Submitted for publication in

March 27, 2015

Bioresource Technology

Combining Treatments to Improve the Fermentation of
Sugarcane Bagasse Hydrolysates by Ethanogenic *Escherichia coli* LY180

Ryan Geddes, Keelnatham T. Shanmugam, and Lonnie O. Ingram

Department of Microbiology & Cell Science, University of Florida, Box 110700, Gainesville, FL
32611

Corresponding Author: Dr. Lonnie O. Ingram

Dept. Microbiology & Cell Science

Bldg. 981, Box 110700

University of Florida, Gainesville FL 32611

Tel.: 352-392-8176 fax: 352-846-0969

E-mail address: ingram@ufl.edu

E-mail address of other authors: rgeddes@ufl.edu, [mailto: shan@ufl.edu](mailto:shan@ufl.edu)

ABSTRACT

Inhibitory side products from dilute acid pretreatment is a major challenge for conversion of lignocellulose into ethanol. Six strategies to detoxify sugarcane hydrolysates were investigated alone, and in combinations (vacuum evaporation of volatiles, high pH treatment with ammonia, laccase, bisulfite, microaeration, and inoculum size). High pH was the most beneficial single treatment, increasing the minimum inhibitory concentration (measured by ethanol production) from 15% (control) to 70% hydrolysate. Combining treatments provided incremental improvements, consistent with different modes of action and multiple inhibitory compounds. Screening toxicity using tube cultures proved to be an excellent predictor of relative performance in pH-controlled fermenters. A combination of treatments (vacuum evaporation, laccase, high pH, bisulfite, microaeration) completely eliminated all inhibitory activity present in hydrolysate. With this combination, fermentation of hemicellulose sugars (90% hydrolysate) to ethanol was complete within 48 h, identical to the fermentation of laboratory xylose (50 g/L) in AM1 mineral salts medium (without hydrolysate).

Keywords: hemicellulose, hydrolysate, ethanol, furfural, inhibitors

Abbreviations: MIC, minimal inhibitory concentration for growth; MIC_{Et100}, minimal inhibitory concentration for ethanol production; IC₅₀, concentration that inhibits growth by 50%; IC_{Et50}, concentration that inhibits ethanol production by 50%; HpH, high pH treatment; V, vacuum treatment; B, bisulfite treatment; L, laccase treatment; dcw, dry cell weight.

Changes in manuscript are shown in red type.

1. Introduction

Lignocellulosic biomass is a non-food source of carbohydrates, and is an attractive feedstock for biorefineries wanting to produce renewable fuels, solvents, plastics, and chemicals. Sources of lignocellulose include agricultural residues, municipal green waste, energy crops, wood processing waste, and others (Hahn-Hagerdal et al., 2006). Unlike starch which is used as an energy storage polymer by green plants, lignocellulose is a structural component that has evolved to resist chemical and biological deconstruction (Jorgensen et al., 2007). Some form of pretreatment is essential for the effective enzymatic digestion of the cellulosic component (Alvira et al., 2010; Jorgensen et al., 2007; Mosier et al., 2005). Dilute acid hydrolysis at elevated temperatures is quite effective at increasing the available surface area for enzymatic hydrolysis of cellulose, while also hydrolyzing most hemicelluloses into monomeric sugars (Alvira et al., 2010).

Major bottlenecks in the conversion of lignocellulose to bioproducts include the generation of fermentation inhibitors during dilute acid pretreatment and the need for high cellulase enzyme loadings (Mills et al., 2009; Palmqvist and Hahn-Hagerdal, 2000). Inhibitory compounds formed include furans, organic acids, aldehydes, alcohols and phenolic compounds (Palmqvist and

1 Hahn-Hagerdal, 2000; Martinez et al., 2001; Alriksson et al., 2011). Although increased
2 pretreatment severity improves enzymatic digestibility, this method also increases the generation
3 of inhibitory side products that inhibit microbial activity and require detoxification (Mussatto
4 and Roberto, 2004).

5
6
7 Many methods have been reported for the mitigation of toxins and improvement of biomass
8 sugar fermentation and can be categorized broadly as biological, chemical and physical
9 treatments (Chandel et al., 2013). *Saccharomyces cerevisiae* NRRL Y-1536 and 424A (LNH-ST)
10 fermentation of steam pretreated mixed hardwood was improved by the removal of phenolic
11 compounds (Kim et al., 2011). Kim *et al.* (2011, 2013) determined that the removal of phenolic
12 compounds through activated charcoal or ethyl acetate, enhanced fermentation and relieved
13 enzyme inhibition. Alkaline pH treatment of hemicellulose hydrolysate with calcium hydroxide
14 (over-liming), sodium hydroxide, potassium hydroxide or ammonium hydroxide has been shown
15 to reduce toxicity of hydrolysates using yeasts and ethanologenic *Escherichia coli* (Martinez et
16 al., 2001; Mohagheghi et al., 2006). In most cases, however, high pH treatment also resulted in
17 significant sugar destruction (Martinez et al., 2001; Mohagheghi et al., 2006). Increasing the pH
18 to pH 9.0 with ammonium hydroxide was demonstrated to decrease hydrolysate toxicity with
19 minimal sugar loss (Alriksson et al., 2005; Geddes et al., 2013; Martinez et al., 2001). In
20 addition, several reduced sulfur compounds have been investigated for their ability to improve
21 growth and fermentation of hemicellulose hydrolysates (Alriksson et al., 2011; Nieves et al.,
22 2011a). Sodium meta-bisulfite (converts to two bisulfite molecules in aqueous solution) has also
23 been shown to decrease hydrolysate toxicity (Alriksson et al., 2011; Nieves et al., 2011a).

1 Some ethanologenic microorganisms have the native ability to partially detoxify hydrolysates
2 (Jonsson et al., 1998). However, even strains engineered with inhibitor detoxifying genes require
3 high levels of inoculum to be effective (Palmqvist and Hahn-Hagerdal, 2000; Zaldivar et al.,
4 1999; Sarks et al., 2014).

5 Non ethanologenic microorganisms also produce enzymes that metabolize some of the toxic
6 chemicals in hydrolysate (Lopez et al., 2004). White rot fungi (able to grow on decaying plant
7 material) produce laccase enzymes that couple the oxidation of various phenolic compounds to
8 the reduction of oxygen (Cambria et al., 2011; Jonsson et al., 1998). Oxidation of phenolic
9 compounds is thought to create free radicals that lead to polymerization, thereby removing these
10 toxic compounds from solution (Aggelis et al., 2002; Jonsson et al., 1998). Studies have shown
11 improved fermentability of hydrolysates treated with laccase enzymes (Cambria et al., 2011;
12 Jonsson et al., 1998).

13 Processes can be configured to include physical steps that reduce hydrolysate toxicity (Frazer
14 and McCaskey, 1989). Volatile inhibitory compounds can be removed from hydrolysate by
15 evaporation under vacuum (Chandel et al., 2013; Frazer and McCaskey, 1989; Mussatto and
16 Roberto, 2004). The resulting hydrolysate is more concentrated in sugar and non-volatile
17 inhibitors but has reduced levels of furfural, hydroxymethyl furfural, acetic acid and vanillin
18 (Chandel et al., 2013; Frazer and McCaskey, 1989; Mussatto and Roberto, 2004). The addition
19 of small amounts of air to the culture or headspace has been shown to promote the fermentation
20 of hemicellulose hydrolysates. Low levels of aeration increased the rate of ethanol production
21 and cell mass, but often decreased ethanol yields (Nieves et al., 2011b; Okuda et al., 2007).

22 In this investigation, we have investigated various combinations of six methods previously
23 shown to individually improve the fermentation of dilute acid hydrolysates of hemicellulose.

Each method was tested under optimal or near optimal conditions for our biocatalysts, conditions intended as a starting point for optimization with other biocatalysts and hydrolysates. A combination of treatments was identified that fully eliminated toxicity for ethanologenic *E. coli*, allowing the fermentation of hemicellulose sugars at a rate equivalent to that of pure xylose in the same mineral salts medium.

2. Materials and methods

2.1. Preparation of sugarcane bagasse hydrolysate

Sugarcane bagasse was generously provided by Florida Crystals Corporation (Okeelanta, FL). Hemicellulose hydrolysates of sugarcane bagasse were prepared at the University of Florida Biofuels Pilot Plant. Bagasse was soaked for 4 h in a 14-fold excess of 0.5% phosphoric acid (w/w including moisture in bagasse), dewatered to 50% moisture using a model CP-4 screw press (Vincent Corporation, Tampa, FL) and loaded into a steam pretreatment reactor designed by Dr. Guido Zacchi (Palmqvist et al., 1996). Dilute acid impregnated bagasse was treated for 5 min at 190°C, as previously described (Nieves et al., 2011a). Pretreated bagasse contained 70% moisture (30% dry weight including fiber and solubles). Hemicellulose hydrolysate (syrup) was separated from pre-treated bagasse by using a CP-4 screw press (Vincent Corporation, Tampa, FL). A glass fiber filter (Whatman GF/D, 15 mm diameter, 27 µm pore size) was used to remove fine particles prior to storage (4°C). Clarified hydrolysates were diluted and used as sugar and nutrient sources during experiments that evaluated toxicity. Multiple batches of hydrolysate were used during the course of this study. The average composition for 5 batches was (g/L): xylose (48 ± 7), glucose (2.7 ± 0.7), arabinose (4.5 ± 0.5), galactose (2.9 ± 0.5), acetate (3.7 ± 1.6), furfural (1.6 ± 0.5), hydroxymethyl furfural (0.0) and total monomer sugars (58.4 ± 8.5).

2.2. Strains, media, and growth conditions

Ethanologenic *E. coli* strain LY180 (*E. coli* W derivative; [Miller et al., 2009]) was used in this study of hemicellulose hydrolysate toxicity. LY180 contains a single set of chromosomally integrated genes from *Zymomonas mobilis* (*pdhA*, *adhA*, *adhB*) for ethanol production. Key genes in competing pathways (*ldhA*, *adhE*, *ackA*, *frdBC*, *mgsA*) have been deleted. A cellulase gene (*celY*) from *Erwinia chrysanthemi* and cellobiose transport and utilization genes from *Klebsiella oxytoca* (*casABC*) have also been added to this strain.

Cultures were grown in AM1 medium (Martinez et al., 2007) containing 20 g/L xylose (plates) or 50 g/L xylose (broth). OD readings were substituted by ethanol production, due to the fact that solutions are obscured by color in the hemicellulose hydrolysate. With LY180 and other ethanologenic *E. coli* strains and low inocula, cell growth is essential for ethanol production. Both parameters follow similar trends (Geddes et al., 2014; Miller et al., 2009; Wang et al., 2011; Zheng et al., 2012). In this study, ethanol production was used as the primary measure of hydrolysate performance.

2.3. Toxicity of untreated and treated hydrolysates

Relative toxicity of hydrolysate was evaluated by measuring ethanol produced after 48 h (37°C) in tube cultures (13 mm by 100 mm) containing 4 ml of AM1 medium (Martinez et al., 2007) (50 g/L xylose) supplemented with various concentrations of hydrolysate (0% hydrolysate to 90% hydrolysate) containing the components of AM1 medium (ammonia neutralization, 1.5 mM MgSO₄, 2 mM KCl, 1 mM betaine and AM1 trace metals; approximately 50 g/L total sugars). Inocula were grown overnight on AM1 xylose plates (solid medium). Cells from fresh colonies were re-suspended in AM1 medium and adjusted to an OD_{550nm} of 1.0. Tube cultures

1 were inoculated to an initial OD_{550nm} of 0.1 (43 mg dcw/L) and incubated in a reciprocating
2 water bath (50 oscillations min⁻¹, 37°C). Since inocula and media components represented up to
3 10% of the volume, the highest concentration of hydrolysate that could be tested was 90%.
4 Hydrolysate adjusted to pH 6.3 with ammonium hydroxide served as a control for maximum
5 toxicity. Relative toxicity was evaluated by determining the hydrolysate concentrations (treated
6 and untreated control) that inhibited ethanol production by 50% (ICEt₅₀) and by 100%
7 (MICEt₁₀₀). All tube cultures and tests were prepared as biological triplicates and all experiments
8 were repeated at least twice.

2.4. Hydrolysate treatments

11 Six approaches were evaluated for their effects on hydrolysate toxicity alone and in
12 combination. The order for these treatments was selected to minimize the impact on processing
13 such as temperature and pH adjustment: 1) vacuum treatment; 2) laccase; 3) high pH treatment
14 (HpH); 4) bisulfite addition; 5) increased inoculum; and 6) aeration. For high pH exposure
15 (HpH) treatment, hydrolysate was adjusted to pH 9.0 by addition of ammonium hydroxide (5 N)
16 and stored at room temperature overnight before inoculation (16 h). Ammonia treated
17 hydrolysate fell to approximately pH 7.5 during incubation (Geddes et al., 2013). For bisulfite
18 treatment, a freshly prepared solution was added to culture tubes immediately before inoculation
19 (1 mM final concentration of bisulfite or 0.5 mM sodium metabisulfite) (Nieves et al., 2011a).
20 Hydrolysate adjusted to pH 6.3 immediately before inoculation served as the untreated control
21 for all experiments.

22 A Buchi Rotavapor R110 evaporator (Flawil, Switzerland) equipped with a Cole Palmer
23 aspirator pump Model 7049-00 (Chicago, Illinois) was used (55°C) to remove volatiles (vacuum

1 treatment) prior to pH adjustment of hydrolysate. Unless otherwise indicated, hydrolysate was
2 evaporated to 50% by weight, restored to original weight by adding deionized water, and
3 adjusted to pH 6.3 before testing for toxicity.

4 Laccase (Novozymes NS-22127) was generously provided by Novozymes North America, Inc.
5 (Franklinton, North Carolina). For laccase treatment, hydrolysate was adjusted to pH 5.0 with
6 ammonium hydroxide (5 N). Laccase (250 U total) was added to 50 ml hydrolysate in a 250 ml
7 flask. This was incubated in a water bath (50°C, 150 rpm) for 3 h. A laccase unit, U, is defined
8 as the amount of laccase that catalyzes the conversion of 1 μ mole (Leonowicz and Grzywnowicz,
9 1981) of syringaldazine per minute under standard conditions (pH 7.5, 30°C). When needed,
10 aeration was provided in tube cultures using an incubator with a rotator (30 rpm, 60° angle,
11 37°C).

12 Conditions tested were optima or near optima for ethanologenic *E. coli* LY180. These should
13 represent an excellent starting point for further optimization with other biocatalysts and
14 hydrolysates.

15 16 2.5. Fermentation

17 Fermentations were conducted in pH-controlled vessels (500 ml) with a 300 ml working
18 volume (37°C, 150 rpm; Geddes et al., 2013). Cultures were maintained at pH 6.5 by the
19 automatic addition of 2M potassium hydroxide. A modified AM1 medium (AM1-90%
20 hydrolysate containing approximately 50 g/L total sugar) was prepared using hemicellulose
21 hydrolysate as the sole source of fermentable sugar. Trace metals, MgSO₄ (1.5 mM final), KCl
22 (2 mM final) and betaine (1 mM final) were added from 1000X stock solutions (Martinez et al.,
23 2007). Hydrolysates were treated in various ways, as indicated, to reduce toxicity. Seed cultures

1 were grown overnight (16 h) in AM1 medium supplemented with 50 g/L xylose. Fermentations
2 were initiated by inoculating to an initial OD_{550nm} of 0.1 (43 mg dcw/L) and monitored for up to
3 120 h. For cultures requiring low level aeration (micro-aeration), subsurface sparging (0.01 vvm,
4 3 ml/min) was provided by using a peristaltic pump.

2.6. Chemical Analysis

5
6
7 Sugars, furans, and organic acids were analyzed using two Agilent Technologies (Santa Clara,
8 CA) 1200 series HPLC systems, one equipped with a BioRad (Hercules, CA) Aminex HPX-87P
9 ion exclusion column and the other equipped with a BioRad Aminex HPX-87H column (Geddes
10 et al., 2013). Ethanol was measured using an Agilent Technologies 6890 N Network gas
11 chromatography system with a wide bore HP-PLOT Q column (J&W Scientific, Folsom, CA;
12 Geddes et al., 2013). Total furans (furfural and hydroxymethyl furfural) were measured by
13 ultraviolet absorbance (Martinez et al., 2000b) and by HPLC. Moisture content was determined
14 using a Kern model MLB 50–3 moisture analyzer (Balingen, Germany).

2.7. Statistical Analysis

15
16
17 Prism software (Graphpad, San Diego, CA) was used to perform two-way ANOVA (analysis of
18 variance) comparisons of data. Differences were judged significant when *P* values for the null
19 hypothesis were ≤ 0.05 .

3. Results and discussion

3.1. Ethanol production (*MICet*₁₀₀ and *ICet*₅₀) as a measure of hydrolysate toxicity

1 A standardized procedure was developed to compare hydrolysate treatments for toxicity using
2 culture tubes containing dilutions of hydrolysate in AM1 mineral salts medium (50 g/L xylose)
3 and a low inoculum. Hydrolysate adjusted to pH 6.3 with ammonium hydroxide immediately
4 before inoculation served as the control, exhibiting maximum toxicity. In previous studies
5 (Zaldivar et al., 1999; Miller et al., 2009), OD_{550nm} , MIC and IC_{50} were used as metrics.
6 However, these measurements of optical density in hydrolysate medium are complicated by dark
7 color, color changes during incubation, and precipitation of lignin in some cases. Since the
8 shapes of curves for cell mass during growth and cumulative ethanol production are similar in
9 AM1 medium without hydrolysate (Fig. 1a), analogous metrics for this study were based on
10 ethanol production: 1) hydrolysate concentration at which ethanol production was completely
11 inhibited ($MIC_{Et_{100}}$); and 2) 50% inhibition of ethanol production ($IC_{Et_{50}}$). Figure 1b illustrates
12 the graphical estimation of $MIC_{Et_{100}}$ and $IC_{Et_{50}}$ using control hydrolysate (adjusted to pH 6.3
13 and inoculated) and hydrolysate after (HpH) as examples. The higher $MIC_{Et_{100}}$ and $IC_{Et_{50}}$
14 values after HpH exposure indicate a reduction in hydrolysate toxicity.

16 *3.2. Single and combination treatments of hydrolysate*

17 Many treatments have been described previously that reduce hydrolysate toxicity for yeasts
18 (Jonsson et al., 1998; Sarks et al., 2014; Okuda et al., 2007) and alcohol-producing bacteria
19 (Frazer and MacCasky, 1989; Martinez et al., 2000a; Nieves et al., 2011b). Six of these
20 treatments were compared for their ability to reduce the toxicity of dilute acid hydrolysates of
21 sugarcane bagasse (Fig. 2a). When tested individually, HpH treatment was the most beneficial
22 for reduction of hydrolysate toxicity. With HpH treatment, $MIC_{Et_{100}}$ and $IC_{Et_{50}}$ concentrations
23 of hydrolysate were increased by at least 5 fold over the control, allowing fermentation (ethanol

production) in 70% hydrolysate. Previous studies have shown that high pH treatments are also effective using sodium hydroxide, potassium hydroxide, and calcium hydroxide (Martinez et al., 2000a; Mohagheghi et al., 2006). Unlike previous reports with these mineral hydroxides, however, HpH treatment using ammonia caused very little sugar destruction (Table 1) (Geddes et al., 2013).

Addition of 1 mM bisulfite (Nieves et al., 2011a) had the second largest benefit when tested alone, increasing MIC_{Et100} from 15% hydrolysate (control) to 25% hydrolysate and IC_{Et50} from 4.8% hydrolysate (control) to 16.3% hydrolysate (3-fold). When tested alone, addition of air, increasing inoculum size by 4-fold, and removal of volatiles with vacuum each caused only a small decrease in toxicity. Laccase treatment alone provided little benefit for fermentation as a single treatment.

All IC_{Et50} values for treated hydrolysates were significantly higher than the untreated hydrolysate control. All MIC_{Et100} values were significantly higher than the control except for laccase alone and air alone. Combination treatments were investigated using HpH, bisulfite, laccase and vacuum evaporation (Fig. 2b). All were beneficial in comparison to the control (Fig. 2a). All treatment combinations that included high pH treatment were better than combinations which lacked high pH treatment. In the absence of high pH treatment, vacuum treatment combined with bisulfite was the most effective binary combination, increasing the MIC_{Et100} by over 2 fold and the IC_{Et50} by over 4 fold as compared to control (Fig. 2a). Neither bisulfite with laccase nor vacuum with laccase increased the MIC_{Et100} or the IC_{Et50} more than bisulfite alone (Fig. 2a). The beneficial effect of laccase was observed only for combinations that included prior vacuum treatment. The combination of laccase, vacuum and bisulfite was significantly better than any binary combination ($p<0.05$).

None of the combinations tested without HpH exposure (Fig. 2b) were as effective as the HpH alone (Fig. 2a). Binary combinations that included HpH treatment (Fig. 2b) were all significantly less toxic than HpH, as indicated by high IC_{Et50} values and MIC_{Et100} values at or above 90% hydrolysate. The combination of vacuum and HpH treatment was more effective than the ternary combination of laccase, HpH, and bisulfite. In most cases, each added treatment caused an incremental decrease in toxicity (increase in IC_{Et50} concentration), consistent with the presence of multiple inhibitors and mechanisms of action. Laccase was ineffective except when combined with prior vacuum treatment or with HpH treatment. Hydrolysate exposed to HpH combined with three other treatments (vacuum, laccase, and bisulfite) had the highest metrics and lowest toxicity, an IC_{Et50} of over 80% hydrolysate and a MIC_{Et100} of greater than 90% hydrolysate.

3.3. Reducing the extent of vacuum evaporation

Half the weight of hydrolysate was evaporated in the initial vacuum evaporation experiments. With this treatment, all furfural and 25% of the acetate were removed (Fig. 3a; Table 1).

However, incomplete furfural removal may be desirable for some applications. For instance, further study with a cost analysis may reveal that 90% of the biological benefit can be derived from evaporation of only 10% of the volume due in part to energy savings and development of a new coproduct (distillate with higher furfural concentration).

Further tests were conducted to determine the extent of furfural removal at different stages of evaporation (Fig. 3a). The initial furfural concentration (20 mM) was reduced by 90% after only 10% evaporation of hydrolysate. Furfural was completely removed after 20% or more evaporation (Fig. 3a).

1 The effect of different vacuum treatments on toxicity was also examined. After each vacuum
2 treatment, deionized water was added to replace lost weight and the hydrolysate was exposed to
3 HpH (Fig. 3a). Toxicity was measured as ethanol production in treated hydrolysate diluted with
4 an equal volume of AM1 medium (50 g/L xylose; with and without bisulfite). Without vacuum
5 treatment, 50% hydrolysate contained sufficient furfural to inhibit ethanol production (10 mM
6 furfural. After a 10% evaporation, ethanol production was increased by more than 3-fold (results
7 with and without bisulfite), concurrent with the removal of 90% of the furfural. A further
8 increase in ethanol production was observed after complete removal of furfural (20%
9 evaporation). This small further increase in ethanol production may also reflect removal of less
10 volatile compounds such as some phenolics and acetic acid (Fig. 3a).

11 In all cases, ethanol production was lower without bisulfite, consistent with independent
12 modes of action. The mechanism of bisulfite action is not known but may include reactions with
13 aldehydes, formation of Schiff bases, and other reactions. Apparently, the direct interaction of
14 bisulfite and furfural was not involved since the benefit of sulfite persisted after furfural removal.
15 Bisulfite may help ethanol production by providing partially reduced sulfur for assimilation
16 (Nieves et al. 2011a).

17 The concurrent reduction of toxicity and removal of furfural by vacuum evaporation suggests
18 that furfural is an important inhibitor in hydrolysate (Frazer and McCaskey, 1989). However,
19 unquantified compounds of equal or greater importance may also be removed by evaporation.
20 Examining the effect of adding 20 mM furfural to vacuum-treated hydrolysate (50%
21 evaporation) tested this possibility (Fig. 3b). Restoring the initial level of furfural to vacuum-
22 treated hydrolysate precisely restored the toxicity for ethanol production, establishing furfural as
23 the dominant inhibitor in HpH treated hydrolysate (with and without bisulfite treatment). These

1 results provide further evidence that furfural plays a central role as an inhibitor of growth and
2 fermentation in dilute acid hydrolysates.

3 4 *3.4. Effect of selected treatments on sugar and inhibitor concentrations*

5 Sugar and inhibitor concentrations were measured in hydrolysates after various combinations
6 of treatments (Table 1). To facilitate comparisons, all hydrolysates were diluted to 90% with
7 AM1 medium analogous to the medium used in tube fermentations. Vacuum treatment (50%
8 evaporation) of hydrolysate reduced furfural to undetectable amounts and also reduced acetate
9 concentrations by 25% compared to the control (pH 6.3). When vacuum evaporation was
10 combined with laccase and bisulfite treatment, no further reductions in acetate were observed.
11 All treatment combinations conducted without HpH caused very little sugar loss (<1%). HpH
12 treatment reduced furfural concentrations by 36% while acetate concentrations remained
13 unchanged. A small amount of sugar was lost during HpH treatment, approximately 0.6 g/L. in
14 contrast to high pH treatments using lime (10% to 20% loss; Geddes et al., 2013; Martinez et al.,
15 2000a). No further loss of sugar was observed when HpH was combined with vacuum
16 evaporation, laccase or bisulfite treatments.

17 18 *3.5. Increasing inoculum size and aeration improved ethanol production.*

19 All hydrolysates exposed to HpH (alone or in combinations) were less toxic than untreated
20 hydrolysate (Fig. 2a and 2b). Two of the least toxic combinations were used to examine the
21 effects of inoculum size and aeration: 1) vacuum evaporation + HpH + bisulfite; and 2) vacuum
22 evaporation + laccase + HpH + bisulfite (Fig. 4a and 4c). Increasing inoculum size by up to 4-
23 fold that of the standard inoculum resulted in a small incremental increase in ethanol production.

1 A similar increase in ethanol production with hydrolysate and high inoculum has been reported
2 for yeasts (Sarks et al., 2014). Increased inoculum size may increase the rate of inhibitor
3 metabolism. However, a threshold of toxicity must be overcome before this increase in inocula
4 can be effective (Palmqvist and Hahn-Hagerdal, 2000; Zaldivar et al., 1999). **Growth and ethanol
5 production would be minimal during inhibitor metabolism at levels above this threshold.**

6 Addition of air (Fig. 4b and 4d) also provided an incremental benefit, increasing ethanol
7 production in 90% hydrolysate by over 2-fold for hydrolysates treated with all four methods
8 (vacuum evaporation + laccase + HpH + bisulfite). Air promotes an increase in cell mass, energy
9 production, and allows redox balance, factors that may minimize damage from toxins in
10 hydrolysate (Nieves et al., 2011b; Okuda et al., 2007). Aeration of the most highly-treated
11 hydrolysate produced more ethanol (17 g/L) than tube cultures of LY180 with laboratory AM1
12 medium containing 50 g/L xylose without hydrolysate (8.5 g/L ethanol; Figure 1a).

14 *3.6. Fermentation of hydrolysate (90%) in pH-controlled vessels*

15 The effect of the various hydrolysate treatments was further investigated using pH-controlled
16 fermenters. LY180 grown in AM1 minimal medium with 50 g/L xylose was able to completely
17 utilize xylose within 48 h (Fig. 5a), achieving a corresponding maximum ethanol titer of 23 g/L
18 (Fig. 5b). In contrast, sugar utilization and ethanol production was completely inhibited in 90 %
19 hydrolysate even after HpH exposure, with or without bisulfite. However, vacuum evaporation
20 combined with HpH treatment allowed fermentation in 90% hydrolysate to proceed, producing
21 20 g/L ethanol after 120 h with nearly complete sugar utilization. Further improvements in
22 productivity were observed when bisulfite and laccase were also included
23 (vacuum+HpH+bisulfite and vacuum+laccase+HpH+bisulfite), producing 21 g/L ethanol in 120

h and 96 h, respectively. Lower final ethanol titers with hydrolysate are due to lower sugar content (90% hydrolysate). Micro-aeration (0.01 vvm) dramatically improved fermentation of treated hydrolysate (vacuum+laccase+HpH+bisulfite). With this combined approach, fermentation was completed within 48 h, equivalent to the fermentation of laboratory xylose in AM1 medium without hydrolysate. All sugars present in the sugarcane bagasse hemicellulose hydrolysate were completely utilized (Fig. 5c). This combination of treatments with micro-aeration fully eliminated the toxicity of hemicellulose hydrolysate for ethanologenic *E. coli* LY180.

4. Conclusions

Simple detoxification strategies were developed for acid hydrolysates of bagasse hemicellulose by using low inocula and measuring ethanol production in tube cultures. High pH treatment (pH 9) provided the best improvement in hydrolysate fermentation (single treatment). Culture tube results were an excellent predictor of trends in pH-controlled fermentations. Combinations of treatments were generally additive, consistent with multiple inhibitory chemicals and multiple sites of action. The best combination of detoxification methods completely eliminated hydrolysate toxicity, allowing sugars in hemicellulose hydrolysates to be fermented as effectively as laboratory xylose in AM1 mineral salts medium (without hydrolysate).

Acknowledgements

This work was supported by grants from the US Department of Agriculture (2011-10006-30358 and 2012-67009-19596), the US Department of Energy's Office of International Affairs (DE-PI0000031), BASF, and the Florida Department of Agriculture and Consumer Services.

References

1. Aggelis, G., Ehaliotis, C., Nerud, F., Stoychev, I., Lyberatos, G., Zervakis, G., 2002. Evaluation of white-rot fungi for detoxification and decolorization of effluents from the green olive debittering process. *Appl. Microbiol. Biotechnol.* 59:353-360.
2. Alriksson, B., Horvath, I.S., Sjode, A., Nilvebrant, N.O., Jonsson, L.J. 2005. Ammonium hydroxide detoxification of spruce acid hydrolysates. Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals ABAB Symposium pp 911-922.
3. Alriksson, B., Cavka, A., Jonsson, L.J. 2011. Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents. *Bioresour. Technol.* 102:1254-1263.
4. Alvira P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresour. Technol.* 101:4851–4861.
5. Cambria, M.T., Ragusa, S., Calabrese, V., Cambria, A. 2011. Enhanced laccase production in white-rot fungus *Rigidoporus lignosus* by the addition of selected phenolic and aromatic compounds. *Appl. Biochem. Biotechnol.* 163:415-422.
6. Chandel, A.K., Silva, S.S., Singh, O.V. 2013. Detoxification of lignocellulose hydrolysates: Biochemical and metabolic engineering towards white biotechnology. *BioEner. Res.* 6:388-401.
7. Frazer, F.R., McCaskey, T.A. 1989. Wood hydrolysate treatments for improved fermentation of sugars to 2, 3-butanediol. *Biomass* 18:31–42.

- 1
2
3
4 1 8. Geddes, C.C., Mullinnix, M.T., Nieves, I.U., Hoffman, R.W., Sagues, W.J., York, S.W.,
5
6 2 Shanmugam, K.T., Erickson, J.E., Vermerris, W.E., Ingram, L.O. 2013. Seed train
7
8 3 development for the fermentation of bagasse from sweet sorghum and sugarcane using a
9
10 4 simplified fermentation process. *Bioresour. Technol.* 128:716-724.
11
12
13
14 5 9. Geddes, R.D., Wang, X., Yomano, L.P., Miller, E.N., Shanmugam, K.T., Ingram, L.O.
15
16 6 2014. Polyamine transporters and polyamines increase furfural tolerance during xylose
17
18 7 fermentation with ethanologenic *Escherichia coli* strain LY180. *Appl. Environ.*
19
20 8 *Microbiol.* Jul 25. pii: AEM.01913-14. [Epub ahead of print]
21
22
23
24 9 10. Hahn-Hagerdal, B., Galbe, M. Gorwa-Grauslund, M.F., Liden, G., Zacchi, G. 2006. Bio-
25
26 10 ethanol – the fuel of tomorrow from the residues of today. *Trends Biotechnol.* 24:549-
27
28 11 556.
29
30
31 12 11. Jonsson, L.J., Palmqvist, E., Nilvebrant, N.O., Hahn-Hagerdal, B. 1998. Detoxification of
32
33 13 wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes*
34
35 14 *versicolor*. *Appl. Microbio. Biotechnol.* 49:691-697.
36
37
38 15 12. Jorgensen H., Kristensen J.B., Felby, C. 2007. Enzymatic conversion of lignocellulose
39
40 16 into fermentable sugars: challenges and opportunities. *Biofuels Bioprod. Bioref.* 1:119–
41
42 17 134.
43
44
45 18 13. Kim Y, Kreke T, Hendrickson R, Parenti J, Ladisch MR. 2013. Fractionation of cellulase
46
47 19 and fermentation inhibitors from steam pretreated mixed hardwood. *Biorefineries* 135:30-
48
49 20 38.
50
51
52 21 14. Kim Y, Ximenes E, Mosier NS, Ladisch MR. 2011. Soluble inhibitors/deactivators of
53
54 22 cellulase enzymes from lignocellulosic biomass. *Enzyme Microb. Technol.* 48:408-415.
55
56
57
58
59
60
61
62
63
64
65

15. Leonowicz, A., Grzywnowicz, K. 1981. Quantitative estimation of laccase form in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb. Technol.* 3:55-58.
16. Lopez, M.J., Nichols, N.N., Dien, B.S., Moreno, J., Bothast, R.J. 2004. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. *Appl. Microbiol. Biotechnol.* 64:125-131.
17. Martinez A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O. 2000a. Effects of $\text{Ca}(\text{OH})_2$ treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. *Biotechnol. Bioeng.* 69:526-536.
18. Martinez A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O. 2000b. Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. *Biotechnology Progress* 16, 637-641.
19. Martinez, A., Rodriguez, M.E., Wells, M.L., York, S.W., Preston, J.F., Ingram, L.O. 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. *Biotechnol. Prog.* 17:287-293.
20. Martinez, A., Grabar, T.B., Shanmugam, K.T., Yomano, L.P., York, S.W., Ingram, L.O. 2007. Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. *Biotechnol. Lett.* 29:397-404.
21. Miller, E.N., Jarboe, L.R., Yomano, L.P., York, S.W., Shanmugam, K.T., Ingram, L.O. 2009. Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. *Appl. Environ. Microbiol.* 75:4315-4323.
22. Mills, T.Y., Sandoval, N.R., Gill, R.T. 2009. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. *Biotechnol. Biofuels* 2:26.

23. Mohagheghi, A., Ruth, M., Schell, D.J. 2006. Conditioning hemicellulose hydrolysates for fermentation: Effects of overliming pH on sugar and ethanol yields. *Process Biochem.* 41: 1806-1811.
24. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96:673–686.
25. Mussatto, S.I., Roberto, I.C. 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. *Bioresour. Technol.* 93: 1-10.
26. Nieves, I.U., Geddes, C.C., Miller, E.N., Mullinnix, M.T., Hoffman, R.W., Fu, Z., Ingram, L.O., 2011a. Effect of reduced sulfur on the fermentation of phosphoric acid pretreated bagasse by ethanologenic *Escherichia coli*. *Bioresour. Technol.* 102:5145-5152.
27. Nieves, I.U., Geddes, C.C., Mullinnix, M.T., Hoffman, R.W., Tong, Z., Castro, E., Shanmugam, K.T., Ingram, L.O. 2011b. Injection of air into the headspace improves fermentation of phosphoric acid pretreated sugarcane bagasse by *Escherichia coli* MM170. *Bioresour. Technol.* 102:6959-6965.
28. Okuda, N., Ninomiya, K., Takao, M., Katakura, Y., Shioya, S. 2007. Microaeration enhances productivity of bioethanol from hydrolysate of waste house wood using ethanologenic *E. coli* KO11. *J. Biosci. Bioeng.* 103:350-357.
29. Palmqvist, E., Hahn-Hagerdal, B., Galbe, M., Larsson, M., Stenberg, K., Szengyel, Z., Tengborg, C., Zacchi, G. 1996. Design and operation of a bench-scale process

- development unit for the production of ethanol form lignocellulosics. Bioresour. Technol. 58:171-179.
30. Palmqvist, E., Hahn-Hägerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition. Bioresour. Technol. 74: 25-33.
31. Sarks, C., Jin, M., Sato, T.K., Balan, V., Dale, B.E. 2014. Studying the rapid bioconversion of lignocellulosic sugars into ethanol using high cell density fermentations with cell recycle. Biotechnol. Biofuels 7:73.
32. Wang X, Miller EN, Yomano LP, Zhang X, Shanmugam KT, Ingram LO. 2011. Increased furfural tolerance due to overexpression of NADH-dependent oxidoreductase FucO in *Escherichia coli* strains engineered for the production of ethanol and lactate. Appl. Microbiol. Biotechnol. 77:5132-5140.
33. Yomano, L.P., York, S.W., Shanmugam, K.T., Ingram, L.O. 2009. Deletion of methylglyoxal synthase genes (*mgsA*) increased sugar co-metabolism in ethanol producing *Escherichia coli*. Biotechnol. Lett. 31:1389-1398.
34. Zaldivar J., Martinez, A., Ingram, L.O. 1999. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 65:24-33.
35. Zheng H, Wang X, Yomano LP, Shanmugam, KT, Ingram LO. 2012. Increase in furfural tolerance in ethanologenic *Escherichia coli* LY180 by plasmid-based expression of *thyA*. Appl. Environ. Microbiol. 78:4346-4352.

1 Table 1. Sugar and inhibitor concentrations after hydrolysate treatments.

Treatment	Sugars (g/L)					Inhibitors (g/L)	
	Glucose	Xylose	Galactose	Arabinose	Total Sugars	Furfural	Acetate
Control	3.2	43.8	3.1	4.1	54.2	2.0	5.1
V ^a	3.2	43.8	3.0	4.1	54.1	ND ^b	3.7
V, B ^c	3.2	43.6	3.0	4.1	53.9	ND	3.7
V, L ^d , B	3.2	43.6	3.0	4.0	53.9	ND	3.7
HpH ^e	3.2	43.4	3.0	4.1	53.6	1.3	5.1
V, HpH	3.2	43.4	3.0	4.0	53.5	ND	3.7
V, HpH, B	3.2	43.4	3.0	4.0	53.5	ND	3.7
V, L, HpH, B	3.2	43.3	3.0	4.0	53.5	ND	3.7
Average Hydrolysate ^f	2.7 ± 0.7	48.0 ± 7.0	2.9 ± 0.5	4.5 ± 0.5	58.4 ± 8.5	1.6 ± 0.5	3.7 ± 1.6

^a vacuum evaporation^b not detectable^c bisulfite^d laccase^e high pH treatment^f Average of 5 batches of hydrolysate

Figure 1. Metrics used to evaluate toxicity in treated hydrolysates. a. Growth and ethanol production in AM1 medium (50 g/L xylose). b. Examples of hydrolysate plots illustrating the estimation of MIC_{Et100} (hydrolysate concentration that completely blocks ethanol production) and IC_{Et50} (hydrolysate concentration that causes a 50% inhibition of ethanol production). These metrics are based on ethanol production rather than OD_{550nm} due to color in the hydrolysate, changes in color during incubation, and precipitation. Ethanol production was measured after 48 h. Two curves are shown as examples: untreated hydrolysate adjusted to pH 6.3 immediately before inoculation (○, control); and high pH treated hydrolysate (Δ).

Figure 2. Effects of chemical and process treatments on ethanol production in hemicellulose hydrolysates using LY180 (MIC_{Et50} and MIC_{Et100} values). a. Single hydrolysate treatments; b. Combinations of hydrolysate treatments. The open triangle above bars indicates that the MIC_{Et100} is greater than 90%, the highest concentration which can be tested. All treatments were beneficial in comparison ($p>0.05$) to the control (hydrolysate adjusted to pH 6.3 with ammonium hydroxide immediately prior to inoculation).

Figure 3. Effect of vacuum evaporation (V) on furfural removal and fermentation of hemicellulose hydrolysate (50% dilution): a. Correlation between furfural removal and ethanol production by LY180 (48 h, 50% hydrolysate; high pH treatment (HpH) with or without bisulfite (B) in tube cultures; and b. LY180 fermentation (48 h, 50% hydrolysate, HpH exposure, with and without bisulfite, with and without vacuum treatment. HpH exposure and bisulfite treatment reduce toxicity. Vacuum treatment removes furfural and causes a further decrease in toxicity

(increase in ethanol production). Restoring furfural to original levels in 50% hydrolysate (10 mM) fully restored toxicity.

Figure 4. Fermentation of treated hydrolysate with LY180. Combinations of increasing inoculum size, and aeration were tested using two combination treatments of hemicellulose hydrolysates (90%). Figure a and b (vacuum, HpH, and bisulfite treatments); figure c and d (vacuum, laccase, HpH, and bisulfite treatments). No air was supplied to a and c. Micro-aeration (0.01 vvm) was provided to b and d.

Figure 5. Fermentation of 90% hydrolysates by LY180. a). Sugar utilization, b). Ethanol production, c). Utilization of individual sugars after V, L, HpH, B, Air treatment. Abbreviations for hydrolysate treatments: V, vacuum evaporation; L, laccase; HpH, high pH treatment; B, bisulfite; and Air, micro-aeration (0.01 vvm).

Figure

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5