**Quantifying pretreatment degradation compounds in solution and
accumulated by cells during solids and yeast recycling in the Rapid
Bioconversion with Integrated recycling Technology process using AFEX™
corn stover**

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Abstract

Effects of degradation products (low molecular weight compounds produced during pretreatment) on the microbes used in the RaBIT (Rapid Bioconversion with Integrated recycling Technology) process that reduces enzyme usage up to 40% by efficient enzyme recycling were studied. Chemical genomic profiling was performed, showing no yeast response differences in hydrolysates produced during RaBIT enzymatic hydrolysis. Concentrations of degradation products in solution were quantified after different enzymatic hydrolysis cycles and fermentation cycles. Intracellular degradation product concentrations were also measured following fermentation. Degradation product concentrations in hydrolysate did not change between RaBIT enzymatic hydrolysis cycles; the cell population retained its ability to oxidize/reduce (detoxify) aldehydes over five RaBIT fermentation cycles; and degradation products accumulated within or on the cells as RaBIT fermentation cycles increased. Synthetic hydrolysate was used to confirm that pretreatment degradation products are the sole cause of decreased xylose consumption during RaBIT fermentations.

Keywords

AFEX™

Cellulosic Ethanol

Pretreatment Degradation Products

Saccharomyces cerevisiae

Cell Recycling

1. Introduction

Ethanol produced using lignocellulosic biomass is a renewable energy option to reduce dependence on petroleum for transportation fuels. Despite the establishment of lignocellulosic

ethanol plants in the U.S., lignocellulosic ethanol is still widely considered uneconomical. The RaBIT process is one potential method to improve process economics. The RaBIT process uses increased biocatalyst (enzymes and microbe) concentrations to reduce processing time and biocatalyst recycle to improve economics (Jin et al., 2012). One problem associated with the RaBIT process is the decrease in cell performance after recycling. This decrease was observed for multiple microbial strains and could not be eliminated by nutrient supplementation (Sarks et al., 2014).

Yeast recycling is a common practice in ethanol fermentations. The Brazilian sugar cane industry uses an initial yeast pitch and recycles the yeast population for the rest of the season (Wheals et al., 1999). Different species of yeast are also introduced naturally during the sugar cane process and may end up dominating the fermentations (da Silva-Filho et al., 2005). The beer industry recycles its yeast for a shorter period of time to eliminate any drift in the beer flavor due to mutation or adaptation (Huuskonen et al., 2010). Although biocatalyst recycling has been used successfully elsewhere, its employment in lignocellulosic processes has challenges. The three main differences are reduced nutrient levels, xylose fermentation, and the presence of degradation products produced during pretreatment and hydrolysis. Previous work reported by Sarks et al. (2014) showed that additional nutrients did not eliminate decreasing xylose consumption and that significant cell death was occurring during the xylose consumption phase. This work highlights pretreatment degradation product effects.

Pretreatment of lignocellulosic biomass is necessary for its efficient conversion to monomeric sugars (Balan, 2014). Pretreatment processes are commonly performed under high

temperature, high pressure, caustic, and/or acidic conditions, which generate degradation compounds that inhibit microorganisms (Balan, 2014; Du et al., 2010). Under acidic conditions, carbohydrates present in the biomass degrade into furfural or hydroxymethylfurfural and the lignin degrades into a variety of phenolic compounds (Du et al., 2010; Klinke et al., 2004). The Ammonia Fiber Expansion (AFEXTM)[†] process produces many ammoniated compounds. A previous comparison of AFEX and dilute acid treated corn stover showed that dilute acid pretreatment produces 316% more acidic compounds, 142% more aromatics, 3555% more furans, but no nitrogenous compounds (Chundawat et al., 2010). Nitrogenous compounds are significantly less inhibitory than their acid counterparts (Tang et al., 2015).

Pretreatment degradation products inhibit microbes in lignocellulosic hydrolysates. Inhibition occurs for both cell growth and sugar consumption (Tang et al., 2015). Previous research has mostly been focused on the effect of degradation compounds during single batch fermentations. Our literature review has found no research on the effects of degradation compounds on fermentations with cell recycling. The biggest concern, in the context of cell recycling, is whether pretreatment degradation products can be accumulated by the cell.

We report the effects of pretreatment degradation products on the performance of recycled cells. We used chemical genomic profiling to determine whether a specific yeast strain could detect differences between RaBIT enzymatic hydrolysis cycles (Serate et al., 2015; Ho et al., 2011). RaBIT fermentations were performed at different concentrations of pretreatment degradation products. Pretreatment degradation product concentrations were quantified before fermentation, after fermentation, and within the yeast cells. Finally, degradation product effects on yeast fermentations with cell recycle were investigated further using synthetic hydrolysate.

[†] AFEX is a trademark of MBI, Lansing, Michigan

2. Materials and Methods

2.1 Biomass and Pretreatment

Great Lakes Bioenergy Research Center (GLBRC) corn stover was used for this study. The corn (Pioneer 36H56) was planted and harvested during 2010 at the Arlington Agricultural Research Station (Columbia County, WI). The biomass was pretreated using the AFEX process as previously described in the literature using a 5 gallon reactor (Balan et al., 2009). AFEX pretreatment conditions were as follows: 1:1 ammonia to biomass ratio, 60% moisture, 100 °C, and 30 min. reaction time. Composition analysis, following the method of Sluiter et al., 2010 showed the glucan, xylan, acid insoluble lignin, and ash contents were 31.4%, 18.6%, 13.08%, and 13.39%, respectively. The pretreated corn stover was stored at 4 °C.

2.2 Microorganism and Seed Culture Preparation

Saccharomyces cerevisiae GLBRCY128 was used for this study having previously been genetically modified and adapted for xylose utilization (Parreiras et al., 2014). Xylose isomerase and xylulokinase genes were introduced to facilitate xylose utilization.

Seed cultures were prepared from glycerol stocks stored at -80°C. Seed culture media contained 100 g/L dextrose, 25 g/L xylose, 20 g/L tryptone, and 10 g/L yeast extract. Erlenmeyer flasks (250 mL) containing 100 mL of seed culture media were inoculated with 0.1 OD₆₀₀. The cultures were incubated at 30 °C and 150 RPM in shaker incubators under microaerobic conditions for 22 h. After 22 h, fresh seed culture media was inoculated with 1% of the first seed culture medium and incubated under the same conditions for another 22 h.

2.3 RaBIT Enzymatic Hydrolysis

Enzymatic hydrolysis at 7% (w/w) glucan loading was performed using the RaBIT process in 250 mL baffled Erlenmeyer flasks using a 100 gm reaction mass (biomass, water, enzymes, and acid). The first cycle enzyme cocktail was composed of 13 mg protein/g glucan Cellic CTec3 and 11.8 mg protein/g glucan Cellic HTec3 (Novozymes). Hydrolysis was performed for 24 h at 50°C and 250 RPM in a shaking incubator. The pH was maintained at 5.0 using 12.1 M hydrochloric acid. After 24 h, the hydrolysate slurry was centrifuged at 6200 RCF. The hydrolysate supernatant was poured off and the solids were recycled back into the next enzymatic hydrolysis cycle. Enzyme loadings were reduced to 60% and 50% of that used in the first cycle for cycle 2 and cycles 3+, respectively. Ampicillin was loaded at 50 mg/L for experiments involving degradation product analysis and chemical genomic profiling.

2.4 RaBIT Fermentations

Fermentations were performed in 125 mL Erlenmeyer flasks using 50 mL of hydrolysate. Cell pellets prepared by centrifugation of seed cultures were used to inoculate first cycle fermentations. The inoculum size was 10 g/L dry cell weight (DCW) concentration determined from an OD₆₀₀ vs. DCW concentration plot. Initial pH was adjusted to 6.0 using 10 M potassium hydroxide. Fermentations were conducted for 24 h at 32°C and 150 RPM in shaking incubators under microaerobic conditions. After 24 h, the fermentation broth was centrifuged at 3250 RCF and the resulting cell pellet was used to inoculate the next fermentation cycle.

OD was measured 600_{nm} using a Beckman Coulter DU 720 spectrophotometer. Samples were diluted to stay within a raw reading of 0.1-1.

2.5 Chemical Genomic Profiling of RaBIT Hydrolysates

Chemical genomic analysis of these hydrolysate and fermentation broth was performed as described previously using a collection of ~4000 yeast deletion mutants (Piotrowski et al., 2015a; Piotrowski et al., 2015b). 200 μ L cultures with the pooled collection of *S. cerevisiae* deletion mutants were grown in the different RaBIT cycles of hydrolysates or yeast extract (10 g/L), peptone (20 g/L), and dextrose (20 g/L) (YPD) medium in triplicate for 48 h at 30 °C under aerobic conditions. Genomic DNA was extracted from the cells and mutant-specific molecular barcodes were amplified using specially designed multiplex primers as described previously (Piotrowski et al., 2015b). The barcodes were sequenced using an Illumina HiSeq2500 in rapid run mode (Illumina, Inc, San Diego, CA). The average barcode counts for each yeast deletion mutant in the replicate hydrolysates were normalized against the YPD control in order to define sensitivity or resistance of individual strains (chemical genetic interaction score). Strains with low read counts were omitted from analysis. A resistant mutant has a positive interaction score, whereas a negative score indicates a sensitive mutant. The pattern of genetic interaction scores for all mutant strains represents the chemical genomic profile or “biological fingerprint” of a sample (Piotrowski et al., 2015a; Piotrowski et al., 2015b). Correlations of the chemical genomic profiles across cycles were calculated using Spotfire 5.5.0 (Tibco, Boston, MA, USA). The clustergram of the chemical genomic profiles were created in Cluster 3.0 (de Hoon et al., 2004), and visualized in Treeview (v1.1.6r4) (Page, 1996). A Bonferroni-corrected hypergeometric distribution test was used to search for significant enrichment of GO terms among the top 10 sensitive deletion mutants (Boyle et al., 2004).

2.6 Degradation Product Analysis

To determine intracellular degradation product concentrations, cells were harvested by centrifugation at 3250 RCF. The cell pellet was then washed using PBS buffer (8 g/L sodium

chloride, 0.2 g/L potassium chloride, 1.44 g/L sodium phosphate dibasic, and 0.24 g/L potassium phosphate monobasic). After a second centrifugation and removal of supernatant, the cell pellet was flash frozen using a dry ice in an ethanol bath. The cell pellet was then dissolved in a 40:40:20 (v/v/v) mixture of acetonitrile, methanol, and water along with 0.1% formic acid.

Quantification of degradation products was performed by HPLC high resolution/accurate mass spectrometry (RP-HPLC-HRAM MS) and headspace solid-phase microextraction gas chromatography-isotope dilution mass spectrometry (HS-SPME/IDMS). Specific methods are described in Keating et al. (2014) and Serate et al. (2015) having been previously adapted from Sharma et al. (2009).

2.6 Degradation Product Binding Assay

To assess the ability of components of the yeast cell wall to bind degradation products, we prepared a dead yeast cell mass from the GLBRCY128 strain by growing 1 L of Y128 in SynHv2.1 medium for 48h before filtration through a Whatman #1 filter and drying overnight at 60 °C. To test the ability of this dead cell mass or purified chitin (a component of the yeast cell wall) to bind and remove degradation products from hydrolysates, we incubated 1 mL of AFEX corn stover hydrolysate with 100 mg of either the dried yeast cells or purified chitin at 30 °C for 24h. The mixtures were then centrifuged and the hydrolysate supernatant removed from the cell and chitin pellets. Quantification of degradation products was conducted as described above.

2.7 Synthetic Hydrolysate

The composition of synthetic hydrolysate was described elsewhere (Tang et al., 2015). Sugar and nutrient concentrations in the original synthetic hydrolysate were based on 6% glucan

loading AFEX corn stover hydrolysate. These concentrations were multiplied by 7/6ths to mimic the 7% glucan loading AFEX corn stover hydrolysate used in this Chapter. Degradation product concentrations were taken from the analysis presented in this work. The concentration of degradation products normally found in 7% glucan loading AFEX corn stover hydrolysate was designated as 1x degradation products. Synthetic hydrolysate designated with 0x contained no degradation products. Synthetic hydrolysate designated with 0.5x and 2x degradation products contained half and double the normal concentration of degradation products, respectively.

2.8 HPLC Analysis

Glucose, xylose and ethanol concentrations were analyzed by HPLC using a Biorad Aminex HPX-87H column. Column temperature was maintained at 50 °C. Mobile phase (5 mM H₂SO₄) flow rate was 0.6 mL/min.

3. Results and Discussion

3.1 Glucan Loading Variation

The effect of degradation products on RaBIT fermentations was tested by preparing hydrolysates with different glucan loadings (4.5%, 6.0%, 7.5%, and 9.0%), thereby creating hydrolysates varying in degradation product, sugar, and nutrient concentrations. To minimize the effects of any sugar concentration difference, glucose and xylose were added based on initial HPLC data to final concentrations of ~60 g/L glucose and ~30 g/L xylose. No nutrients were added to address varying nutrient concentrations.

The results in Fig. 1 suggest that degradation products strongly affected xylose consumption both before and after cell recycle during RaBIT fermentations. Xylose

consumption during specific cycles (cycles 1, 2, and 3) showed a linear correlation ($R^2 > 0.96$) between glucan loading and percent xylose consumption (Fig. S1). This was not the case for glucan loading and xylose consumption change between cycles (Fig. S1). This observation suggests that the potential xylose consumption within a cycle depends on degradation product concentrations, while the observed decrease in xylose consumption decrease between cycles is the product of a different mechanism, possibly degradation product accumulation within cells. These conclusions assumed that glucan loading can be directly correlated to degradation product concentration and that varying nutrient level did not significantly affect the results.

3.2 Chemical-genomic profiling of RaBIT Hydrolysates

To further understand the RaBIT enzymatic hydrolysis process, variability in hydrolysate composition between cycles was tested. Variation in hydrolysate composition was a potential cause for the decreased xylose consumption upon cell recycle during RaBIT fermentations. Comparing work of Jin et al. (2012) and Sarks et al. (2014) shows that hydrolysate variation is not the sole reason for decreased xylose consumption, but it could not be ruled out as a contributing cause. To answer whether hydrolysate variation between cycles occurred, chemical-genomic profiling was performed using a genome-wide deletion mutant collection of *S. cerevisiae*. Chemical-genomics is the study of chemical compound interactions with specific genes within an organism. This approach determined whether hydrolysate variability existed in RaBIT enzymatic hydrolysis using a biological “sensor” (individual gene mutants) to create a genome-wide, biological “fingerprint” (Serate et al., 2015).

Enzymatic hydrolysis was performed for 5 cycles using the RaBIT process. Triplicate chemical genomic profiles of the 5 RaBIT hydrolysates revealed a striking similarity of response across all gene deletions; the resulting clustergram of gene deletion response is shown in Fig. 2c.

Selected sensitive and resistant genes are highlighted in Fig. 2d and Fig. 2e, respectively.

Among the top 10 sensitive mutants across all hydrolysates, we found significant enrichment ($p=0.002$) for “Tryptophan biosynthetic process” (GO:0000162), driven by mutants of *TRP3* and *TRP4*. Interestingly, the mutant of *YIL015C* (aka *YIL014C-A*), was resistant across all conditions. *YIL015C-A* is a gene of unknown function, whose deletion is known to increase stress resistance (North et al., 2012). Mutants of *SPH1* were also resistant. This gene encodes a GPI-anchored cell wall protein involved in weak acid resistance, and its expression is induced under stress and during the diauxic shift.

Quantitative analysis of the clustergram data is shown in Fig. 2b. Overall, there appears to be no significant difference between hydrolysis cycles. When comparing all hydrolysate cycles to each other, the average correlation value (R) was 0.90 (Fig. 2b). Fig. 2a shows an example (comparison of cycles 1 and 5) of how correlation between cycles was performed. This eliminates hydrolysate variability as a cause of reduced xylose consumption upon cell recycle.

3.3 Hydrolysate Degradation Products Quantification

Degradation product concentrations both before and after fermentation were quantified. Results from the chemical-genomic study suggested that degradation product concentrations do not change between cycles. Quantifying the degradation compounds would confirm this result, and also give insight into the dynamics of degradation products concentrations during the RaBIT process. Fig. 1 shows that increased glucan loading, and therefore increased degradation product concentration, reduced the ability of the yeast cells to consume xylose. The subsequent decrease in xylose consumption by the recycled yeast populations could be caused by reduced cellular detoxification capability or by accumulation of degradation products within the cells.

The results of this study are shown in Table 1. In general, there was no significant difference in degradation product concentrations when comparing hydrolysate produced during different RaBIT enzymatic hydrolysis cycles (Fig. 3; Table 1); the same is true when comparing the fermentation broth after different RaBIT fermentation cycles (Fig. 3; Table 1). These results are significant for two reasons. First, they eliminate the variation of degradation products between hydrolysis cycles as a possible cause for fermentation performance decrease in recycled cells. Second, they show that the yeast population retains the potential to detoxify hydrolysate when recycled (Fig. 3). Detoxification potential was correlated to aldehyde oxidation/reduction; *S. cerevisiae* strains can reduce aldehydes into alcohols or oxidize them into acids, thereby decreasing inhibition (Liu, 2011; Klinker et al., 2004). The results did not show whether detoxification rates decreased during successive cycles, which could be significant to RaBIT fermentation performance.

This study was also performed to determine if any degradation products were disappearing from the hydrolysate during fermentation or accumulating within the cell. Vanillin was the only compound disappearing that could not be accounted for in a reduced or oxidized form due to detoxification and was outside the standard deviation. To our knowledge, *S. cerevisiae* strains are not known to metabolize vanillin. However, there are reports which demonstrated vanillin production using genetic engineered yeast (Brochado et al. 2010). Other fungi such as *Coniochaeta ligniaria* (Nichols et al. 2008) are capable of metabolizing vanillin. Other degradation products could have accumulated within the cells but at concentrations below detection.

3.4 Degradation Product Accumulation by Cells

The question of whether degradation products were accumulated by the cell was answered next. Cells were harvested after cycles 1, 3, and 5 by centrifugation. The cells were then washed with PBS before submersion in an ethanol and dry ice bath to flash freeze the cells before storage at -80°C. The cells were then thawed before dissolved in a mixture of acetonitrile, methanol, water, and formic acid. The resulting solution was analyzed using RP-HPLC-HRAM MS and HS-SPME/IDMS. The results in Table 2 show that some specific degradation products associated with the cell and some did not. This experiment could not elucidate whether accumulation was occurring in the cytosol, cell wall, membranes, mitochondria, etc. Different acids, alcohols, and amides were found to either accumulate or not accumulate indicating specific molecular features aren't required for accumulation. To attempt to explain the results, acid dissociation constants were compared for each accumulating compound. If a compound has an acid dissociation constant between 5.0-6.0 (fermentation pH) and 7.0 (intracellular pH), a compound could diffuse into the cell, protonate, and then not be able to diffuse back out. No correlation of acid dissociation constants with intracellular accumulation was found. An experiment was performed to see if degradation products bind to cellular components. The results in Table 3 showed that all degradation products bind to either chitin or yeast lysate. The accumulating degradation products did not show stronger binding on average compared to non-accumulating degradation products. Previous work by Piotrowski et al. (2015a) showed that p-coumaric acid, a diferulate, binds and targets beta-1,3-glucan in fungal cell walls.

Fig. 4 shows a potential correlation between increasing intracellular degradation product concentrations and the final xylose concentration. These results, while strongly suggestive of this relationship, do not prove conclusively that accumulation of degradation products causes

decreasing xylose consumption upon cell recycle. To better understand this potential relationship, experiments using synthetic hydrolysate were performed.

3.5 Synthetic Hydrolysate Experiments

Synthetic hydrolysate was used to further investigate the effect of degradation products on RaBIT fermentations. The original synthetic hydrolysate recipe was developed for AFEX corn stover as outlined in Tang et al. (2015). Ongoing work on synthetic hydrolysate yielded the recipe used for these experiments (Tables S1 and S2).

The first synthetic hydrolysate experiment compared different degradation product concentrations. For this experiment, degradation product concentrations of 0x, 0.5x, 1x, and 2x in relation to 7% glucan loading AFEX hydrolysate concentrations were added to the synthetic hydrolysate. The degradation product concentrations for 1x are shown in Table S2.

Concentrations were based off the analysis performed in this work on 7% glucan loading AFEX hydrolysate. All degradation products previously analyzed in this work were not included due to availability. The results in Fig. 5 showed that eliminating degradation products (0x) in hydrolysate enables complete xylose consumption and no cell performance decrease during RaBIT fermentations. When degradation product concentrations were increased to half the normal concentration (0.5x), residual xylose doubled from ~0.6 g/L to ~ 1.2 g/L. With the normal level of degradation products (1x), xylose consumption began to decrease upon cell recycle. The results for synthetic hydrolysate with 1x degradation products (Fig. 5) were also very comparable to RaBIT fermentations in AFEX hydrolysate (Fig. 6). The 1x degradation products synthetic hydrolysate did show better initial xylose consumption when compared to AFEX hydrolysate (2.71 g/L residual xylose vs 5.31 g/L residual xylose, respectively). However, the performance decrease after 5 fermentation cycles was more pronounced with 1x

degradation product synthetic hydrolysate compared to AFEX hydrolysate (9.47 g/L residual xylose vs 8.28 g/L residual xylose, respectively). Missing nutrients in the synthetic hydrolysate recipe may have a greater impact on fermentation performance than missing degradation products. Increasing degradation product concentrations to 2x in synthetic hydrolysate, caused less than 50% xylose consumption in cycles 2 through 4 (Fig. 5).

Further experiments are needed to completely elucidate the effects of degradation products in RaBIT fermentations. It appears likely that degradation product accumulation by cells causes decreased xylose consumption upon cell recycle. Determining how and how much of each major degradation product accumulates should be studied. Fluorescent tagging of degradation products could help determine where products are accumulated by the cell.

Conclusions

Degradation product concentrations between RaBIT cycles do not vary between RaBIT enzymatic hydrolysis cycles. Chemical genomics was used to verify that the biological fingerprint between cycles was also unchanged. Quantification of degradation products after fermentation showed that aldehyde detoxification by the cell population did not decrease with increased RaBIT fermentation cycles. Further work showed that specific degradation products are accumulated by the cell during RaBIT fermentation cycles. Synthetic hydrolysate experiments showed that the presence of degradation products is the primary cause of decreased xylose consumption during RaBIT fermentations.

Supplementary information is provided detailing correlations, mol balance calculations around aldehyde detoxification, and the complete synthetic hydrolysate recipe.

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References

1. Balan, V., 2014. Current challenges in commercially producing biofuels from lignocellulosic biomass. *ISRN Biotechnol.* 2014
2. Balan, V., Bals, B., Chundawat, S.P.S., Marshall, D., Dale, B.E., 2009. Lignocellulosic Biomass Pretreatment Using AFEX, in: Mielenz, J.R. (Ed.), *Biofuels: Methods and Protoc.* Humana Press, Totowa, NJ, pp. 61-67
3. Balan, V., Sousa, L., 2009. Enzymatic digestibility and pretreatment degradation products of AFEX-treated hardwoods (*Populus nigra*). *Biotechnol. Prog.* 25, 365-375
4. Boyle E. I., Weng S., Gollub J., Jin H., Botstein D., Cherry J. M., Gerlock G., 2004. GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinform.* 20(18):3710-3715
5. Chundawat, S., Vismeh, R., 2010. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresour. Technol* 101, 8429-38

6. da Silva-Filho, E.A., Brito dos Santos, S.K., Resende, A.D.M., De Moraes, J.O.F., De Moraes, M.A., Ardaillon Simões, D., 2005. Yeast population dynamics of industrial fuel-ethanol fermentation process assessed by PCR-fingerprinting. *Antonie van Leeuwenhoek* 88, 12-23
7. de Hoon MJL, Imoto S, Nolan J, Miyano S., 2004. Open source clustering software. *Bioinforma.* 20(9):1453-4
8. Du, B., Sharma, L.N., Becker, C., Chen, S.-F., Mowery, R. a, Van Walsum, G.P., Chambliss, C.K., 2010. Effect of varying feedstock-pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates. *Biotechnol. and Bioeng.* 107, 430-40
9. Ho C. H., Piotrowski J. S., Dixon S. J., Baryshnikova A., Costanzo M., Boone C., 2011. Combining functional genomics and chemical biology to identify targets of bioactive compounds. *Curr. Opin. in Chem. Biol.* 15:66-78
10. Huuskonen, A., Markkula, T., Vidgren, V., Lima, L., Mulder, L., Geurts, W., Walsh, M., Londesborough, J., 2010. Selection from industrial lager yeast strains of variants with improved fermentation performance in very-high-gravity worts. *Appl. and Environ. Microbiol.* 76, 1563-73
11. Jin, M., Gunawan, C., Uppugundla, N., Balan, V., Dale, B.E., 2012. A novel integrated biological process for cellulosic ethanol production featuring high ethanol productivity, enzyme recycling and yeast cells reuse. *Energy & Environ. Sci.* 5, 7168
12. Keating, D.H., Zhang, Y., Ong, I.M., McIlwain, S., Morales, E.H., Grass, J. a, Tremaine, M., Bothfeld, W., Higbee, A., Ulbrich, A., Balloon, A.J., Westphall, M.S., Aldrich, J., Lipton, M.S.,

13. Kim, J., Moskvina, O. V., Bukhman, Y. V., Coon, J.J., Kiley, P.J., Bates, D.M., Landick, R.,
2014. Aromatic inhibitors derived from ammonia-pretreated lignocellulose hinder bacterial
ethanogenesis by activating regulatory circuits controlling inhibitor efflux and detoxification.
Front. in Microbiol. 5, 402
14. Klinke, H.B., Thomsen, A. B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and
bacteria by degradation products produced during pre-treatment of biomass. Appl. and Environ.
Microbiol. and Biotechnol. 66, 10-26
15. Liu, Z. L., 2011. Molecular mechanisms of yeast tolerance and in situ detoxification of
lignocellulose hydrolysates. Appl. Microbiol. Biotechnol. 90:809-825
16. North, M., Steffen, J., Loguinov, A. V., Zimmerman, G. R., Vulpe, C. D., Eide, D. J., 2012.
Genome-Wide Functional Profiling Identifies Genes and Processes Important for Zinc-Limited
Growth of *Saccharomyces cerevisiae*. PLoS Genet. 8(6)
17. Page RD., 1996. TreeView: an application to display phylogenetic trees on personal
computers. Comput. Appl. Biosci. 12(4), 357-8
18. Parreiras L. S., Breuer R. J., Avanesi Narasimha, R., Higbee A. J., La Reau A., Tremaine M.,
et al. 2014. Engineering and Two-Stage Evolution of a Lignocellulosic Hydrolysate-Tolerant
Saccharomyces cerevisiae Strain for Anaerobic Fermentation of Xylose from AFEX Pretreated
Corn Stover. 9(9)
19. Piotrowski, J.S., Okada, H., Lu, F., Li, S.C., Hinchman, L., Ranjan, A., Smith, D.L., Higbee,
A.J., Ulbrich, A., Coon, J.J., Deshpande, R., Bukhman, Y. V., McIlwain, S., Ong, I.M., Myers,

C.L., Boone, C., Landick, R., Ralph, J., Kabbage, M., Ohya, Y., 2015a. Plant-derived antifungal agent poacic acid targets β -1,3-glucan. *Proc. of the Natl. Acad. of Sci.* 112(12), 1490-97

20. Piotrowski JS, Simpkins SW, Li SC, Deshpande R, McIlwain SJ, Ong IM, et al. (2015b) Chemical Genomic Profiling via Barcode Sequencing to Predict Compound Mode of Action. *Chemical Biology. Methods and Protoc.* 1260, 299-318

21. 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. for Biofuels* 7:73

22. Serate J., Xie D., Pohlmann E., Donald Jr. C., Shabani M.; Hinchman L., Higbee A., Mcgee M., La Reau A., Klinger G., Li S., Myers C. L., Boone C., Bates D., Cavalier D., Eilert D., Oates L. G., Sanford G., Sato T. K., Dale B. E., Landick R., Piotrowski J. S., Garlock-Ong R., Zhang Y., 2015. Controlling microbial contamination during hydrolysis of AFEX-pretreated corn stover and switchgrass: effects on hydrolysate composition, microbial response and fermentation. *Biotechnol. for Biofuels.* 8:180

23. L. N. Sharma, C. Becker and C. K. Chambliss., 2009. Analytical characterization of fermentation inhibitors in biomass pretreatment samples using liquid chromatography, UV-visible spectroscopy, and tandem mass spectrometry. *Methods Mol Biol.* 581, 125-43

24. Sluiter, J.B., Ruiz, R.O., Scarlata, C.J., Sluiter, A.D., Templeton, D.W., 2010. Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods. *J. of Agric. and Food Chem.* 58, 9043-53

25. Tang, X., Da Costa Sousa, L., Jin, M., Chundawat, S.P., Chambliss, C.K., Lau, M.W., Xiao, Z., Dale, B.E., Balan, V., 2015. Designer synthetic media for studying microbial-catalyzed biofuel production. *Biotechnol. for Biofuels* 8(1)
26. Wheals, A.E., Basso, L.C., Alves, D.M., Amorim, H. V, 1999. Fuel ethanol after 25 years. *Trends in Biotechnol.* 17, 482-7

Fig. 1 Glucan loading effect on RaBIT fermentations. Initial glucose and xylose concentrations were 59.2 ± 1.2 g/L and 30.5 ± 1.0 g/L, respectively. Final concentrations for glucose (blue), xylose (orange), and ethanol (green) are shown after 24 h fermentation along with OD (purple triangles).

Fig. 2 Chemical genomic profiling of RaBIT hydrolysates across 5 cycles. Correlation of the chemical genomic profile of sensitive and resistant yeast mutants between cycle 1 and cycle 5 hydrolysates was highly similar, with a correlation of $R=0.92$ (A). The chemical genomic profiles were highly correlated across all cycles (B). A clustergram showing the entire chemical genomic profile of sensitive (blue) and resistant (yellow) yeast mutants across all hydrolysates (C). Examples of common highly sensitive (D) and resistant (E) yeast mutants across the 5 RaBIT cycles.

Fig. 3 Key degradation product levels in hydrolysate before and after RaBIT fermentation.

Fig. 4 Degradation product accumulation and fermentation results for multiple RaBIT cycles. Final glucose (blue), xylose (orange), and ethanol (green) concentrations are shown after three 23 h RaBIT fermentations. Concentration of accumulating degradation products in the cell pellet are also shown (red circles).

Fig. 5 Synthetic hydrolysate experiments for varying concentration of degradation products. Concentration multipliers are relative to degradation product concentrations in 7% glucan loading AFEX hydrolysate. Concentrations for xylose (orange) and ethanol (green) along with OD (purple triangles) are reported. Original glucose and xylose concentrations were 60 g/L and 34 g/L, respectively. All glucose was consumed during each cycle.

Fig. 6 RaBIT fermentation using 7% glucan loading AFEX hydrolysate. Concentrations for glucose (blue), xylose (orange) and ethanol (green) along with OD (purple triangles) are reported. Original glucose and xylose concentrations were 59 g/L and 32 g/L, respectively

Table 1 Degradation product levels in hydrolysate before and after fermentation

	Pre-fermentation (μmols/L)					Post-fermentation (μmols/L)				
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Phenolic Acids										
4-Hydroxybenzoic Acid	50.65 ± 5.99	53.96 ± 2.49	51.02 ± 1.64	40.89 ± 10.67	50.56 ± 4.39	72.21 ± 2.78	104.39 ± 4.16	107.50 ± 6.17	100.56 ± 5.08	99.50 ± 3.51
3-Hydroxybenzoic Acid	0.41 ± 0.04	0.45 ± 0.01	0.44 ± 0.03	0.43 ± 0.01	0.44 ± 0.05	0.39 ± 0.03	0.45 ± 0.01	0.45 ± 0.03	0.48 ± 0.02	0.53 ± 0.05
3,4-Dihydroxybenzoic Acid	3.03 ± 0.44	3.36 ± 0.15	3.31 ± 0.10	3.35 ± 0.19	3.62 ± 0.44	3.14 ± 0.05	3.72 ± 0.24	3.63 ± 0.15	3.75 ± 0.18	4.11 ± 0.26
2,3-Dihydroxybenzoic Acid			Not Detected					Not Detected		
Syringic Acid	7.73 ± 0.56	8.08 ± 0.30	7.76 ± 0.30	7.35 ± 0.17	7.75 ± 0.64	7.51 ± 0.39	8.08 ± 0.35	7.82 ± 0.48	7.62 ± 0.25	7.67 ± 0.44
Coumaric Acid	1768.72 ± 144.38	1810.45 ± 199.76	1552.14 ± 78.03	1509.55 ± 119.36	1389.91 ± 8.54	1634.03 ± 297.75	1765.65 ± 228.50	1452.29 ± 76.26	1534.41 ± 32.17	1421.67 ± 96.63
Ferulic Acid	48.24 ± 1.23	53.27 ± 2.97	49.14 ± 2.03	43.00 ± 1.43	44.21 ± 3.96	47.17 ± 3.70	53.41 ± 2.94	50.23 ± 3.60	59.68 ± 20.12	45.97 ± 2.19
Sinapic Acid	0.70 ± 0.19	0.54 ± 0.02	0.45 ± 0.03	0.41 ± 0.00	0.40 ± 0.02	1.45 ± 0.11	1.09 ± 0.12	1.07 ± 0.08	1.04 ± 0.14	1.05 ± 0.03
Vanillic Acid	33.44 ± 3.47	34.49 ± 0.31	32.73 ± 1.29	31.21 ± 1.05	32.81 ± 2.48	33.95 ± 1.44	44.19 ± 1.19	43.21 ± 2.01	39.69 ± 1.65	38.99 ± 1.57
DiFA 8-5-O	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.04	0.15 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
DiFA 8-8-C	0.16 ± 0.01	0.18 ± 0.00	0.18 ± 0.00	0.16 ± 0.00	0.15 ± 0.01	0.19 ± 0.00	0.22 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
DiFA 8-O-4	0.59 ± 0.02	0.64 ± 0.02	0.60 ± 0.01	0.55 ± 0.03	0.56 ± 0.02	0.58 ± 0.02	0.70 ± 0.02	0.66 ± 0.03	0.60 ± 0.04	0.59 ± 0.03
DiFA 8-8-O	0.64 ± 0.02	0.71 ± 0.02	0.76 ± 0.03	0.76 ± 0.04	0.82 ± 0.04	1.00 ± 0.02	1.11 ± 0.07	1.04 ± 0.04	1.01 ± 0.04	1.06 ± 0.07
DiFA 8-8-THF			<0.1					<0.1		
DiFA 5-5			<0.1					<0.1		
DiFA 8-5-C			<0.1					<0.1		
DiFA 8-5-DC			<0.1					<0.1		
Phenolic Amides										
4-Hydroxybenzamide	27.54 ± 3.26	30.90 ± 1.68	28.71 ± 1.99	27.43 ± 1.17	28.34 ± 3.38	27.01 ± 2.37	31.82 ± 1.86	30.66 ± 2.23	28.71 ± 2.25	30.16 ± 1.70
Vanillamide	121.36 ± 5.72	138.95 ± 4.09	128.59 ± 5.99	122.29 ± 3.42	127.89 ± 11.19	121.51 ± 6.41	136.49 ± 6.90	132.16 ± 9.41	127.81 ± 8.06	130.11 ± 7.47
Coumaroyl Amide	6167.75 ± 500.83	6145.77 ± 570.72	5596.44 ± 236.19	5697.35 ± 472.44	5637.09 ± 222.62	5772.61 ± 980.13	5974.08 ± 534.08	5448.49 ± 154.40	5441.64 ± 133.66	5354.84 ± 213.54
Feruloyl Amide	2617.75 ± 171.48	2698.47 ± 293.46	2456.97 ± 124.99	2525.05 ± 29.90	2416.35 ± 32.45	2540.59 ± 414.23	2649.23 ± 206.50	2281.34 ± 214.45	2388.26 ± 89.53	2385.31 ± 34.96
Syringamide	34.74 ± 3.73	37.40 ± 1.28	33.52 ± 2.14	32.58 ± 1.26	33.93 ± 3.50	38.72 ± 3.30	41.19 ± 2.82	37.52 ± 3.16	35.29 ± 2.70	35.84 ± 2.35
Benzamide			Not Detected					Not Detected		
Phenolic Alcohols										
4-Hydroxybenzyl Alcohol	6.62 ± 1.66	5.86 ± 1.36	8.56 ± 0.00	6.23 ± 0.92	4.90 ± 0.14	94.92 ± 5.35	84.56 ± 8.18	81.43 ± 5.57	84.96 ± 3.69	97.89 ± 6.40
Vanillyl Alcohol			Not Detected			89.60 ± 8.29	97.04 ± 5.58	91.71 ± 7.54	93.96 ± 11.16	91.55 ± 5.69
Phenolic Aldehydes										
Coniferaldehyde			<0.25					<0.25		
Syringaldehyde	4.52 ± 0.58	5.98 ± 0.37	5.61 ± 0.25	5.51 ± 0.12	5.80 ± 0.67	0.93 ± 0.08	0.96 ± 0.02	0.87 ± 0.02	0.70 ± 0.03	0.63 ± 0.02
4-Hydroxybenzaldehyde	104.04 ± 6.18	128.98 ± 3.38	122.21 ± 7.08	122.76 ± 5.05	128.80 ± 16.54			Not Detected		
Vanillin	198.92 ± 1.61	198.32 ± 5.43	196.71 ± 7.74	199.92 ± 4.93	200.90 ± 4.28	7.11 ± 0.03	8.16 ± 0.40	7.40 ± 0.32	6.71 ± 0.20	6.11 ± 0.28
Phenolics										
4-Hydroxyacetophenone	2.27 ± 0.21	2.51 ± 0.08	2.38 ± 0.10	2.30 ± 0.10	2.40 ± 0.24	2.13 ± 0.11	2.46 ± 0.08	2.36 ± 0.14	2.26 ± 0.08	2.37 ± 0.11
Acetovanillone	4.19 ± 0.29	4.34 ± 0.19	4.08 ± 0.27	3.91 ± 0.07	4.11 ± 0.49	3.81 ± 0.24	4.35 ± 0.11	4.21 ± 0.23	3.94 ± 0.17	4.15 ± 0.26
Acetosyringone	5.72 ± 0.61	6.07 ± 0.41	5.20 ± 0.16	5.29 ± 0.43	5.31 ± 0.42	5.35 ± 0.43	6.31 ± 0.16	5.62 ± 0.95	5.15 ± 0.46	5.74 ± 0.29
Other										
5-Hydroxymethylfurfural	0.63 ± 0.02	0.72 ± 0.04	0.73 ± 0.08	0.66 ± 0.09	0.84 ± 0.10	0.41 ± 0.04	0.44 ± 0.03	0.44 ± 0.04	0.43 ± 0.02	0.43 ± 0.05
Azelaic Acid	30.66 ± 1.92	32.16 ± 0.54	32.16 ± 0.56	30.29 ± 0.65	30.85 ± 1.65	27.55 ± 1.31	31.44 ± 0.85	31.42 ± 1.02	29.25 ± 0.68	30.21 ± 0.95

Abbreviations: DiFA (Diferulic Acid); THF (tetrahydrofuran form); DC (decarboxylated form); C (carboxylated form)

Table 2 Degradation product concentrations in post-fermentation cell pellet

	Cycle 1			Cycle 3			Cycle 5		
	mmol/gm DCW								
<i>Non-accumulating degradation products</i>									
Vanillin	2.36	±	0.06	2.00	±	0.13	2.37	±	0.07
4-Hydroxyacetophenone	1.49	±	0.04	2.60	±	0.43	1.72	±	0.23
Acetovanillone	3.64	±	0.18	4.98	±	0.64	2.89	±	0.37
Hydroxymethylfurfural	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Gamma-Valerolactone	0.05	±	0.05	0.03	±	0.04	0.00	±	0.00
Benzoic Acid	52.42	±	7.48	69.04	±	10.52	49.70	±	7.54
Ferulic Acid	65.12	±	0.92	68.59	±	10.90	45.03	±	1.88
Vanillic Acid	57.53	±	1.32	58.69	±	7.43	38.32	±	1.56
3-Hydroxybenzoic Acid	0.55	±	0.02	0.65	±	0.10	0.41	±	0.06
4-Hydroxybenzoic Acid	91.85	±	2.02	118.15	±	15.47	113.32	±	2.51
Sinapic Acid	2.43	±	0.07	2.30	±	0.66	1.74	±	0.12
Syringic Acid	8.49	±	0.44	8.06	±	1.30	6.71	±	0.32
Azelic Acid	73.74	±	0.79	59.81	±	7.96	43.71	±	1.22
4-Hydroxybenzaldehyde	0.61	±	0.00	0.46	±	0.05	0.29	±	0.03
Syringaldehyde	0.54	±	0.01	0.39	±	0.02	0.18	±	0.00
4-Hydroxybenzamide	21.03	±	0.92	21.8	±	2.77	20.70	±	0.69
<i>Accumulating degradation products</i>									
Vanillyl Alcohol	22.77	±	0.49	41.54	±	10.27	66.01	±	12.76
4-Hydroxybenzyl Alcohol	23.46	±	1.25	25.59	±	6.63	54.33	±	8.41
3,4-Dihydroxybenzoic Acid	4.16	±	0.42	4.81	±	1.13	8.26	±	0.32
Benzamide	0.13	±	0.02	0.48	±	0.22	0.71	±	0.13
Syringamide	21.02	±	1.74	26.63	±	5.93	30.41	±	3.49
Vanillamide	68.32	±	3.76	80.37	±	17.22	101.72	±	0.59
Ferulylamide	787.27	±	62.84	846.97	±	194.85	Over Detection Limit		

Table 3 Percent change of degradation products concentration in hydrolysate after exposure to chitin or yeast lysate

	Chitin			Yeast Lysate		
4-Hydroxybenzamide	-7.38	±	0.01	-27.38	±	0.02
Hydroxymethylfurfural	2.25	±	0.05	-13.51	±	0.08
4-Hydroxybenzyl Alcohol	7.50	±	0.14	-10.76	±	0.06
3,4-Dihydroxybenzoic Acid	-14.28	±	0.22	19.16	±	0.00
Vanillamide	-11.42	±	0.07	-11.75	±	0.10
Benzamide	-2.88	±	0.01	-10.99	±	0.02
Vanillyl Alcohol	-5.44	±	0.02	-13.41	±	0.04
4-Hydroxybenzoic acid	-5.42	±	0.01	-9.71	±	0.00
Syringamide	-8.32	±	0.02	-18.27	±	0.01
Coumaramide	-3.84	±	0.01	-8.47	±	0.04
4-Hydroxybenzaldehyde	-7.77	±	0.02	-17.85	±	0.02
3-Hydroxybenzoic Acid	-8.99	±	0.02	-12.18	±	0.02
Vanillic Acid	-9.09	±	0.04	-19.38	±	0.01
Ferulamide	-4.43	±	0.02	-6.61	±	0.05
Syringic Acid	-7.67	±	0.01	-18.84	±	0.01
4-Hydroxyacetophenone	-5.80	±	0.00	-17.17	±	0.01
Vanillin	-5.35	±	0.03	-6.74	±	0.01
Coumaric Acid	-2.67	±	0.02	-7.90	±	0.02
Syringalddehyde	-4.40	±	0.00	-14.91	±	0.02
Acetovanillone	-3.70	±	0.02	-13.24	±	0.02
Ferulic Acid	-14.81	±	0.08	-22.85	±	0.04
Acetosyringone	-8.36	±	0.05	-13.96	±	0.01
Benzoic acid	-3.61	±	0.06	-9.06	±	0.03
Sinapic acid	-11.40	±	0.03	-13.80	±	0.07
Azelic acid	-2.47	±	0.01	-11.36	±	0.02
DiFA 8-O-4	-3.55	±	0.04	-7.77	±	0.01

Figure 1

Fig. 1

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5

Figure 6

Fig. 6