Co-Hydrolysis of Lignocellulosic Biomass for Microbial Lipid Accumulation

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ABSTRACT: The herbaceous perennial energy crops miscanthus, giant reed, and switchgrass, along with the annual crop residue corn stover, were evaluated for their bioconversion potential. A co-hydrolysis process, which applied dilute acid pretreatment, directly followed by enzymatic saccharification without detoxification and liquid–solid separation between these two steps was implemented to convert lignocellulose into monomeric sugars (glucose and xylose). A factorial experiment in a randomized block design was employed to optimize the co-hydrolysis process. Under the optimal reaction conditions, corn stover exhibited the greatest total sugar yield (glucose + xylose) at 0.545 g g⁻¹ dry biomass at 83.3% of the theoretical yield, followed by switchgrass (0.44 g g⁻¹ dry biomass, 65.8% of theoretical yield), giant reed (0.355 g g⁻¹ dry biomass, 64.7% of theoretical yield), and miscanthus (0.349 g g⁻¹ dry biomass, 58.1% of theoretical yield). The influence of combined severity factor on the susceptibility of pretreated substrates to enzymatic hydrolysis was clearly discernible, showing that co-hydrolysis is a technically feasible approach to release sugars from lignocellulosic biomass. The oleaginous fungus *Mortierella isabellina* was selected and applied to the co-hydrolysate mediums to accumulate fungal lipids due to its capability of utilizing both C5 and C6 sugars. Fungal cultivations grown on the co-hydrolysates exhibited comparable cell mass and lipid production to the synthetic medium with pure glucose and xylose. These results elucidated that combining fungal fermentation and co-hydrolysis to accumulate lipids could have the potential to enhance the utilization efficiency of lignocellulosic biomass for advanced biofuels production.

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Introduction

Lignocellulosic biomass represents the most abundant natural polymer in the biosphere, and interest in its use as a feedstock for the production of advanced biofuels has gained momentum in recent years as shown by various government directives. The Renewable Fuels Standard 2 provision in the United States Energy Independence and Security Act of 2007 mandates the production of 36 billion gallons of biofuels by 2022, with 16 billion gallons coming from lignocellulosic sources while capping conventional biofuels (i.e., corn starch-based ethanol) at 15 billion gallons (Coyle, 2010). Support for lignocellulosic fuels is owed not only to its large supply, but also its ability to mitigate greenhouse gas emissions, avoid competition with food resources, stimulate rural economies, and provide a stable and secure source of energy production (Coyle, 2010). Many sources of lignocellulosic biomass exist, including municipal solid wastes, pulp and paper wastes, forest and agricultural residues, and dedicated woody and herbaceous perennial energy crops. Herbaceous perennial energy crops in particular, have been the focus of much attention due to their positive environmental characteristics. Compared to annual crops, perennials, once established, do not need reseeding, and require lower inputs (i.e., water, fertilizer, pesticide, tillage). Additionally they can often be grown on uncultivated or marginal lands where their deep root systems act to reduce soil erosion, increase soil fertility, and accumulate greater soil organic carbon than their annual counterparts (i.e., corn or canola) (Williams et al., 2009). Among the twenty perennial grasses studied by the European Union, the most promising in terms of biofuel production were: miscanthus, giant reed, switchgrass, and reed canarygrass (Lewandowski et al., 2003). The first three crops, along with corn stover will be the primary focus of this investigation.

Many studies have been devoted to the pretreatment and enzymatic hydrolysis of the herbaceous perennial energy crop switchgrass, however, information about the recalcitrance and bioconversion potential of miscanthus and giant

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reed is still limited. Wet explosion (Sorensen et al., 2008), ammonia fiber expansion (Murnen et al., 2007), one-step extrusion/NaOH (de Vrije et al., 2002), and aqueous-ethanol organosolvent treatments were applied for the bioconversion of miscanthus to fermentable sugars (Brosse et al., 2010). Dilute acid pretreatment (Anderson et al., 2008; Dien et al., 2006; Scordia et al., 2011; Shatalov and Pereira, 2012) and ethanol-alkaline treatment (Shatalov and Pereira, 2005) were carried out for giant reed bioconversion. All of the aforementioned studies employed traditional methods of biomass processing where, after pretreatment, the solids are separated from the liquid stream, washed to neutralize and detoxify the remaining solids, then subjected to enzymatic hydrolysis in order to extract glucose monomers (Decker et al., 2009). Depending on the particular pretreatment method, the discarded liquid stream will often contain a large percentage of the solubilized xylose, and to a lesser extent, glucose monomers, and soluble lignin. Since xylose represents a significant fraction of the lignocellulosic biomass, it is critical from an engineering and economic standpoint to retain this fraction in order to improve pretreatment efficiency. Additionally, such a process requires large amounts of water to adjust the pH and detoxify the solid biomass, and separation of the solid and liquid streams is timely and introduces the possibility of contamination (Studer et al., 2011). Therefore, development of a novel process (co-hydrolysis), which eliminates liquid–solid separation, detoxification or washing of the pretreated solids, and directly carries out enzymatic hydrolysis after pretreatment would make a significant contribution to the production of advanced biofuels. To date, comprehensive evaluations of co-hydrolysis of lignocellulosic biomass are sparse. A few researchers have studied the effects of higher solids loading for un-detoxified pretreated wheat straw (Georgieva et al., 2008; Jorgensen et al., 2007), the effects of increasing enzyme dosage in comparing washed-solids of wheat straw versus whole-slurry hydrolysis (Felby et al., 2003), and the combined effects of severity, and enzyme and solid loadings on co-hydrolysis performance of populus (Studer et al., 2011).

Realizing the potential benefits of co-hydrolysis comes with its own challenges, such as utilizing microbes that are robust enough to withstand elevated levels of toxins specific to each biomass and pretreatment method, while containing the ability to ferment both hexose and pentose sugars into biofuels. There are few studies on the bioconversion of both the hexose and pentose fractions of herbaceous perennial energy crops like switchgrass, miscanthus, and giant reed. The extreme thermophilic bacterium *Thermotoga elfii* metabolized undetoxified miscanthus hydrolysate containing glucose and xylose for hydrogen production, while the yeast *Scheffersomyces stipitis* CBS6054 was able to convert giant reed hydrolysate to ethanol (Scordia et al., 2012). Our previous study found that the oleaginous mold *Mortierella isabellina*, when grown on corn stover co-hydrolysate, could produce lipid yields comparable to those from synthetic hydrolysate without toxins (Ruan et al., 2012). In addition,

there is still limited information regarding its lipid production from herbaceous perennial energy crops, even though lipid accumulation by *M. isabellina* from lignocellulosic biomass has been studied (Economou et al., 2011; Xing et al., 2012). Thus, the aim of this study is to provide a comparative evaluation of *M. isabellina* lipid accumulation on the co-hydrolysates of four different lignocellulosic feedstocks: switchgrass, giant reed, miscanthus, and corn stover, using a co-hydrolysis process for bioconversion.

Materials and Methods

Lignocellulosic Biomass

Corn stover and switchgrass were collected from the Michigan State University Crop and Soil Science Teaching and Research Field Facility. Miscanthus and giant reed were obtained from Werks Management, LLC (Fishers, IN). Each feedstock was air-dried and ground using a mill (Willey Mill, Standard Model No. 3; Arthur H. Thomas, Philadelphia, PA) with a 2 mm size screen; all materials were then sieved to make a particle size distribution of <30 mesh (<1.6 mm) but >80 mesh (>1 mm). The biomass samples were analyzed for cellulose, xylan, and lignin content according to the National Renewable Energy Laboratory's (NREL) analytical procedure for determination of structural carbohydrates and lignin in biomass (Sluiter and National Renewable Energy Laboratory (U.S.), 2008).

Dilute Acid Pretreatment

To evaluate the combined glucose and xylose recovery from cellulose and xylan in each biomass sample, dilute acid pretreatment was performed with a factorial randomized block design. Dilute acid concentration, pretreatment time, and temperature were the factors investigated. Eighteen treatments with two replicates were applied to each lignocellulosic biomass for a total of 36 individual samples per feedstock. Each sample was treated in a screw cap 125 mL serum bottle and placed in an autoclave (Brinkmann 2540M; Tuttnauer USA Co. Ltd., Hauppauge, NY). Dilute acid pretreatments were carried out at sulfuric acid concentrations of 1%, 2%, and 3% (w/w), respectively. Retention times of 1, 2, and 3 h were applied once the reaction temperature reached its predetermined level of 110, 120, or 130°C, respectively. Biomass concentration for pretreatment was fixed at 10% dry matter. After dilute acid pretreatment, biomass slurries were titrated to a pH of 4.5–5 with 5 mol L⁻¹ sodium hydroxide.

Enzymatic Saccharification

After dilute acid pretreatment and pH adjustment of the biomass slurry, various amounts of 0.1 mol L⁻¹ citrate buffer (pH 4.8) and an enzyme mixture consisting of 34.85 mg

cellulase (Accellerase 1500[®], protein content 69.7 mg mL⁻¹, lot number 3016295230; Genencor, Palo Alto, CA) and 4.31 mg xylanase (Accellrase XY, protein content 43.1 mg mL⁻¹, lot number 4900667792; Genencor) per gram of initial dry biomass were added to the slurry to achieve a final dry matter concentration of 8% for all pretreated solutions. All of the resulting samples were incubated at 50°C in a shaking incubator at 150 rpm for 72 h. All enzymatic hydrolysis experiments were carried out in duplicates. After hydrolysis, the samples were removed from the shaker and put on ice to stop the reaction; the hydrolysate was separated by centrifugation at 7,025g for 5 min to obtain a clear sugar solution, which was then filtered through a 0.22 µm polyethersulfone membrane filter for HPLC analysis. The clear enzymatic hydrolysate solutions were stored in a 4°C refrigerator for further use.

Microorganisms and Culture Conditions

M. isabellina ATCC 42613 was obtained from the American Type Culture Collection (Manassas, VA). This strain was first cultured on potato dextrose agar (Sigma–Aldrich, St. Louis, MO) to produce spores at 30°C. After 14 days cultivation, the spores were washed with sterilized distilled water to obtain a spore suspension (stored at 4°C). Seed cultures were grown with 24 g L⁻¹ potato dextrose broth (Sigma–Aldrich) with 8 g L⁻¹ yeast extract at 25°C and 180 rpm for 2 days in a rotary shaker (Thermal Scientific, Odessa, TX) with a spore concentration of 1–2 × 10⁷ spore mL⁻¹. The salt medium of submerged batch cultures contained: KH₂PO₄ (1 g L⁻¹; Mallinckrodt Bakker, St. Thomas, US Virgin Islands), MgCl₂·6H₂O (0.5 g L⁻¹; Mallinckrodt Bakker), ZnSO₄·7H₂O (0.0014 g L⁻¹; Sigma–Aldrich), MnSO₄·H₂O (0.0016 g L⁻¹; Sigma–Aldrich), CoCl₂·6H₂O (0.0036 g L⁻¹; Sigma–Aldrich), and FeSO₄·7H₂O (0.00275 g L⁻¹; Sigma–Aldrich). 2.74 g L⁻¹ yeast extract (DOT Scientific Inc., Burton, MI) was used as the sole nitrogen source. The carbon sources were synthetic sugars (composed of a mixture of glucose and xylose using similar concentrations found in the co-hydrolysates), and lignocellulosic biomass co-hydrolysates. Corn stover and switchgrass co-hydrolysates were diluted to obtain similar sugar concentrations to those found in miscanthus and giant reed co-hydrolysates. The pH of the medium was adjusted to 6.0 ± 0.1 before autoclaving. Two hundred fifty milliliter Erlenmeyer flasks were filled with 50 mL of growth medium and sterilized at 121°C for 15 min. The growth medium was inoculated with a 10% (v/v) seed culture and cultivated at 25 ± 0.5°C on a rotary shaker (Thermal Scientific) with an agitation speed of 180 rpm.

Mass Balance

Mass balance analysis was based on the co-hydrolysis and fermentation data. Lipid production was calculated by the

amount of lipid accumulated during fermentation divided by the dry weight of initial lignocellulosic biomass.

Analytical Methods

The sugar yield from combined dilute acid pretreatment and enzymatic hydrolysis was calculated to evaluate the performance of co-hydrolysis and conduct the mass balance analysis. The sugar yield was determined by the ratio of the measured amount of sugars (glucose and xylose) in co-hydrolysates to the dry weight of initial biomass and also calculated as the percent of theoretical combined glucose and xylose yield.

The combined severity factor (log CS), which couples reaction conditions of time, temperature, and acid concentration into a single variable, was used to compare sugar yields. Combined severity is calculated as follows: $\log CS = t \exp[(T_H - T_R)/14.75] - \text{pH}$, where t is reaction time in minutes, T_R is the hydrolysis temperature in °C, and T_H is a reference temperature of 100°C (Lloyd and Wyman, 2005); after pretreatment, the pH measurement was determined by a pH meter (Fisher Scientific, Pittsburgh, PA).

Mycelia were collected by filtration and washed twice with distilled water. Cell mass was determined by drying under 105 ± 1°C to obtain a consistent weight. Glucose, xylose, acetic acid, formic acid, furfural, and hydroxymethylfurfural (HMF) in the co-hydrolysates and fermentation broths were determined by High Performance Liquid Chromatography (Shimadzu Prominence, Kyoto, Japan) equipped with a Bio-rad Aminex HPX-87H analytical column and a refractive index detector. The mobile phase was 0.005 mol L⁻¹ sulfuric acid with a flow rate of 0.6 mL min⁻¹. Column temperature was set at 65°C. HPLC standards including glucose (Catalog Number: 49158), xylose (Catalog Number: 95729), sodium acetate (Catalog Number: S8750), sodium formate (Catalog Number: 17841), HMF (Catalog Number: 53407), and furfural (Catalog Number: 185914) were purchased from Sigma–Aldrich. Dried mycelia were ground in a mortar and used for lipid extraction. Total lipid was determined gravimetrically (Ruan et al., 2012).

Statistical Analysis

A general linear model using R software (R Version 2.15.0, Vienna, Austria) was applied to the experimental data in order to perform an analysis of variance (ANOVA) and multiple comparisons. Tukey's test, using a comparison-wise type I error rate of 0.05, was adopted to compute honestly significant differences among different feedstocks regarding sugar yield and inhibitor generation. Within each feedstock, the significance of individual factors, as well as the interactions between factors on sugar conversion and inhibitors generation, was identified using ANOVA analysis. Bonferroni's test was carried out at an experimental type I error rate of 0.05 to conduct multiple comparisons of both

Table I. Structural carbohydrate and lignin of four raw lignocellulosic biomass.*

Biomass	Cellulose (w/w, %)	Xylan (w/w, %)	Lignin (w/w, %)
Corn stover	36.3	22.0	18.6
Switchgrass	37.4	22.1	20.5
Miscanthus	34.2	19.0	22.9
Giant reed	29.7	19.2	22.1

*The particle size of raw biomass was between 1 and 1.6 mm; compositional data were the average of two replicates.

sugar and inhibitor production under different co-hydrolysis conditions.

Results and Discussion

Degradation of Carbohydrate Into Sugars and Inhibitors

The composition of lignocellulosic biomass indicated that cellulose was the most abundant fraction in each feedstock (Table I). Corn stover and switchgrass exhibited similar but higher cellulose content (36.3% and 37.4%, respectively) compared to miscanthus (34.2%) and giant reed (29.7%), respectively. The hemicellulose fraction in each feedstock was of a xylan type as indicated by the relatively high amounts of xylose in the polysaccharide. The xylan content for corn stover (22.0%) and switchgrass (22.1%) were equivalent; while miscanthus (19.0%) and giant reed (19.2%) had comparatively lower levels. In addition, miscanthus and giant reed contained more lignin (22.9% and 22.1%, respectively), compared to switchgrass (20.5%), and corn stover (18.6%).

Dilute acid pretreatment and enzymatic hydrolysis using both the conventional and co-hydrolysis processes were represented in Figure 1. Results from the co-hydrolysis process at various pretreatment conditions were listed in Tables II–V and summarized in Figure 2. ANOVA and Tukey's HSD analysis indicated that there were significant ($P < 0.0001$) mean differences on sugar yields between feedstocks, with the exception of miscanthus and giant reed ($P > 0.05$).

The sugar yield for corn stover co-hydrolysis was dependent on the main factors of pretreatment temperature and sulfuric acid concentration ($P < 0.05$), but independent of treatment time ($P > 0.05$). There were also significant two-way interactions between time and temperature ($P < 0.05$), time and sulfuric acid concentration ($P < 0.05$), and temperature and sulfuric acid concentration ($P < 0.05$). A pretreatment condition of 130°C, 2% H_2SO_4 for 1 h led to the greatest sugar yield of 0.545 g g^{-1} dry initial biomass and 83.3% of theoretical combined glucose and xylose yield for corn stover, producing a total of $52.09 \pm 0.13 \text{ g L}^{-1}$ of fermentable sugars, including $31.1 \pm 0.38 \text{ g L}^{-1}$ glucose and $21.0 \pm 0.51 \text{ g L}^{-1}$ xylose (Table II and Fig. 2A).

ANOVA analysis indicated that the sugar yield of switchgrass co-hydrolysis was independent of pretreatment temperature, time, and sulfuric acid concentration. The effects of two-factor interactions between time and sulfuric acid concentration ($P < 0.05$), as well as temperature and sulfuric acid concentration ($P < 0.05$) were significant. The treatments of 2% H_2SO_4 at 130°C for 2 h had the highest sugar yield of 0.44 g g^{-1} dry initial biomass and 65.8% of theoretical combined glucose and xylose yield (Table III). It generated $22.8 \pm 1.22 \text{ g L}^{-1}$ glucose and $19.23 \pm 0.16 \text{ g L}^{-1}$ xylose (Fig. 2B).

Figure 1. Conventional and co-hydrolysis process of dilute acid pretreatment and enzymatic saccharification operation for microbial lipid production (modified from Studer et al., 2011).

Table II. Fermentable sugars yield and inhibitory compounds derived from co-hydrolysate of corn stover.

Dilute acid pretreatment parameters			Sugar yields			Inhibitory compounds		
Acid (w/w %)	Temp (°C)	Time (h)	Log CS	Glu + Xyl (g g ⁻¹ , dry biomass)	Glu + Xyl (% of theoretical yield)	Acetate (g L ⁻¹)	HMF (mg L ⁻¹)	Furfural (g L ⁻¹)
1	120	1	1.29	0.455	69.5	3.49	53.05	0.07
2	120	1	1.65	0.503	76.8	3.83	77.57	0.17
3	120	1	1.85	0.510	78.0	3.88	82.39	0.3
1	120	2	1.57	0.475	72.6	3.53	65.14	0.14
2	120	2	1.90	0.516	78.8	3.94	92.53	0.43
3	120	2	2.10	0.505	77.1	4.01	72.36	0.64
1	120	3	1.97	0.507	77.4	3.66	86.14	0.3
2	120	3	2.22	0.527	80.5	4.21	96.76	0.8
3	120	3	2.35	0.491	75.0	4.18	93.32	0.91
1	130	1	1.62	0.513	78.4	3.64	89.98	0.25
2	130	1	1.94	0.545	83.3	4.08	109.7	0.63
3	130	1	2.18	0.513	78.5	4.02	98.69	0.59
1	130	2	1.92	0.519	79.4	3.5	114.5	0.3
2	130	2	2.18	0.512	78.3	3.97	110.8	0.7
3	130	2	2.37	0.474	72.4	4.17	107.2	0.97
1	130	3	2.04	0.512	78.2	3.78	99.73	0.33
2	130	3	2.50	0.521	79.6	4.04	102.0	0.72
3	130	3	1.98	0.507	77.5	4.43	95.29	0.99
Mean	—	—	—	0.506 ± 0.021	77.3 ± 3.2	3.92 ± 0.29	91.8 ± 17.8	0.54 ± 0.31

(1) NREL's analysis of structural carbohydrate and lignin: cellulose: 36.3% (w/w); xylan: 22.0% (w/w); lignin: 18.6% (w/w).

(2) Dilute acid pretreatment was carried out at 10% (w/w) dry solids. Enzymatic saccharification was studied at 8% (w/w) dry solids with (35 mg Accellerase 1500 and 2.15 mg Accellerase XY) per gram dry biomass.

(3) All the sugar and inhibitor values are the average of two replicates.

The sugar yield for giant reed co-hydrolysis was shown to be dependent on the main effects of pretreatment temperature ($P < 0.0001$), sulfuric acid concentration ($P < 0.05$), and pretreatment time ($P < 0.05$). Two-way interactions of time and sulfuric acid concentration ($P < 0.0025$), temperature and time ($P < 0.0001$) were also significant. Two different pretreatment conditions (3% H₂SO₄ at 130°C for 1 h, and 2% H₂SO₄ at 130°C for 1 h) were both shown to have the highest sugar yield of 0.355 g g⁻¹ dry initial biomass at 64.7% of the theoretical combined glucose and xylose yield (Table IV). However, the concentrations of individual sugars from these two pretreatment conditions were significantly different ($P < 0.05$). The treatment condition of 3% H₂SO₄, 130°C, and 1 h generated 18.35 ± 0.67 g L⁻¹ glucose and 15.56 ± 0.46 g L⁻¹ xylose, while the treatment condition of 2% H₂SO₄ at 130°C for 1 h released 16.71 ± 0.76 g L⁻¹ glucose and 17.2 ± 0.14 g L⁻¹ xylose, respectively (Fig. 2C).

For the miscanthus co-hydrolysis combined ANOVA analysis showed that the main pretreatment factors of temperature ($P < 0.0001$), time ($P < 0.0001$), and sulfuric acid concentration ($P < 0.05$) had significant impacts on the sugar yield, while the effects of two-factor interaction terms: temperature and time ($P < 0.0001$), temperature and sulfuric acid concentration ($P < 0.05$), time and sulfuric acid concentration ($P < 0.05$) as well as the three-factor interaction of temperature, time, and sulfuric acid concentration ($P < 0.05$) were also considered statistically significant. The highest sugar yield was 0.349 g g⁻¹ dry

initial biomass at 58.1% of the theoretical combined sugar yield from the treatment of 2% H₂SO₄ at 130°C for 2 h (Table V), corresponding to 17.76 ± 0.94 g L⁻¹ glucose and 15.55 ± 0.08 g L⁻¹ xylose, respectively (Fig. 2D).

Comparison of co-hydrolysis of the four feedstocks demonstrated that sugar yields of miscanthus and giant reed were significantly ($P < 0.0001$) lower than corn stover and switchgrass. The lower yields were possibly due to those feedstocks having relatively higher lignin contents which likely hindered the enzyme action due to steric actions or absorbing active enzymes during the enzymatic hydrolysis step in the co-hydrolysis process (Liao et al., 2005; Yang and Wyman, 2006).

The co-hydrolysis of corn stover generated the lowest mean concentration of acetic acid (3.92 ± 0.29 g L⁻¹; Table II), followed by switchgrass (4.04 ± 0.32 g L⁻¹; Table III), miscanthus (5.12 ± 0.65 g L⁻¹; Table V) and giant reed (5.66 ± 0.86 g L⁻¹; Table IV). Tukey's HSD analysis showed that corn stover and switchgrass had no significant ($P > 0.05$) differences for acetate generation at the comparison-wise type I error rate of 0.05, while miscanthus and giant reed were significantly different from corn stover and switchgrass ($P < 0.0001$). Under the best co-hydrolysis conditions of sugar production, the acetic acid concentrations reached 6.43 g L⁻¹ in miscanthus hydrolysate (Table V) and 6.79 or 6.31 g L⁻¹ for giant reed (Table IV), respectively. In contrast, an acetate concentration of 4.08 g L⁻¹ was obtained from co-hydrolysis of corn stover (Table II) and 4.2 g L⁻¹ from switchgrass, respectively (Table III).

Figure 2. Glucose and xylose production from corn stover (A), switchgrass (B), giant reed (C), and miscanthus (D).

Furfural and HMF are generated by thermal conversion of pentoses and hexoses. The experimental results (Tables II–V) demonstrated that, among the four feedstocks, corn stover co-hydrolysate exhibited the lowest mean concentrations of furfural ($0.54 \pm 0.31 \text{ g L}^{-1}$) and HMF ($0.0918 \pm 0.018 \text{ g L}^{-1}$), switchgrass co-hydrolysate had the highest mean furfural concentration ($0.77 \pm 0.41 \text{ g L}^{-1}$), and giant reed co-hydrolysate had the highest mean HMF concentration ($0.24 \pm 0.12 \text{ g L}^{-1}$). Tukey's HSD analysis exhibited that there were significant differences in HMF concentrations between corn stover and giant reed ($P < 0.0001$), miscanthus and giant reed ($P < 0.0001$), as well as switchgrass and giant reed ($P < 0.0001$). However, miscanthus and corn stover ($P > 0.05$), switchgrass and corn stover ($P > 0.05$), switchgrass and miscanthus ($P > 0.05$) were not significantly different in regards to HMF concentrations. Tukey's HSD also indicated that there

were no significant differences on furfural concentrations among the four feedstocks ($P > 0.05$).

Combined Severity Factor Analysis

The effects of combined severity factor on the co-hydrolysis of the four feedstocks were presented in Figure 3. The concentrations of glucose, xylose, acetate, HMF, and furfural were chosen as the responses in order to evaluate the effects of the combined severity factor. The general trend for glucose production in the feedstocks was an initial increase with greater combined severity factor followed by a decrease at higher severity factor levels. Corn stover was an exception to this trend showing no decrease in glucose production even at higher severity levels. It was clear that xylose release was negatively associated with the combined severity factor for all materials due to the degradation

Table III. Fermentable sugars yield and inhibitory compounds derived from co-hydrolysate of switchgrass.

Dilute acid pretreatment parameters			Sugar yields			Inhibitory compounds		
Acid (w/w, %)	Temp. (°C)	Time (h)	Log CS	Glu + Xyl (g g ⁻¹ , dry biomass)	Glu + Xyl (% of theoretical yield)	Acetate (g L ⁻¹)	HMF (mg L ⁻¹)	Furfural (g L ⁻¹)
1	120	1	1.33	0.388	57.9	3.47	98.54	0.22
2	120	1	1.65	0.421	62.9	3.8	165.8	0.28
3	120	1	1.84	0.428	64.0	4.02	164.1	0.39
1	120	2	1.57	0.42	62.7	3.78	165.9	0.31
2	120	2	1.93	0.436	65.2	3.81	170.6	0.43
3	120	2	2.09	0.422	63.1	4.08	140.8	0.87
1	120	3	1.89	0.432	64.6	3.91	183.2	0.52
2	120	3	2.22	0.426	63.7	4.13	135.6	1.03
3	120	3	2.32	0.422	63.1	4.5	100.8	1.32
1	130	1	1.85	0.434	64.9	3.67	187.4	0.48
2	130	1	2.04	0.438	65.5	4.05	168.6	0.88
3	130	1	2.18	0.421	62.9	4.11	140.4	0.81
1	130	2	2.00	0.433	64.7	3.79	191.4	0.57
2	130	2	2.14	0.44	65.8	4.20	146.9	0.96
3	130	2	2.37	0.415	62.1	4.4	136.3	1.17
1	130	3	2.13	0.437	65.3	3.88	198.7	0.56
2	130	3	2.50	0.433	64.7	4.41	155	1.29
3	130	3	2.65	0.399	59.6	4.61	132.8	1.56
Mean	—	—	—	0.425 ± 0.015	63.5 ± 2.05	4.04 ± 0.32	155.4 ± 28.3	0.77 ± 0.41

(1) NREL's analysis of structural carbohydrate and lignin: cellulose: 37.4% (w/w); xylan: 22.1% (w/w); lignin: 20.5% (w/w).

(2) Dilute acid pretreatment was carried out at 10% (w/w) dry solids. Enzymatic saccharification was studied at 8% (w/w) dry solids with (35 mg Accellerase 1500 and 2.15 mg Accellerase XY) per gram dry biomass.

(3) All the sugar and inhibitor values are the average of two replicates.

Table IV. Fermentable sugars yield and inhibitory compounds derived from co-hydrolysate of giant reed.

Dilute acid pretreatment parameters			Sugar yield			Inhibitory compounds		
Acid (w/w, %)	Temp. (°C)	Time (h)	Log CS	Glu + Xyl (g g ⁻¹ , dry biomass)	Glu + Xyl (% of theoretical yield)	Acetate (g L ⁻¹)	HMF (mg L ⁻¹)	Furfural (g L ⁻¹)
1	120	1	1.22	0.240	43.8	3.87	270.6	0.04
2	120	1	1.62	0.301	54.8	5.27	352.2	0.18
3	120	1	1.84	0.327	59.6	5.84	307.6	0.31
1	120	2	2.00	0.32	58.3	6.34	199.5	0.75
2	120	2	1.50	0.267	48.6	4.52	351.1	0.15
3	120	2	1.77	0.314	57.2	5.45	331.4	0.3
1	120	3	1.84	0.313	57.1	5.46	347.2	0.4
2	120	3	2.10	0.334	60.8	6.27	209.9	0.65
3	120	3	2.37	0.328	59.8	6.74	111.1	1.02
1	130	1	1.66	0.327	59.5	5.5	424.3	0.44
2	130	1	1.98	0.355	64.7	6.31	263.1	0.71
3	130	1	2.18	0.355	64.7	6.79	123.5	0.83
1	130	2	2.16	0.339	61.7	4.61	124.2	0.5
2	130	2	2.33	0.336	61.2	4.97	78.0	0.92
3	130	2	2.52	0.339	61.8	5.19	74.65	0.81
1	130	3	2.04	0.315	57.4	5.47	420.6	0.56
2	130	3	2.39	0.325	59.2	6.25	200	1.0
3	130	3	2.61	0.302	55.0	6.98	105	1.01
Mean	—	—	—	0.319 ± 0.028	58.1 ± 5.18	5.66 ± 0.86	238.6 ± 117.2	0.59 ± 0.33

(1) NREL's analysis of structural carbohydrate and lignin: cellulose: 34.2% (w/w); xylan: 19.0% (w/w); lignin: 22.9% (w/w).

(2) Dilute acid pretreatment was carried out at 10% (w/w) dry solids. Enzymatic saccharification was studied at 8% (w/w) dry solids with (35 mg Accellerase 1500 and 2.15 mg Accellerase XY) per gram dry biomass.

(3) All the sugar and inhibitor values are the average of two replicates.

Table V. Fermentable sugars yield and inhibitory compounds derived from co-hydrolysate of miscanthus.

Dilute acid pretreatment parameters			Sugar yields			Inhibitory compounds		
Acid (w/w, %)	Temp (°C)	Time (h)	Log CS	Glu + Xyl (g g ⁻¹ , dry biomass)	Glu + Xyl (% of theoretical yield)	Acetate (g L ⁻¹)	HMF (mg L ⁻¹)	Furfural (g L ⁻¹)
1	120	1	1.36	0.262	43.8	4.22	102.8	0.06
2	120	1	1.67	0.305	50.9	4.79	125.8	0.17
3	120	1	1.87	0.311	51.9	4.96	116.8	0.26
1	120	2	1.58	0.303	50.6	4.55	134.6	0.18
2	120	2	1.89	0.319	53.3	4.86	121	0.37
3	120	2	2.08	0.314	52.4	5.0	86.8	0.62
1	120	3	1.95	0.319	53.2	4.69	109.2	0.32
2	120	3	2.23	0.332	55.3	4.98	73.8	0.76
3	120	3	2.42	0.315	52.6	5.21	66.47	1.06
1	130	1	1.74	0.341	56.9	4.81	155.6	0.37
2	130	1	1.99	0.344	57.3	5.21	85.13	0.96
3	130	1	2.17	0.327	54.5	5.02	68.68	0.91
1	130	2	1.94	0.332	55.5	5.12	201.8	0.37
2	130	2	2.25	0.349	58.1	6.43	174.2	0.89
3	130	2	2.46	0.342	56.9	6.93	114.7	0.88
1	130	3	2.24	0.328	54.8	4.67	126.3	0.41
2	130	3	2.47	0.319	53.3	5.14	74.69	1.11
3	130	3	2.63	0.304	50.6	5.52	72.83	1.49
Mean	—	—	—	0.32 ± 0.02	53.4 ± 3.35	5.12 ± 0.56	111.7 ± 37.7	0.62 ± 0.40

(1) NREL's analysis of structural carbohydrate and lignin: cellulose: 29.7% (w/w); xylan: 19.2% (w/w); lignin: 22.1% (w/w).

(2) Dilute acid pretreatment was carried out at 10% (w/w) dry solids. Enzymatic saccharification was studied at 8% (w/w) dry solids with (35 mg Accellerase 1500 and 2.15 mg Accellerase XY) per gram dry biomass.

(3) All the sugar and inhibitor values are the average of two replicates.

of xylose into byproducts, mainly furfural, under harsh conditions. This corresponded well to the fact that furfural generation was positively correlated with the combined severity factor in all samples. Acetic acid likewise showed a positive correlation with combined severity factor, particularly for miscanthus and giant reed. However, HMF production appeared to be independent of the combined severity factors for the range being studied.

The maximum overall sugar concentrations for corn stover, switchgrass, giant reed, and miscanthus were obtained in the combined severity factor ranges of 1.94, 2.14, 1.98, or 2.18, and 2.25, respectively (Tables II–V). This indicated that the three herbaceous perennial energy crops required harsher pretreatment conditions compared to corn stover. The combined severity analysis effectively allowed for the observation of sugar and inhibitor trends for co-hydrolysis with regards to the dilute acid pretreatment parameters.

Microbial Lipid Accumulation From Lignocellulosic Hydrolysates

The co-hydrolysates of corn stover, switchgrass, miscanthus, and giant reed obtained from the most effective hydrolysis conditions were evaluated for their potential uses as carbon sources to cultivate *M. isabellina* for lipid accumulation. The cell mass, lipid, and lipid productivity of *M. isabellina* cultivation, along with sugar concentrations and carbon/nitrogen molar ratios in the culture medium were shown in Table VI.

The deleterious effects of acetic acid, furfural, and HMF on cell growth and lipid accumulation of yeasts has been studied (Chen et al., 2009; Hu et al., 2009; Huang et al., 2009, 2011), however, information regarding their effects on oleaginous fungal lipid accumulation is quite limited. The experimental results from this study demonstrated that *M. isabellina* cultured on different co-hydrolysates, with the exception of giant reed co-hydrolysate, exhibited comparable lipid accumulation compared to synthetic hydrolysate (Table VI). The switchgrass co-hydrolysates accumulated 4.4 ± 0.44 g L⁻¹ lipid at a culture time of 118 h with initial acetic acid, HMF, and furfural concentrations at 3.25 ± 0.088 , 0.07 ± 0.002 , and 0.97 ± 0.02 g L⁻¹, respectively, in the fermentation broth. Miscanthus co-hydrolysate produced 3.71 ± 0.45 g L⁻¹ lipid at the same culture time with initial concentrations of acetic acid, HMF, and furfural in the fermentation broth of 5.01 ± 0.039 , 0.07 ± 0.0009 , and 0.92 ± 0.02 g L⁻¹, respectively. The corn stover co-hydrolysate produced 3.18 ± 0.02 g L⁻¹ lipid at 88 h with initial acetic acid, HMF, and furfural concentrations of 2.4 ± 0.004 , 0.02 ± 0.0004 , and 0.19 ± 0.0097 g L⁻¹, respectively, in the culture broth. The synthetic hydrolysate produced 3.15 ± 1.13 g L⁻¹ lipid at 68 h. While giant reed co-hydrolysate generated 3.02 ± 0.31 g L⁻¹ lipid at 136 h accompanied by initial acetic acid, HMF and furfural concentrations of 6.2 ± 0.0735 , 0.19 ± 0.0004 , and 0.51 ± 0.019 g L⁻¹ in culture media, respectively. Compared to the culture on synthetic hydrolysate, the increased cell mass from co-hydrolysates could be attributed to the strain's

Figure 3. Effect of combined severity factor on the conversion of overall glucose and xylose, acetic acid, HMF, and furfural from corn stover (A), switchgrass (B), giant reed (C), and miscanthus (D).

capability of utilizing acetic acid and minor sugars such as galactose and arabinose (Chen et al., 2009; Cheng et al., 2008; Fei et al., 2011; Ruan et al., 2012) in co-hydrolysates. Experimental data also indicated that there was a correlation between the period of lag phase and inhibitor concentrations which lead to the difference of lipid productivity among various co-hydrolysates. It was found that the higher acetic acid, HMF and furfural concentrations in the fermentation

broth, such as giant reed, the longer the lag phase and vice versa.

Material Balances

Mass balance analysis demonstrated the effects of co-hydrolysates on utilization efficiency of different lignocellulosic biomass for microbial lipid production (Fig. 4). One

Table VI. Cell mass and microbial lipid accumulation of *M. isabellina* using corn stover, switchgrass, miscanthus, and giant reed co-hydrolysates.

Carbon source	CN ^{-1*} (mol mol ⁻¹)	Glucose (g L ⁻¹)	Xylose (g L ⁻¹)	Cell mass (X, g L ⁻¹)	Lipid (L, g L ⁻¹)	% Yield _{L/X} (g g ⁻¹)	Lipid productivity (g L ⁻¹ day ⁻¹)
Synthetic hydrolysate	70.3 ± 4.8	13.66 ± 0.37	14.42 ± 0.78	10.4 ± 0.23	3.15 ± 1.13	30.66 ± 11.77	1.11 ± 0.4
Corn stover hydrolysate	68.9 ± 2.2	15.0 ± 0.04	12.6 ± 0.04	12.84 ± 0.23	3.18 ± 0.02	24.82 ± 0.75	0.87 ± 0.01
Switchgrass hydrolysate	71.5 ± 0.3	13.3 ± 0.1	15.3 ± 0.5	12.55 ± 0.37	4.4 ± 0.44	35.62 ± 3.3	0.90 ± 0.09
Miscanthus hydrolysate	71 ± 0.3	13.2 ± 0.1	15.2 ± 0.1	12.28 ± 0.02	3.71 ± 0.45	32.21 ± 3.18	0.76 ± 0.09
Giant reed hydrolysate	68.4 ± 2.6	12.1 ± 0.1	15.1 ± 0.1	13.75 ± 0.87	3.02 ± 0.31	21.18 ± 0.96	0.53 ± 0.05

(1) Dilute acid pretreatment was carried out at 10% (w/w) dry solids, pretreatment of corn stover was under 2% (w/w) sulfuric acid, 1 h, 130°C; switchgrass pretreatment was carried out at 2% (w/w) sulfuric acid, 2 h, 130°C; giant reed was treated under 3% (w/w) sulfuric acid, 1 h, 130°C; and miscanthus was pretreated using 2% (w/w) sulfuric acid, 2 h, 130°C.

(2) Enzymatic saccharification was studied at 8% (w/w) dry solids with (35 mg Accellerase 1500 and 2.15 mg Accellerase XY) per gram dry mass.

(3) All the values are the average of three replicates plus standard deviation.

*For C:N molar ratio in the medium, it was assumed that yeast extract contained 12% (w/w) of carbon source and 7% (w/w) of nitrogen source.

Figure 4. Flow chart indicating mass balance of microbial lipid accumulation from corn stover, switchgrass, miscanthus, and giant reed co-hydrolysates.

kilogram of dry corn stover contains 0.363 kg cellulose, 0.22 kg xylan, and 0.186 kg lignin. After co-hydrolysis, 0.33 kg of glucose and 0.20 kg of xylose were obtained resulting in the production of 0.0628 kg lipid from fungal fermentation (0.0628 g lipid g⁻¹ corn stover). One kilogram of dry switchgrass contains 0.374 kg cellulose, 0.221 kg xylan, and 0.205 kg lignin. Cellulose and xylan were converted to 0.25 kg glucose and 0.20 kg xylose enabling *M. isabellina* to accumulate 0.0722 kg lipid (0.0722 g lipid g⁻¹ switchgrass). One kilogram of dry miscanthus and giant reed were able to produce 0.0499 and 0.0421 kg lipid, respectively (0.0499 and 0.0421 g lipid g⁻¹ biomass).

The mass balance analysis demonstrated that the *M. isabellina* lipid accumulation on co-hydrolysates were much superior to other widely studied lipid producing strains such as yeasts grown on similar feedstocks. It has been reported that the oleaginous yeast *Trichosporon fermentans* grown on un-detoxified sulfuric acid pretreated rice straw hydrolysate containing glucose (5.1 g L⁻¹), xylose

(25.5 g L⁻¹), arabinose (4.6 g L⁻¹), acetic acid (1.4 g L⁻¹), furfural (0.5 g L⁻¹), and HMF (0.08 g L⁻¹) resulted in a lipid yield of 0.017 g lipid g⁻¹ dry biomass (Huang et al., 2009). The comparison of lipid yield elucidated that oleaginous filamentous fungi could be a better microbial option to accumulate lipid from lignocellulosic biomass.

Conclusion

This study indicated that the co-hydrolysis process was a technically feasible method to maximize biomass conversion (generate C5/C6 sugars and acetic acid), and eliminate the need of a large amount of water for washing and detoxification. The oleaginous fungus *M. isabellina* ATCC 42613 further demonstrated its unique capacity to not only utilize glucose, xylose and acetic acid in the co-hydrolysates to accumulate fungal lipids, but also tolerate relatively high concentrations of inhibition products. These results

conclude that combining co-hydrolysis of lignocellulosic feedstocks and *M. isabellina* cultivation for lipid accumulation could be a promising solution for advanced lignocellulosic fuel production.

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