose hydrolysate. This pretreatment was conducted inside of a 200-mL PARR reactor. The original hemicellulose hydrolysate collected after pretreatment cannot be fermented by either the engineered *E coli.* strains. Therefore, a detoxification method of the hemicellulose hydrolysate was implemented by using an over-liming method (over-limed hydrolysate) followed by the activated carbon treatment (combined hydrolysate). The composition of the hemicellulose hydrolysate detoxification treatment is shown in **Table 2**. The combined hydrolysate showed significant reduction in furfural, HMF, and total phenolic contents. However, the combined hydrolysate was limited in reducing acetate and the sugar concentrations were unchanged.

After pretreatment, the recovery of the gluconate was approximately 75.6±0.59%. Roughly 4.2±0.14% of gluconic acid may have been lost from degradation from the pretreatment condition. Hence, we estimate that roughly 20% of gluconic acid was unaccounted for in the hemicellulose hydrolysate. Similar observations of organic acid loss after pretreatment have been discussed where the organic acid was found to be somehow selectively bound to the pretreated solid residue, leading to lower acid

concentration in the hydrolysate (31). We suspect that 20% of the gluconic acid could also be bound to the straw particles.

Table 2. Comparison of composition of gluconic acid hydrolysate before and after detoxification. Standard deviations are denoted after "±-sign" from sample triplicate runs.

	Inhibitory Compounds (g/L)			
Detoxification Method	Acetic acid	HMF	Furfural	Total Phenols
Original hemicellulose hydrolysate (oR)	2.46±0.05	0.32±0.03	2.55±0.45	1.68±0.00
Overliming (oV)	3.34±0.00	0.16±0.04	1.30±0.13	1.01±0.01
Overliming+activated charcoal (cO)	3.10±0.05	0.04±0.01	0.36±0.03	0.16±0.00
	Carbon Source (g/L)			
Detoxification Method	Glucose	Xylose	Arabinose	Gluconic acid
Original hemicellulose hydrolysate (oR)	1.05±0.06	5.21±0.24	1.16±0.04	18.80±0.15
Overliming (oV)	0.97±0.02	5.08±0.02	1.15±0.06	19.21±0.46
Overliming+activated charcoal (cO)	0.95±0.15	5.06±0.15	1.11±0.04	18.33±0.61

4.2. Fermentation of the hemicellulose hydrolysate to ethanol using *E. coli* AH003

The strain *E. coli* AH003 utilized gluconate, glucose, xylose, and arabinose simultaneously as shown in **Figure 9**. Glucose was consumed after 7.5 hours, gluconate and arabinose were both consumed after 18.5 hours, and xylose was fully consumed after 30 hours. The starting hydrolysate contained an initial 38 mM of acetate. The strain produced an additional 26.5 mM of acetate throughout the fermentation time period. Another prominent fermentation by-product was 2.21 mM of lactate produced by the strain. The yield of the ethanol from the consumed gluconate and sugars were about 107.5±1.2% of the theoretical maximum (**APPENDIX EQUATION 10.1**). The theoretical maximum of over 100% may be due to the use of LB media which contains nutrients such as casamino acids that could support cell growth and ethanol production in addition to the fermentation substrates (60,72).

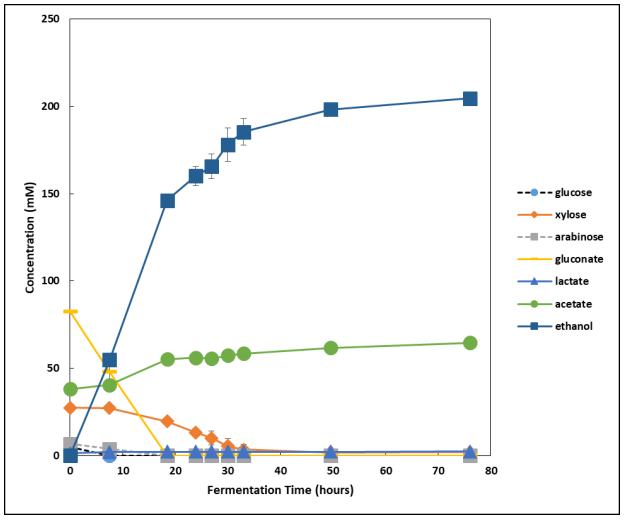


Figure 9: Ethanol production from combined hydrolysate by *E. coli* AH003. Error bars denote the standard deviations from sample triplicate runs.

4.3. Fermentation of the hemicellulose hydrolysate to ethanol using *E. coli* SL100

E. coli SL100 utilized gluconate, glucose, xylose, and arabinose simultaneously as shown in **Figure 10**. The strain consumed glucose after 8 hours, xylose after 49 hours, arabinose after 26.5 hours and gluconate after 31 hours. The starting hydrolysate contained an initial 33 mM of acetate. However, the presence of acetate and low inhibitors in the fermentation hydrolysate did not hinder the ability of *E. coli* SL100 to consume the substrates. By the end of the fermentation process, the acetate concentration increased by 5mM, even though 78mM of gluconate was consumed, which is significantly lower than the theoretical maximum. It is possible that *E. coli* can utilize acetate as a carbon source for cell growth in this case (75). Another prominent by-product is 1.93mM of lactate that was produced by the strain. The yield of the ethanol from consumed gluconate and sugars was about 90.4±1.8% of the theoretical maximum (**APPENDIX Equation 10.1**).

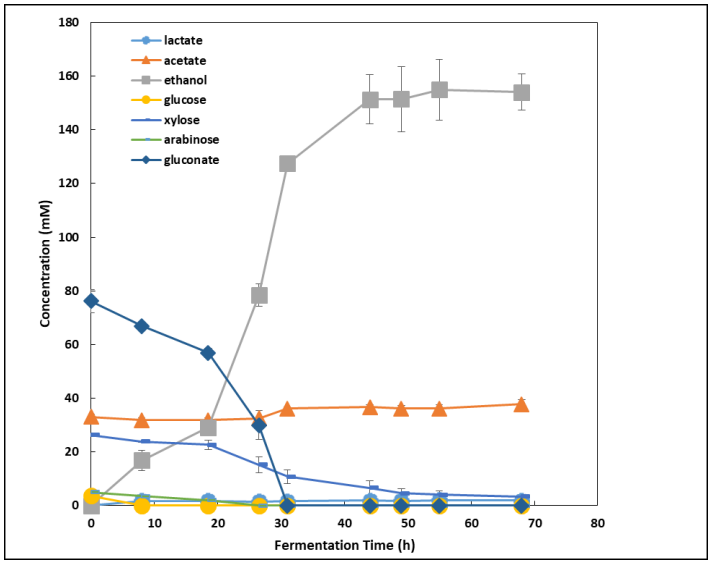


Figure 10: Ethanol production from combined hydrolysate by *E. coli* SL100. Error bars denote the standard deviations from sample triplicate runs.

4.4. Fermentation of pretreated solids to ethanol from SSF using *E. coli* AH003

The pretreated solids contained roughly 52.8±0.7% of glucan and 5.9±0.2% of xylan. The pretreated solid residue mixed with fermentation broth was added with cellulase enzymes of 20 FPU/g cellulose and 20 Unit/g of b-glucosidase. The metabolites production was tracked over 8 days. As shown in **Figure 11**, about 162.8 mM of ethanol were produced. The main by-products from the fermentation were acetate (11.4 mM) and lactate (0.36 mM). The cellulose conversion of the pretreated solids was roughly 69.4±1.0% and the xylan conversion was about 54.7±2.0% from the start of 1.06±0.01 grams of pretreated solid residue (dry basis) in a total volume of 30.6-mL. The maximum theoretical yield of ethanol from the fermentation cannot be calculated since the gluconate stuck to the pretreated solid residue was hydrolyzed and

consumed. Furthermore, as stated prior, nutrients in LB media could promote cell growth and ethanol yield that could increase the theoretical maximum.

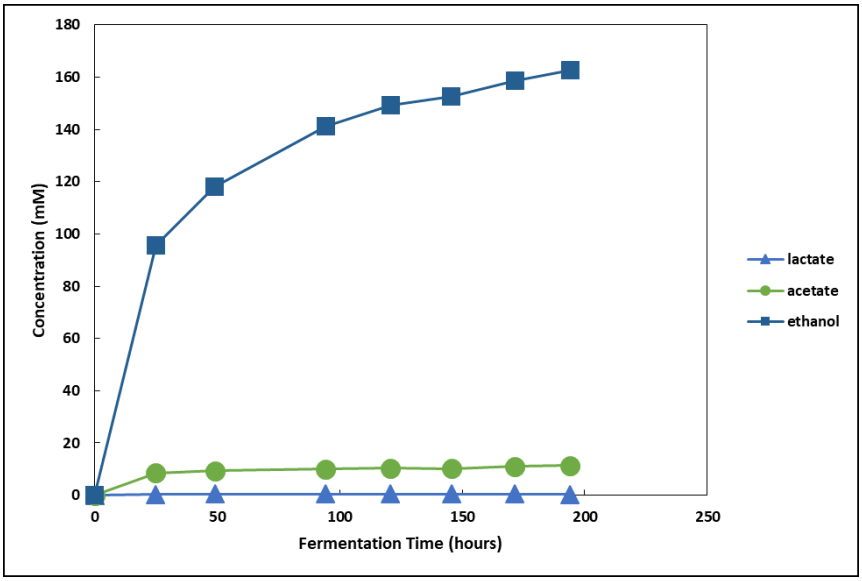


Figure 11: SSF of *E. coli* AH003 in LB media ran for 194.08 hours. Error bars denote the standard deviations from sample triplicate runs.

4.5. Fermentation of pretreated solids to ethanol from SSF using *S. cerevisiae* D5A

The pretreated solid residue contains roughly 52.8±0.7% of glucan and 5.9±0.2% of xylan using mass composition analysis by NREL. The production of metabolites were tracked over 8 days. As shown in Figure 12, about 138.4 mM of ethanol were produced. The main by-products from the fermentation were acetate (1.5 mM) and lactate (3.2 mM) which were very low yield throughout the fermentation. The cellulose conversion of the pretreated solids were roughly 70.8±0.8% from the start of 1.06±0.01 grams of wheat straw (dry basis) in a total volume of 30.6 mL. The maximum theoretical yield of

ethanol from the fermentation was about 92.8±2.0% of the theoretical maximum and 0.475±0.01 (g ethanol/g glucose). Xylose and a tiny bit of gluconate were also hydrolyzed, but neither substrates were consumed by the yeast strain.

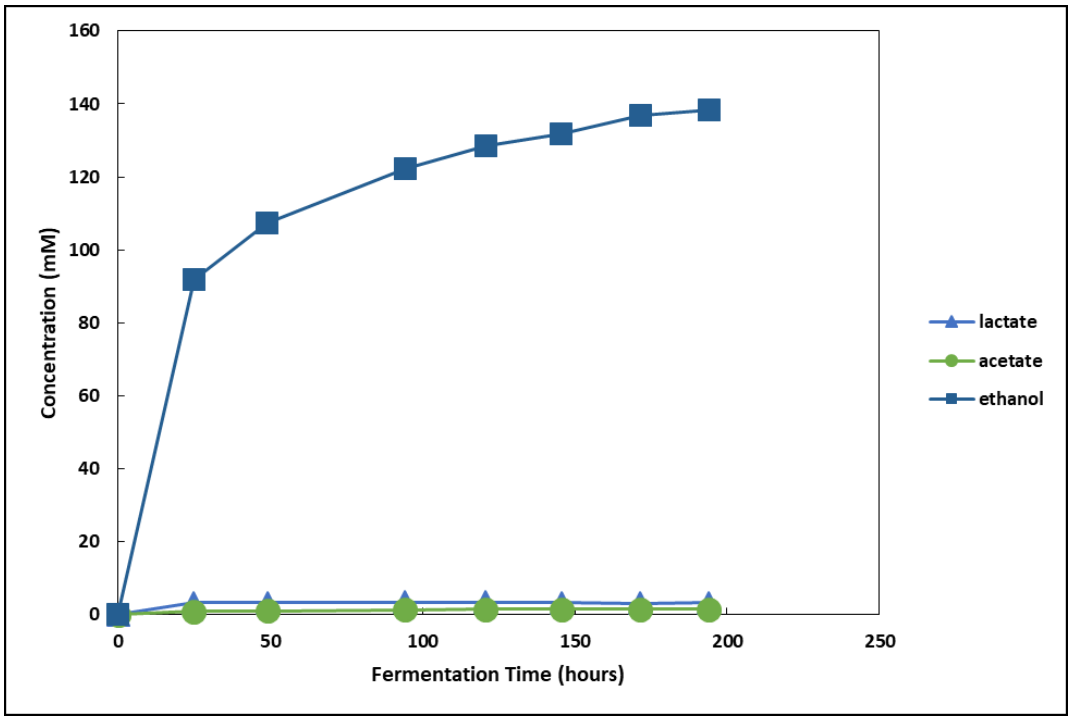


Figure 12: SSF of *S. cerevisiae* D5A in lean media ran for 194.08 hours. Error bars denote the standard deviations from sample triplicate runs.

4. Conclusion and future work recommendations

In conclusion, the study investigated the pretreatment of gluconic acid on wheat straw and fermentation of the resulting hemicellulose hydrolysate and pretreated solid residue. Gluconic acid decomposes as temperature increases. For the pretreatment condition used in the PARR reactor (170°C, 0.125M gluconic acid concentration, and 60-minutes), the degradation of gluconic acid during pretreatment was found to have

lost 4.2±0.14% and 75.6±0.59% of gluconate was recovered in the hemicellulose hydrolysate. The effects of pretreatment conditions, including acid concentration, reaction time, and temperature on sugar yields were investigated. The most optimum pretreatment condition investigated that 0.5M concentration at 170°C, and 30-minutes to have the best overall sugar yield. However, a lower gluconic acid concentration should be used to decrease acid cost and usage. The highest overall xylose yield was achieved with a gluconic acid concentration of 0.125 M, a temperature of 170°C, and a reaction time of 30-minutes. For glucose, the highest overall yield was attained at a temperature of 190°C, a gluconic acid concentration of 0.5 M, and reaction time of 30-minutes.

The ethanol fermentation from hemicellulose hydrolysate was conducted using AM1 media and LB media on strains *E. coli* SL100 and AH003, respectively. Both *E. coli* strains utilized gluconate, glucose, xylose, and arabinose simultaneously in their respective media. Furthermore, the pretreated solid residue can be fermented by *E. coli* AH003 or *S. cerevisiae* D5A using LB or lean media. Both strains were able to utilize hydrolyzed sugars in SSF in their respective media.

Future recommendations for this project should analyze the economic analysis of upscaling of gluconic acid pretreatment. The analysis should include the cost of gluconic acid, ethanol, and detoxification of hemicellulose hydrolysate. Additionally, engineered microorganisms that can consume gluconate and sugars in a cost-effective fermentation medium should be used for both the hemicellulose hydrolysate fermentation and SSF. In general, LB media should be avoided since tryptone and yeast extract are not economically viable for upscale processing. Shortcomings of this study

shows that *E. coli* SL100 was able to consume gluconate but was not able to produce efficient ethanol in SSF based on fermentation experiments analyzed (data not shown). The *E. coli* AH003 would be the best strain but it relies heavily on the use of LB media. Additionally, a prior study showed that ethanol fermentation of *E. coli* AH003 performed very poorly in low-salt M9 media (59). Overall, the prospect of this study is to utilize gluconic acid as a pretreatment agent on wheat straw. Furthermore, this showcases the unique properties of recoverable gluconate in the hemicellulose hydrolysate that was able to be utilized simultaneously with sugars. In addition, the pretreated solid residue was utilized in the subsequent SSF.

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Appendix - Supporting Information

Terminologies:

Sugar analysis in hemicellulose hydrolysate

$GCHH = glucose\ concentration\ in\ hemicellulose\ hydrolysate$

$HHV = hemicellulose\ hydrolysate\ volume$

$GMY = glucose\ monomer\ yield$

$GM = glucose\ monomer$

$GOY = glucose\ oligomer\ yield$

$GO = glucose\ oligomer$

$GCHHAP = glucose\ concentration\ in\ hemicellulose\ hydrolysate\ after\ post\ hydrolysis$

$GCHHBP = glucose\ concentration\ in\ hemicellulose\ hydrolysate\ before\ post\ hydrolysis$

$XCHH = xylose\ concentration\ in\ hemicellulose\ hydrolysate$

$XMY = xylose\ monomer\ yield$

$XM = xylose\ monomer$

$XOY = xylose\ oligomer\ yield$

$XO = xylose\ oligomer$

$XCHHAP = xylose\ concentration\ in\ hemicellulose\ hydrolysate\ after\ post\ hydrolysis$

$XCHHBP = xylose\ concentration\ in\ hemicellulose\ hydrolysate\ before\ post\ hydrolysis$

Sugar analysis in solid residue

$GY = glucose\ yield$

$GCEH = glucose\ concentration\ in\ enzymatic\ hydrolysis$

$EHV = enzymatic\ hydrolysis\ volume$

$XY = xylose\ yield$

$XCEH = xylose\ concentration\ in\ enzymatic\ hydrolysis$

Sugar yields

$OGY = overall\ glucose\ yield$

$OXY = overall\ xylose\ yield$

$TSY = total\ sugars\ yield$

$OG = overall\ glucose$

$OX = overall\ xylose$

Mass comp

$AV = 4\%\ H2SO4\ volume$

Fermentation

$TE = Theoretical\ ethanol$

$EY = Ethanol\ yield$

$PSFB = pretreated\ solid\ in\ fermentation\ bottle\ before\ SSF$

$PSFA = pretreated\ solid\ in\ fermentation\ bottle\ after\ SSF$

List of Equations:

In this paper, stage 1 refers to pretreatment of the milled wheat straw to produce hemicellulose hydrolysate and stage 2 refers to the enzymatic hydrolysis of the pretreated solids. The following yield equations were calculated using the data collected from the HPLC, in units of (g/L). The concentrations are converted into grams and divided over an equivalent sugar content that existed in the raw wheat straw, based on data composition from Idaho National Lab.

Stage 1 sugars exist in the hemicellulose hydrolysate as monomers and oligomers. Glucose and xylose monomers refer to glucose or xylose. While the glucose and xylose oligomers are the soluble polymers with the chain length equal or greater than two.

Equation 1: Stage 1 glucose monomer yield (GMY)

$$GMY = \frac{GM\ (g)}{equivalent\ glucose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

Stage 1 glucose monomer (GM)

$$GM\ (g) = GCHH\ (g/L) \times HHV\ (L)$$

Equation 2: Stage 1 glucose oligomer yield (GOY)

$$GOY = \frac{GO\ (g)}{equivalent\ glucose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

Stage 1 glucose oligomer (GO)

$$GO\ (g) = [GCHHAP\ (g/L) - GCHHBP\ (g/L)] \times HHV\ (L)$$

Equation 3: Stage 1 xylose monomer yield (XMY)

$$XMY = \frac{XM\ (g)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

Stage 1 glucose monomer (GM)

$$XM\ (g) = XCHH\ (g/L) \times HHV\ (L)$$

Equation 4: Stage 1 xylose oligomer yield (GOY)

$$XOY = \frac{XO\ (g)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

Stage 1 xylose oligomer (XO)

$$XO\ (g) = [XCHHAP\ (g/L) - XCHHBP\ (g/L)] \times HHV\ (L)$$

Equation 5: Stage 2 glucose yield (GY)

Stage 2 glucose and xylose remains as unhydrolyzed carbohydrates in pretreated solids. These carbohydrates required the use of enzymes to further yield monomeric sugar forms. The enzyme hydrolysis of pretreated solid wheat straw sugar yield are as follows:

$$GY = \frac{stage\ 2\ glucose\ (g)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

$$stage\ 2\ glucose\ (g) = GCEH\ (g/L) \times EHV\ (L)$$

Equation 6: Stage 2 xylose yield (GY)

$$GY = \frac{stage\ 2\ xylose\ (g)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

$$stage\ 2\ xylose\ (g) = XCEH\ (g/L) \times EHV\ (L)$$

Equation 7: Total sugar yield

Given the definitions of yields for the stage 1 and stage 2 sugars, we will calculate the overall glucose and xylose yields to see how much glucose and xylose were yielded as the result of the pretreatment. Afterwards, we will combined these to calculate the total sugar yields as follows:

$$GY = \frac{OG\ (g)}{equivalent\ glucose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

$$OG\ (g) = stage\ 1\ GM\ (g) + stage\ 1\ GO\ (g) + stage\ 2\ glucose\ (g)$$

$$XY = \frac{XG\ (g)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

$$OX\ (g) = stage\ 1\ XM\ (g) + stage\ 1\ XO\ (g) + stage\ 2\ xylose\ (g)$$

$$TSY = \frac{OG\ (g) + OX\ (g)}{equivalent\ glucose\ content\ in\ raw\ wheat\ straw\ (g) + equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g)} \times 100\%$$

Equation 8: Glucan and xylan yields

A mass composition was done on the pretreated wheat straw in 4% H2SO4 before (pre-SSF) and after (post-SSF) the SSF process to determine the glucan and xylan content.

$$Glucan\ yield\ = \frac{GCEH\ (g/L) \times AV\ (L) \times 0.90}{pretreated\ wheat\ straw\ in\ enzymatic\ hydrolysis\ (g)} \times 100\%$$

$$Xylan\ yield\ = \frac{XCEH\ (g/L) \times AV\ (L) \times 0.88}{pretreated\ wheat\ straw\ in\ enzymatic\ hydrolysis\ (g)} \times 100\%$$

Equation 9: Sugar degradation product yields

Inhibitory compounds that are generated during the pretreatment process are HMF and furfural. HMF is the degradation of glucose/glucan while furfural is the degradation of xylose/xylan. The yield of each degradation products are calculated as follows:

$$HMF\ yield\ = \frac{HMF\ concentration\ (g/L) \times HHV\ (L)}{equivalent\ glucose\ content\ in\ raw\ wheat\ straw\ (g) \times \frac{126}{180}} \times 100\%$$

$$Furfural\ yield\ = \frac{Furfural\ concentration\ (g/L) \times HHV\ (L)}{equivalent\ xylose\ content\ in\ raw\ wheat\ straw\ (g) \times \frac{96}{150}} \times 100\%$$

Equation 10: Fermentation theoretical maximum

Ethanol theoretical maximum yield was determined based on a theoretical metric of sugar consumption to form ethanol. Theoretically, 1.5 moles of ethanol and 0.5 mol of acetate can be produced from 1 mole of gluconate. 1.67 moles of ethanol can be produced from 1 mole of xylose and arabinose, while 2 moles of ethanol are produced from consuming 1 mole of glucose (41,76,77). The definitions are reported in concentrations as follows:

$$Glucose\ (mM) = \frac{Glucose\ concentration\ (g/L)}{180.156\ (g/mol)} \times 1000$$

$$Xylose\ (mM) = \frac{Xylose\ concentration\ (g/L)}{150.13\ (g/mol)} \times 1000$$

$$Arabinose\ (mM) = \frac{Arabinose\ concentration\ (g/L)}{150.13\ (g/mol)} \times 1000$$

$$Gluconate\ (mM) = \frac{Sodium\ gluconate\ concentration\ (g/L)}{218.14\ (g/mol)} \times 1000$$

$$Acetate\ (mM) = \frac{Sodium\ acetate\ concentration\ (g/L)}{82.03\ (g/mol)} \times 1000$$

$$Ethanol\ (mM) = \frac{Ethanol\ concentration\ (g/L)}{46.07\ (g/mol)} \times 1000$$

Equation 10.1: Hemicellulose Hydrolysate Fermentation (HHF)

$$TE\ (mM)\ =\ Glucose(mM)\times 2\ +\ [Xylose(mM)\ +\ Arabinose(mM)]\times 1.67\ +\ Gluconate\ (mM)\times 1.5$$

$$EY\ =\ \frac{Ethanol\ (mM)}{TE\ (mM)}\times 100\%$$

Equation 10.2: Simultaneous Saccharification and Fermentation (SSF)

$$Glucose\ preSSF\ (mM)\ =\ \frac{Glucan\ yield\ (\%)\times PSFB\ (g)}{0.90\times 180.156\ (g/mol)}\times 1000$$

$$Glucose\ postSSF\ (mM)\ =\ \frac{Glucan\ yield\ (\%)\times PSFA\ (g)}{0.90\times 180.156\ (g/mol)}\times 1000$$

$$Xylose\ preSSF\ (mM)\ =\ \frac{Xylan\ yield\ (\%)\times PSFB\ (g)}{0.88\times 150.13\ (g/mol)}\times 1000$$

$$Xylose\ postSSF\ (mM)\ =\ \frac{Xylan\ yield\ (\%)\times PSFA\ (g)}{0.88\times 150.13\ (g/mol)}\times 1000$$

$$Glucose\ consumed\ (mM)\ =\ Glucose\ preSSF\ (mM)\ -\ Glucose\ postSSF\ (mM)$$

$$Xylose\ consumed\ (mM)\ =\ Xylose\ preSSF\ (mM)\ -\ Xylose\ postSSF\ (mM)$$

$$TE\ (mM)\ =\ Glucose(mM)\times 2\ +\ Xylose(mM)\times 1.67$$

$$EY\ =\ \frac{Ethanol\ (mM)}{TE\ (mM)}\times 100\%$$

Equation 11: Combined Severity Factor (CSF)

Combined Severity Factor: Initially, for pretreatment studies, the severity factor was used. The severity factor was described by Overend and Chornet as following the assumption of first-order kinetics and Arrhenius law (70). The severity factor is a function of time and temperature in minutes and degrees celsius respectively. Additionally, constants such as 100 represent the reference temperature in degrees celsius without solubilization and 14.75 represents the activation energy constant as a function of temperature. Chum et al. applied the concept of acid hydrolysis to the equation which applies the acid concentration in pH (71). Lastly, a derivation of the combined severity factor is further discussed by Wyman and Yang (26). The main combined severity factor equation that will be used is as follows:

$$log\ CS\ = log(\ t \times EXP(\ \frac{T-100}{14.75}))\ -\ pH$$

The time t in minutes, and T in °C, and pH were measured using the post-pretreatment hydrolysate.

Table A1. Data corresponding to **Figure 4.**

Reaction Time (hours)	Gluconic Acid Degradation (%)					
	170°C		180°C		190°C	
	0.125M	1M	0.125M	1M	0.125M	1M
0	100.26±0.20	99.94±0.08	100.00±0.03	100.00±0.08	100.08±0.11	100.08±0.12
0.5	97.55±0.00	97.15±1.67	97.03±0.07	96.94±1.09	95.06±0.22	95.33±0.89
1	95.80±0.14	95.42±0.75	94.06±0.71	93.61±1.70	89.81±0.04	87.77±1.90
2	93.10±0.56	92.57±0.68	88.13±0.21	85.65±0.55	78.06±0.51	77.84±1.34
2.5	91.83±0.28	89.51±0.09	85.56±0.11	82.98±0.00	73.63±0.46	75.46±1.37

*data reported as percentage of gluconic acid loss (mM) /gluconic acid (mM) at time 0-hour. Formatted as AVERAGE ± range.

Table A2. Data corresponding to **Figure 5.**

STDEV = standard deviation of triplicate measurements

	Acid Concentrations (M)	Reaction Time (minutes)	Glucose yield from stage 1	Glucose oligomers from stage 1	Xylose yield from stage 1	Xylose Oligomers yield from stage 1
160°C	0.125	30	2.4±0.1%	4.7±0.3%	11.3±0.2%	45.0±1.0%
	0.125	90	2.7±0.1%	5.2±1.0%	30.4±1.5%	37.0±1.8%
	0.125	120	3.6±0.1%	3.2±0.1%	35.1±0.2%	21.6±1.2%
170°C	0.125	30	2.4±0.2%	7.1±0.6%	13.0±0.4%	58.6±3.0%
	0.125	60	3.3±0.4%	4.7±0.6%	27.0±1.1%	44.8±1.3%
	0.125	80	3.5±0.3%	3.9±0.4%	29.8±0.6%	26.6±0.5%
	0.500	30	3.2±0.07%	5.1±0.2%	28.2±2.4%	37.7±1.6%
	1.000	30	4.7±0.6%	4.6±0.4%	37.3±1.0%	29.6±1.2%
180°C	0.125	30	2.2±0.2%	7.3±0.8%	24.8±0.1%	23.4±0.4%
	0.500	30	5.2±0.5%	2.2±0.6%	36.0±0.5%	6.9±1.5%
	1.000	30	6.5±0.7%	2.5±0.5%	39.3±0.2%	6.4±0.2%
190°C	0.125	30	3.4±0.2%	4.1±0.2%	22.9±0.6%	7.3±0.4%
	0.500	30	4.7±0.06%	2.5±0.0%	19.5±0.7%	3.1±0.0%
	1.000	30	6.2±0.08%	1.4±0.3%	17.2%±0.4%	1.1±0.7%

*data reported as percentage of sugar yields g/100 g of raw wheat straw. Formatted as AVERAGE ± STDEV.

Table A3. Data corresponding to **Figure 6.**

STDEV = standard deviation of triplicate measurements

	Acid Concentrations (M)	Reaction Time (min)	HMF yield	Furfural yield
160°C	0.125	30	0.21±0.03%	1.19±0.2%
	0.125	90	0.48±0.05%	6.58±0.05%
	0.125	120	0.62±0.02%	10.3±0.4%
170°C	0.125	30	0.59±0.08%	4.3±0.3%
	0.125	60	0.84±0.07%	11.6±0.2%
	0.125	80	1.01±0.04%	16.6±0.6%
	0.500	30	1.1±0.08%	10.0±0.2%
	1.000	30	1.7±0.1%	14.1±0.8%
180°C	0.125	30	1.1±0.07%	11.8±1.1%
	0.500	30	2.5±0.2%	19.2±0.6%
	1.000	30	2.8±0.5%	30.4±1.5%
190°C	0.125	30	1.5±0.03%	21.0±0.4%
	0.500	30	3.7±0.3%	27.6±4.3%
	1.000	30	4.0±0.2%	28.4±1.2%

*data reported as percentage of sugar degradation products calculated using EQUATION 10. Formatted as AVERAGE ± STDEV.

Table A4. Data corresponding to **Figure 7.**

STDEV = standard deviation of triplicate measurements

	Acid Concentrations (M)	Reaction Time (min)	Glucose yield from stage 2	Xylose yield from stage 2
160°C	0.125	30	39.0±1.2%	19.8±0.6%
	0.125	90	53.6±1.2%	16.8±0.3%
	0.125	120	58.0±0.4%	15.3±0.8%
170°C	0.125	30	53.9±1.4%	18.5±0.5%
	0.125	60	70.3±0.1%	14.0±0.2%
	0.125	80	72.2±0.9%	13.5±0.5%
	0.500	30	74.5±0.8%	19.2±0.8%
	1.000	30	76.3±0.09%	15.0±1.1%
180°C	0.125	30	63.1±1.2%	18.2±0.6%
	0.500	30	79.1±2.0%	9.0±0.8%
	1.000	30	81.0±1.6%	8.0±0.5%
190°C	0.125	30	81.4±0.6%	8.5±0.6%
	0.500	30	84.1±1.7%	7.3±0.1%
	1.000	30	81.8±0.2%	5.2±0.1%

*data reported as percentage of sugar yields g/100 g of raw wheat straw. Formatted as AVERAGE ± STDEV.

Table A5. Data corresponding to **Figure 8.**

STDEV = standard deviation of triplicate measurements

Temperature (°C)	Reaction Time (min)	Concentration (M)	log CS	Glucose yield	STDEV	Xylose yield	STDEV	Combined sugar yield	STDEV
160	90	0.125	0.31	62.10%	0.80%	82.90%	1.70%	69.40%	0.06%
	120	0.125	0.46	64.90%	0.00%	74.20%	0.90%	69.60%	1.70%
170	30	0.125	0.22	63.00%	0.40%	89.60%	3.40%	73.60%	2.40%
	30	0.500	0.80	84.70%	3.20%	83.70%	1.70%	83.50%	2.40%
	30	1.000	1.15	85.10%	0.60%	79.50%	1.10%	81.50%	2.60%
	60	0.125	0.40	78.20%	0.50%	84.90%	0.80%	81.10%	0.20%
	80	0.125	0.53	80.30%	0.10%	70.80%	0.70%	78.40%	1.10%
180	30	0.125	0.42	72.80%	1.60%	74.70%	4.70%	72.10%	0.90%
	30	0.500	1.03	87.10%	4.10%	53.80%	1.00%	79.70%	0.50%
	30	1.000	1.36	89.60%	1.30%	52.80%	0.50%	77.00%	0.50%
190	30	0.125	0.73	88.90%	0.70%	39.70%	2.00%	69.70%	0.30%
	30	0.500	1.21	91.50%	2.00%	30.50%	1.60%	69.20%	2.00%
	30	1.000	1.54	89.20%	0.30%	23.60%	0.80%	66.20%	0.60%

Table A6. Data corresponding to **Figure 9.**

Ave = average of samples from triplicate measurements
SD = standard deviation of triplicate measurements

	Fermentation Substrates & Metabolites (mM)													
	Glucose		Xylose		Arabinose		Gluconate		Lactate		Acetate		Ethanol	
Fermentation time (hours)	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0	4.85	0.11	27.44	0.11	6.65	0.18	82.59	0.81	1.12	0.008	37.99	1.45	0	0
7.5	0	0	27.23	0.45	3.69	0.68	47.80	4.23	1.96	0.007	40.38	0.19	54.72	0.87
18.5	0	0	19.56	1.11	0	0	0	0	2.02	0.03	55.20	0.24	146.17	2.25
23.9	0	0	13.15	2.42	0	0	0	0	2.13	0.006	55.97	0.15	160.11	5.33
26.9	0	0	9.96	4.03	0	0	0	0	2.07	0.02	55.51	0.62	165.66	7.12
30	0	0	5.42	4.19	0	0	0	0	2.11	0.02	57.24	0.22	178.17	9.63
33	0	0	3.43	2.85	0	0	0	0	2.11	0.02	58.33	0.34	185.43	7.67
49.5	0	0	1.81	0.18	0	0	0	0	2.10	0.06	61.71	0.21	198.32	0.90
76	0	0	1.83	0.12	0	0	0	0	2.21	0.09	64.51	0.29	204.70	0.34

Table A7. Data corresponding to **Figure 10.**

Ave = average of samples from triplicate measurements
SD = standard deviation of triplicate measurements

	Fermentation Substrates & Metabolites (mM)													
	Glucose		Xylose		Arabinose		Gluconate		Lactate		Acetate		Ethanol	
Fermentation time (hours)	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0	3.75	0.15	26.32	0.27	5.08	0.11	76.20	4.34	0	0	33.08	0.92	0	0
8	0	0	23.93	0.25	3.50	0.51	66.96	0.71	1.74	0.68	31.81	0.83	16.90	3.75
18.5	0	0	22.66	1.71	2.02	0.53	56.94	1.76	1.84	0.46	31.81	0.83	29.15	2.86
26.5	0	0	15.22	2.89	0	0	30.00	5.32	1.55	0.23	32.59	0.02	78.42	4.12
31	0	0	10.78	2.59	0	0	0	0	1.72	0.03	36.16	0.68	127.50	2.72
44	0	0	6.63	2.67	0	0	0	0	1.97	0.30	36. 70	1.06	151.25	9.16
49	0	0	4.65	1.73	0	0	0	0	1.86	0.11	36.19	1.12	151.36	12.05
55	0	0	4.10	1.43	0	0	0	0	2.06	0.69	36.19	1.35	154.97	11.33
68	0	0	3.36	0.71	0	0	0	0	1.93	0.02	37.77	1.68	154.03	6.82

Table A8. Data corresponding to **Figure 11.**

Ave = average of samples from triplicate measurements

SD = standard deviation of triplicate measurements

	Fermentation Substrates & Metabolites (mM)					
Fermentation time (hours)	Lactate		Acetate		Ethanol	
	Ave	SD	Ave	SD	Ave	SD
0	0	0	0	0	0	0
24.92	0.33	0.037	8.45	0.14	95.57	0.66
48.97	0.36	0.04	9.32	0.23	118.07	0.76
94.35	0.43	0.02	10.03	0.14	141.17	0.55
120.67	0.46	0.01	10.38	0.14	149.35	0.99
145.58	0.37	0.09	10.13	0.63	152.66	0.68
171.5	0.36	0.10	11.03	1.07	158.73	1.83
194.08	0.36	0.11	11.38	1.34	162.81	2.74

Table A9. Data corresponding to **Figure 12.**

Ave = average of samples from triplicate measurements

SD = standard deviation of triplicate measurements

	Fermentation Substrates & Metabolites (mM)					
Fermentation time (hours)	Lactate		Acetate		Ethanol	
	Ave	SD	Ave	SD	Ave	SD
0	0	0	0	0	0	0
24.92	3.18	0.04	0.82	0.006	91.85	1.94
48.97	3.17	0.04	1.03	0.02	107.23	2.02
94.35	3.32	0.06	1.26	0.02	122.36	2.32
120.67	3.43	0.06	1.39	0.03	128.39	2.47
145.58	3.16	0.003	1.44	0.02	131.69	2.33
171.5	3.14	0.007	1.49	0.005	136.74	2.30
194.08	3.16	0.02	1.50	0.05	138.42	2.61