Bioresource Technology 100 (2009) 3532–3539

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Fermentation of soybean hulls to ethanol while preserving protein value

Jonathan R. Mielenz [a,b,*], John S. Bardsley [a,c], Charles E. Wyman [a,d]

[a] Thayer School of Engineering, Dartmouth College, Hanover, NH 03755, United States
[b] BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States
[c] Mascoma Corporation, Lebanon, NH 03766, United States
[d] Department of Chemical and Environmental Engineering, University of California Riverside, Riverside, CA 92507, United States

ARTICLE INFO

Article history:
Received 12 August 2008
Received in revised form 11 February 2009
Accepted 11 February 2009
Available online 27 March 2009

Keywords:
Ethanol
SSF
Biomass
Agricultural residue
Animal feed

ABSTRACT

Soybean hulls were evaluated as a resource for production of ethanol by the simultaneous saccharification and fermentation (SSF) process, and no pretreatment of the hulls was found to be needed to realize high ethanol yields with *Saccharomyces cerevisiae* D_5A. The impact of cellulase, β-glucosidase and pectinase dosages were determined at a 15% biomass loading, and ethanol concentrations of 25–30 g/L were routinely obtained, while under these conditions corn stover, wheat straw, and switchgrass produced 3–4 times lower ethanol yields. Removal of carbohydrates also concentrated the hull protein to over 25% w/w from the original roughly 10%. Analysis of the soybean hulls before and after fermentation showed similar amino acid profiles including an increase in the essential amino acids lysine and threonine in the residues. Thus, eliminating pretreatment should assure that the protein in the hulls is preserved, and conversion of the carbohydrates to ethanol with high yields produces a more concentrated and valuable co-product in addition to ethanol. The resulting upgraded feed product from soybean hulls would likely to be acceptable to monogastric as well as bovine livestock.

1. Introduction

Agricultural residues such as corn stover and wheat straw are important starting materials (feedstocks) for development of a cellulosic biomass ethanol industry because they are inexpensive sources of complex carbohydrates that can be used to produce ethanol. To obtain high bioconversion levels of ethanol from these biomass sources requires a thermochemical process, called pretreatment, prior to enzymatic hydrolysis of complex carbohydrates to simpler sugars that can be fermented to ethanol (Mielenz, 2001). Numerous pretreatment processes have been evaluated that range from use of just hot water and steam to addition of alkali, solvents, and dilute acid (Hsu, 1996; Wyman et al., 2005; Mosier et al., 2005). For example, dilute sulfuric acid pretreatment successfully hydrolyzes much of the hemicellulose to monomeric sugars and short chained sugar oligomers (Lee et al., 1999). After pretreatment, neutralization, and conditioning, fermentation can be initiated by addition of cellulase and β-glucosidase enzymes plus a fermentation microorganism in a process called simultaneous saccharification and fermentation (SSF) (Gauss et al., 1976; Philippidis et al., 1993; Olofsson et al., 2008). Typically commercial enzymes are used to complete the depolymerization process to simple sugars which are fermented to ethanol by microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, or *Klebsiella oxytoca*, some of which have been genetically engineered to convert all or most of the sugars to ethanol (Karhumaa et al., 2006; Sedlak and Ho, 2004; Kuyper et al., 2005; Asghari et al., 1996; Mohagheghi et al., 2004; Doran et al., 1994). Ethanol is purified by removal from the solids left after fermentation by distillation/dehydration of the fermentation broth or beer.

Soybean hulls (SBH) are an agricultural residue produced during processing of soybeans. The hard shell or hull of the soybean is removed mechanically and accounts for about 5–8% of the ~95 million tons ($\sim 3.2 \times 10^9$ bushels) per year soybean crop in the United States (USDA 2006: www.nass.usda.gov). Considered a waste product from the production of soy oil, soybean meal, and other high-protein products, soybean hulls are typically sold as is or as compressed pellets and fed to cows (Blasi et al., 2000). Conversion of the carbohydrates in soybean hulls to ethanol could result in significant quantities of ethanol. However since there is a viable valuable market for soybean hulls as animal feed, little attention to ethanol production has resulted. Recently *Candida guilliermondii* was used to produce ethanol or xylitol from an acid hydrolysate of soybean hulls (Schirmer-michel et al., 2008) . Similarly, Corredor et al. (2008) studied dilute acid and modified steam-explosion pretreatments of soybean hulls, but did not produce ethanol. Alternately, we report here an evaluation of soybean hulls as a

* Corresponding author. Present address: BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831, United States. Tel.: +1 865 576 8522; fax: +1 865 574 6442.
E-mail address: mielenzjr@ornl.gov (J.R. Mielenz).

0960-8524/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.biortech.2009.02.044

possible fermentation feedstock for production of ethanol in a high-density SSF process, in the absence of any thermochemical pretreatment, thus producing ethanol while permitting preservation of the soybean hull protein.

2. Methods

2.1. Organisms and enzymes

S. cerevisiae D_5A was provided by the National Renewable Energy Laboratory (NREL) and is available from ATCC as No. 200062, as was *Z. mobilis* 8b (Mohagheghi et al., 2004) through a materials transfer agreement from NREL to Dartmouth College. *E. coli* KO11 ATCC 55,124 (Asghari et al., 1996) was obtained from the American Type Culture Collection, Manassas, VA. Enzymes used were cellulase Spezyme CP® (Genencor-Danisco, Beloit, WI) at 23 or 59 filter paper units (FPU)/mL, depending upon the lot; β-glucosidase (Sigma–Aldrich Corp., St. Louis, MO, Novozyme 188®, C6105) at 340 U/mL; pectinase (Sigma–Aldrich Corp., St. Louis, MO, P2611) at 28,482 U/mL, and hemicellulase (Sigma–Aldrich Corp., St. Louis, MO, H2125) at 0.3–3 U/mg. Enzyme units are defined by the manufacturer, which include filter paper units for cellulases (Zhang et al., 2006). Enzymes are added as units per gram soybean hulls (SBH) cellulose on a dry basis (DB).

2.2. Small scale fermentations

Soybean hulls were obtained in pellet form from Ag Processing Inc.; Hastings NE. Biomass samples (wheat straw, corn stover, and switchgrass) were obtained from Richard Hess at the Idaho National Laboratory, Idaho Falls, ID. Hull pellets and biomass samples were ground in a Wiley mill through a 1 mm screen prior to fermentation. Fermentations containing approximately 5–20% DB by weight SBH were conducted in sealed 70 mL reusable BBL Septi-Chek bottles using 3 g SBH per bottle in 20 mL unless noted otherwise. *S. cerevisiae* D_5A was grown in YEPD medium (Difco, Detroit, MI), and *E. coli* KO11 and *Z. mobilis* 8b were grown in Luria broth to stationary phase to provide fermentation inoculum at 1.5% v/v. The medium for the *S. cerevisiae* fermentation contained SBH, enzymes, yeast cells, and water to reach the biomass loading target. The limited number of *E. coli* KO11 fermentations contained a final concentration of: 100 mM NaCl, 1 mM $MgCl_2 \cdot H_2O$, and 1 mM $CaCl_2 \cdot 2H_2O$ at pH 7, with no added nitrogen. *Z. mobilis* fermentations contained 1 mM $MgCl_2 \cdot H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, and 10 mM $NaHPO_4$ at pH 7 with no added nitrogen. For typical fermentations all components except enzymes and cells were added to the vials and autoclaved for 30 min. and cooled. The cells, sterile water and enzymes were added, and the vials were sealed without flushing with nitrogen. Bottles were weighed to the nearest 10 mg as tare, after components were added, after autoclaving, and throughout the fermentations. No samples were taken during the fermentations. Fermentations were conducted with shaking @ 36 °C using a New Brunswick C24 shaker (New Brunswick Instrument Company, New Brunswick, NJ) at 150 rpm. Bottles were vented with a sterile needle to release CO_2 prior to weighing on days 1, 2, 3, 6, 9, and 13. Fermentations contained 50 μg/mL streptomycin to minimize mesophilic anaerobe contamination during the research. Larger-scale *S. cerevisiae* fermentations were also conducted in a 2 L Ehrlenmeyer flask at 36 °C with an agitation speed of 150 rpm using unmilled SBH pellets at 15% DB loading (w/w) with a total weight of 1200 g. After autoclaving and cooling, cells, water, streptomycin, and enzymes were added, and the flasks were degassed with ultra-pure nitrogen prior to incubation. At the end of the fermentation, final weights were recorded, and samples were taken and frozen at −70 °C for later analysis. All tests were conducted with technical duplicate replicates.

2.3. Analysis

Fermentation performance was determined by HPLC as described (Yang and Wyman, 2004) after samples were centrifuged in a Sorvall Biofuge microcentrifuge at 13,000 rpm for 2 min, and the supernatant filtered through a 0.45 μm filter to remove solids. A high performance liquid chromatography system (HPLC) equipped with a refractive index (RI) detector (Waters Model 2695, Milford, MA, at Dartmouth College, or Hitachi Model 2490, Pleasanton, CA, at ORNL, was used for both sugar and ethanol analysis. For analysis of fermentation products such as ethanol and acetic acid, a Biorad Aminex HPX-87H 300 × 7.8 mm column was used. Residual sugars in the solid residues were assayed per the NREL "Enzymatic Saccharification of Lignocellulosic Biomass" method (Ehrman, 1994, www.nrel.gov/biomass/analytical_procedures.html), and the resulting sugars analyzed by HPLC using a Biorad Aminex HPX-87P 300 × 7.8 mm. Protein analysis from bottle fermentations were determined by combustion of dry samples with a ThermoFinnigan FlashEA 1112 N/Protein Analyzer (CE Elantech, Lakewood, NJ). A similar procedure was used to determine protein content by Rock River Laboratory, Watertown, WI using a Carlo Erba nitrogen analyzer, and in all cases protein levels were calculated as 6.25 times the sample nitrogen content (Delorme et al., 1981). Amino acid analysis was conducted by Eurofins Central Analytical Laboratory, Metairie LA by acid hydrolysis and HPLC analysis using method AOAC 994.12.

3. Results

3.1. Soybean hull ethanol fermentation

Soybean hulls contain significant levels of carbohydrate that could be hydrolyzed and converted into ethanol. However, the carbohydrate types and distribution are somewhat unique compared to carbohydrates from other non-food plant sources, called lignocellulosic biomass, as shown in Table 1. Unlike other plant materials, soybean hulls typically have approximately 9–14% protein, with significant amounts of pectin and low amounts of lignin (Strombaugh et al., 2000; Smith and Greenfield, 1979; Lee et al., 2007; Mullin and Weili, 2001; Ipharraguerre and Clark, 2003; Corredor et al., 2008).

Initial tests with the soybean hulls were conducted to determine their potential for ethanol production with limited pretreatment to minimize inhibitor formation. In small-scale tests, the soybean hulls received either no treatment, 60 min. autoclaving, or 60 min. autoclaving with 1% sulfuric acid. The samples were adjusted to pH 5 prior to SSF fermentation at 16.6% (w/w) solids loading with 47.3 FPU Spezyme CP®/g SBH cellulose, 100 FPU β-glucosidase/g SBH cellulose, and *S. cerevisiae* D_5A. Ethanol concentrations after six days were similar for all conditions: 30.8 g/L for no autoclaving or acid use, 30.1 g/L for autoclave without acid use, and yielded 28.7 g/L for autoclaving with acid. Residual glucan concentrations in the solids for these conditions were 1.8%, 1.1%, and 2.1%, respectively, compared to the initial glucan levels of 35% by weight, in part due to the high level of enzymes used. The ethanol yields and residual glucose indicated that dilute acid pretreatment may not be needed for SSF production of ethanol by *S. cerevisiae*.

3.2. Enzymes required during ethanol fermentation

To confirm that enzymes alone were sufficient to permit effective SSF conversion of soybean hulls to ethanol, levels of the primary source of cellulase, Spezyme CP®, were varied for SSF of soybean hulls without any acid treatment at a 15% biomass loading

Table 1
Composition of soybean hulls and selected herbaceous biomass. Data as percent of biomass on a dry basis from Strombaugh et al. (2000), Smith and Greenfield (1979), Lee et al. (2007), Mullin and Weili (2001), Ipharraguerre and Clark (2003), Corredor et al. (2008). Measured values for SBH are in (X). nd = not determined. SBH contained 4.3% galactan.

	Cellulose	Hemicellulose	Lignin	Pectin	Protein	Ash
Soybean hulls	29–51 (38.4)	10–20 (10.2)	1–4 (2.8)	6–15 (nd)	9–14 (10.7)	1–4 (5.8)
Corn stover	31–41	20–34	16–23	0	4–9	4–8
Switchgrass	31–45	22–35	18–22	0	2–4	4–9
Wheat straw	32–49	23–39	5–19	0	2–6	1–10

at 2.9, 5.8, 11.6, and 23.2 FPU/g cellulose SBH for 13 days. The second critical enzyme, β-glucosidase, was held constant at 41 U/g SBH cellulose at the 15% biomass loading. Fig. 1 shows the rate of weight loss during fermentation improved until the bottles with 11.6 and 23.2 FPU Spezyme CP®/g cellulose proceeded in similar manner. Ethanol concentrations at the end of the fermentation were similar to weight loss with Spezyme CP® dosages of 2.9, 5.8, 11.6, and 23.2 FPU/g cellulose producing 29.0 ± 0.01, 30.1 ± 0.6, 31.9 ± 1.4, 30.3 ± 1.1 g/L ethanol. A new Genencor/Danisco cellulase Accellerase 1000® was tested and also found to be effective for SSF ethanol production from SBH (data not shown).

The impact of varying β-glucosidase supplementation on ethanol yields was determined with fixed Spezyme CP® dosages of 11.6 FPU/g cellulose along with evaluation of no enzyme and single enzyme use, as shown in Fig. 2. β-glucosidase loadings of 13, 27, and 82 U β-glucosidase/g cellulose resulted in ethanol production of 31.2 ± 0.3, 32.1 ± 0.3, 31.9 ± 0.3 g/L ethanol respectively. However, Spezyme CP® alone resulted in a slow weight loss that ceased at a lower level than with added β-glucosidase of 27.4 ± 3.4 g/L ethanol, while fermentations with β-glucosidase alone at 82 U/g produced 2.5 ± 0.02 g/L ethanol. Without *S. cerevisiae* cells, no ethanol was produced and virtually no weight change was observed (0.01 g loss). Fig. 2 shows that the β-glucosidase loadings used were sufficient at all three levels tested.

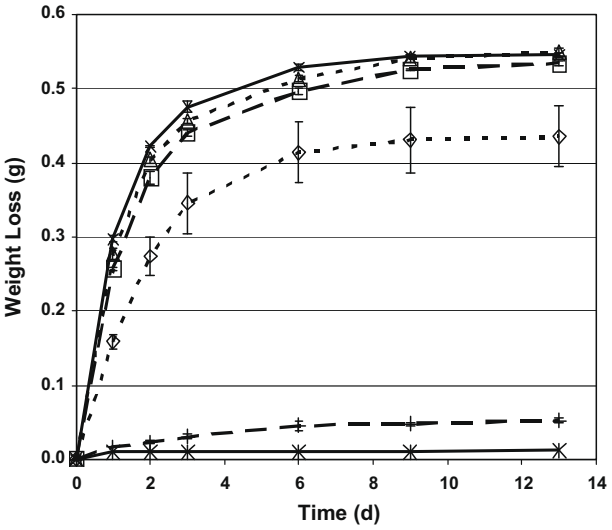

Fig. 2. Impact of β-glucosidase dosages on fermentation of soybean hulls as measured by fermentation bottle weight change. Enzymes used are listed in activity units per gram cellulose in the order Spezyme CP®, β-glucosidase, followed by day 13 ethanol concentration in g/L. Solid line X: 11.7, 82, 31.9 ± 0.3; dots open triangle: 11.6, 27, 32.1 ± 0.3; dash open square: 11.6, 13, 31.3 ± 0.3; dots open diamond: 22, 0, 27.4 ± 3.4; dash + symbol: 0, 82, 2.5 ± 0.02; solid line double X, no cells: 0, 0, 0, all with SD error bars.

3.3. Other biomass feedstock for ethanol

To confirm that soybean hulls are unique in not requiring pretreatment for high yield ethanol production, corn stover, wheat straw, and switchgrass were subjected to the same fermentation process without any prior pretreatment besides autoclaving. In two experiments, these milled feedstocks were prepared in bottles with a biomass loading of 15% (g/g), and *S. cerevisiae* D_5A was used as the fermentation ethanologen at two different enzyme dosages, one being the level selected above. It was not know if the enzyme dosages determined for SBH were sufficient for these other feedstocks, so in a second experiment the enzyme levels were increased to 11 or 22.3 FPU Spezyme CP®/g cellulose and 13 or 39 U/g cellulose β-glucosidase, respectively, in separate experiments. Fig. 3 shows enzyme dosages, weight loss, and final ethanol concentration comparisons for these four biomass sources during SSF incubations. SSF of non-pretreated SBH yielded 29.1 ± 0.8 g/L ethanol for both enzyme dosages, while other non-pretreated corn stover, switchgrass and wheat straw reproducibly resulted in much lower-ethanol levels regardless of the enzyme dosage: 11.3 ± 0.3, 8.3 ± 0.2, and 7.4 ± 0.2 g/L ethanol, combined for both conditions, for corn stover, switchgrass and wheat straw, respectively. The fermentations were highly reproducible at these conditions, and soybean hulls yielded between 2.6- and 4-fold more ethanol concentrations than the other biomass sources tested.

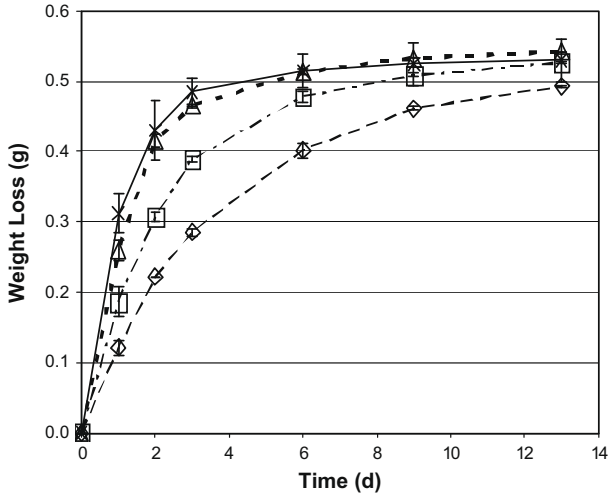

Fig. 1. Impact of Spezyme CP® dosages on fermentation of soybean hulls as measured by fermentation bottle weight change. Enzymes used are listed in activity units per gram cellulose in the order Spezyme CP®, β-glucosidase, followed by day 13 ethanol concentration in g/L. Dash open diamond: 2.9, 41, 29.0 ± 0.01; dash dots open squares: 5.8, 41, 30.1 ± 0.6; dots open triangle: 11.6, 41, 31.9 ± 1.4; solid line X: 23.2, 13, 30.3 ± 1.1. Error bars for all figures are standard deviation of duplicates.

3.4. Biomass loading tests

Industrial operations typically apply high biomass loadings of 15% (w/w) or higher to increase the titer of ethanol while minimizing the fermentor volume required in spite of materials handling difficulties. Therefore, tests were conducted to determine the impact of varying the biomass loading from 5% to 20% DB of SSF

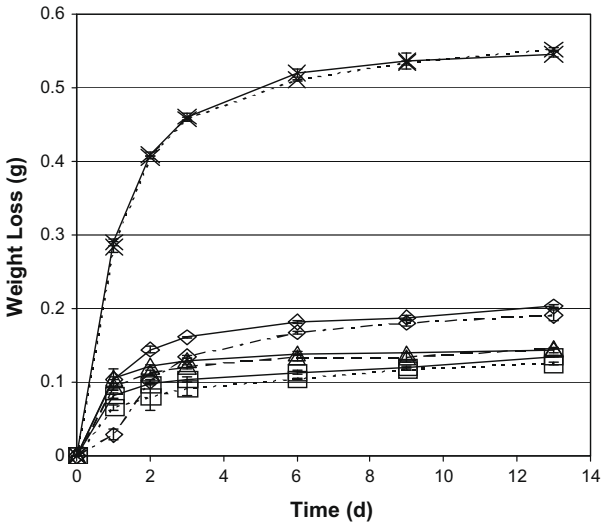

Fig. 3. SSF processing of soybean hulls, corn stover, switchgrass, and wheat straw with no pretreatment at two different enzyme dosages yielding fermentation bottle weight changes. Enzymes used are listed by units per gram cellulose. Solid line fermentations contain Spezyme CP®, β-glucosidase dosages of 11, 13, respectively; dotted line fermentations contain Spezyme CP®, β-glucosidase dosages: 22, 39, respectively. Ethanol concentrations at day 13: X, soybean hulls, low enzyme 28.6 ± 0.2, high enzyme: 31.9 ± 1.4; open diamond, corn stover, low enzyme 11.5 ± 0.1, high enzyme 11.1 ± 0.1; open triangle, switchgrass, low enzyme 8.4 ± 0.03, high enzyme 8.1 ± 0.1; open square, wheat straw, low enzyme 7.4 ± 0.2, high enzyme 7.4 ± 0.2, all with SD error bars.

processing of soybean hulls. Fig. 4 shows the weight loss for SSF with 5%, 10%, 15%, and 20% DB soybean hull loadings using identical enzyme dosages of 11 FPU Spezyme CP®/g cellulose and 13.0 U β-glucosidase/g cellulose in a fixed volume of 20 mL. Fermentation results yielded 8.0 ± 0.1, 16.9 ± 0.1, 25.2 ± 0.04, and 32.5 ± 0.3 g/L ethanol for the 5% 10%, 15%, and 20% w/w loadings, respectively, reflecting ethanol concentrations were essentially proportional to the biomass loading. To examine if the lower biomass loadings fermented more rapidly, 5%, 10%, 15% and 20% DB soybean hull

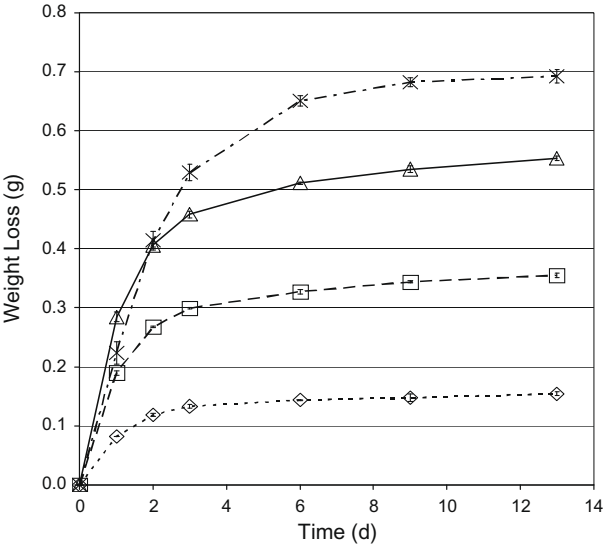

Fig. 4. Impact of biomass loading on fermentation results as measured by fermentation bottle weight changes. Soybean hulls SSF with biomass loading of 5%: dots, open diamond; 10%, dashes, open square; 15%, solid line, open triangle, and 20%, dash-dots, X, produced 8.0 ± 0.1, 16.9 ± 0.1, 25.2 ± 0.04, and 32.5 ± 0.3 g/L ethanol by day 13, respectively. Spezyme CP®, β-glucosidase dosages were 11 and 13 U/g cellulose, all with SD error bars.

loadings reached 90% conversion levels in 5, 5.2, 5.2 and 5.3 days, respectively, suggesting limited retardation in conversion by higher solids concentrations.

3.5. Bacterial ethanol fermentation

Three different ethanologens (*Zymomonas*, *E. coli* and *Saccharomyces*) were used in SBH fermentations to verify that the ease of fermentation of SBH was not specific to *Saccharomyces*. Also, individual bottles were used for each time-point to evaluate the use of weight to estimate progress in the ethanol fermentation. Thirty-six identical vials were prepared containing approximately one gram SBH at about 8.4% (g/g) biomass loading, as described in the methods section, with differences only in added salts, enzymes, and inoculum. Twelve vials were charged with *S. cerevisiae* D_5A, and 12 with *E. coli* KO11 or *Z. mobilis* 8b, both bacteria buffered to pH 7. Enzymes were loaded at 5.1 FPU Spezyme CP®/g cellulose, 15.5 U β-glucosidase/g cellulose, and 13.8 U hemicellulase/g cellulose for all vials. In addition, 500 U pectinase/g cellulose was used with *S. cerevisiae* (Spezyme CP® dosages are different than that determined above as these tests preceded the dosage determination). Hemicellulase was used for this test because *E. coli* KO11 and *Z. mobilis* 8b are genetically engineered to ferment xylose derived from hemicellulase activity to ethanol. Two bottles were removed randomly from each set of 12 bottles at various times, vented, weighed, and frozen at −80 °C. For analysis, the samples were thawed and assayed for ethanol production.

The results of the weight loss versus time for each ethanologen are shown in Fig. 5a while Fig. 5b shows the ethanol yield from all three microorganisms Fig. 6 shows the proportionality of ethanol concentration and weight loss for the data in Fig. 5a and b. Analysis of the samples by HPLC showed a significant reduction in residual soluble materials detectable with a RI detector for *E. coli* KO11 compared to the other ethanologens (data not shown), presumably due to the greater capability of *E. coli* KO11 for fermenting multiple sugars. No additional experiments were conducted with either genetically engineered *E. coli* or *Zymomonas*.

3.6. Impact of pectinase on SSF conversion of SBH

Soybean hulls contain 6–15% pectin as part of its structure (Table 1) so tests were conducted to determine the impact of addition of pectinase at three levels of 2850, 7120, and 14,240 U pectinase per gram cellulose in combination with set levels of 11 FPU/g cellulose Spezyme CP® and 13 U/g cellulose β-glucosidase. Control SSF conversions had either no pectinase or only the highest level of pectinase with neither cellulase nor β-glucosidase. All levels of pectinase increased the rate of fermentation, as evidenced by the weight loss shown in Fig. 7. The lowest and middle pectinase additions did not impact ethanol concentrations, with 30.2 ± 0.01, and 30.2 ± 0.001 g/L ethanol produced, respectively, but the highest pectinase level produced a lower-ethanol level of 29.2 ± 0.7 g/L. However, all were higher than for the control of 28.6 ± 0.2 g/L ethanol. Other experiments not shown showed that the pectinase enzyme preparation was highly inhibitory to bacterial fermentations and may also impact yeast fermentations. Addition of pectinase by itself at the highest level produced 11.1 ± 0.3 g/L ethanol.

3.7. Large scale fermentation tests

To more fully optimize the fermentation and provide sufficient material for further analysis, large batch flask fermentations were conducted in duplicate with 180 g DB SBH at 15% biomass loading in 1200 g SSF conversion. Flask fermentations were chosen because the low fluidity of SBH at a 15% loading prevented agitation in a conventional fermentor during the first 48 h. Enzyme loadings of

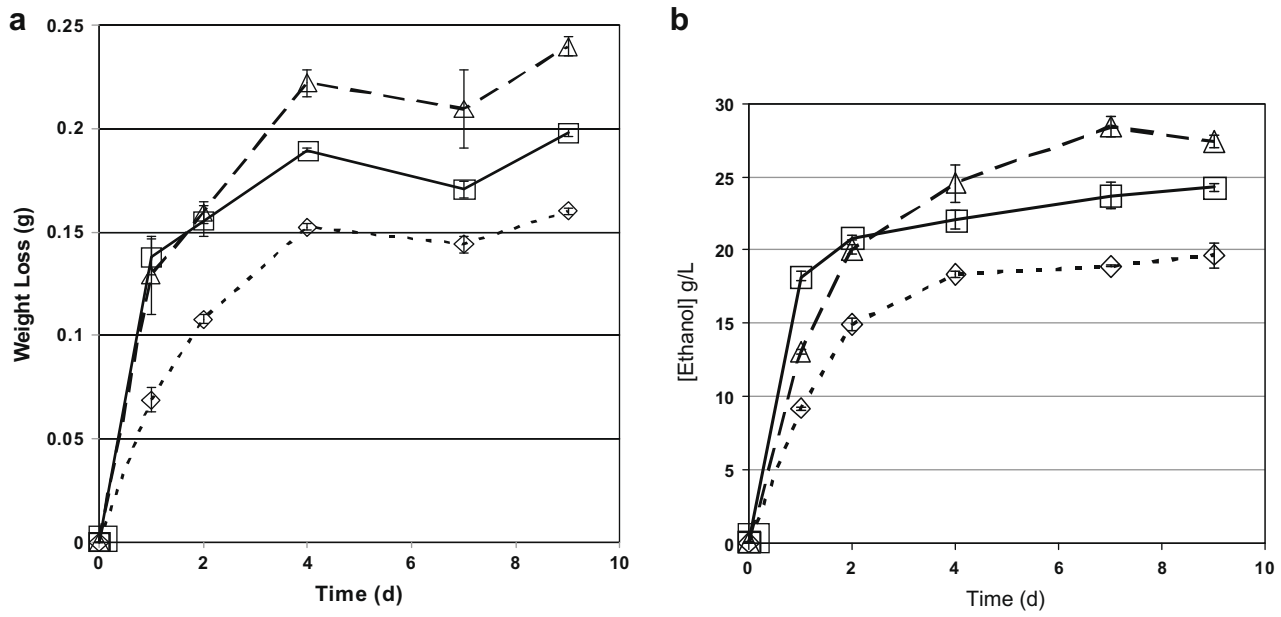

Fig. 5. Results from 36 individual bottle fermentation for comparison of three ethanologens and correlation of weight loss and ethanol concentration. Duplicate bottles were removed at each time-point to determine weight loss and ethanol concentration: (a) weight loss; (b) ethanol concentration, for *Escherichia coli* KO11, dashed line, open triangle; *Saccharomyces cerevisiae* D5A, solid line, open square; and *Zymomonas mobilis* 8b, dotted line, open diamond, over nine day SSF, all with SD error bars.

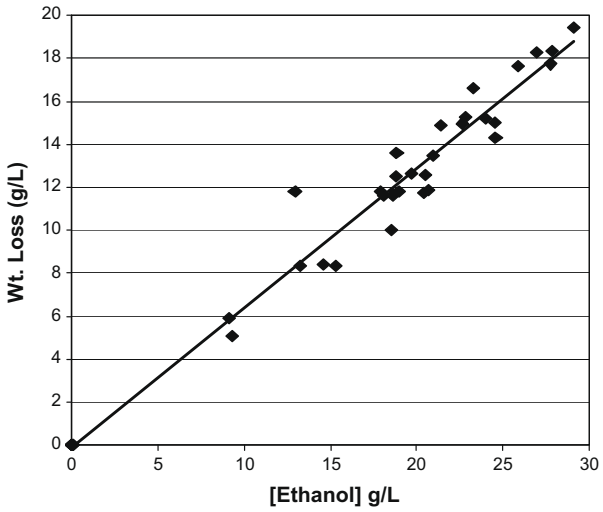

Fig. 6. Correlation of weight loss and ethanol concentration for the 30 bottles from Fig. 5, including six zero time samples. Samples are for data from all three ethanologen cultures. Line is a linear calculated fit.

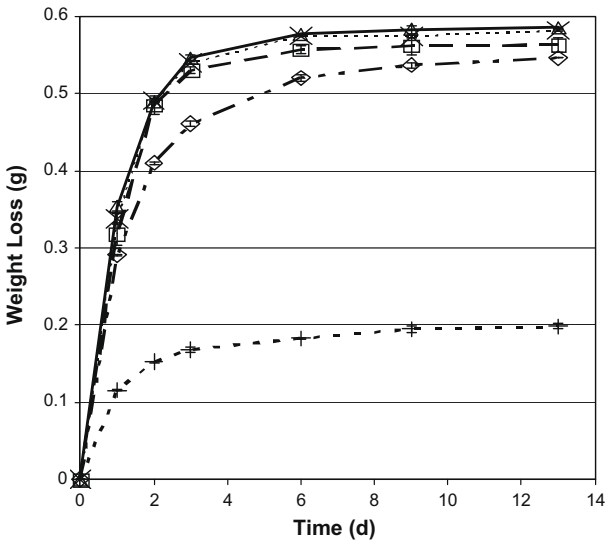

Fig. 7. Analysis of the impact of pectinase enzyme compared to baseline SBH SSF. Spezyme CP®, β-glucosidase dosages at 11 and 13 U/g cellulose, respectively. Pectinase unit (U) additions and ethanol concentrations (g/L): Dashed line, open square: 14,240 U, 29.2 ± 0.7; solid line, open triangle: 7120 U, 30.2 ± 0.01; small dotted line X: 2850 U, 30.2 ± 0.001; dash-dots open diamond: no pectinase, 28.6 ± 0.2; dots + symbol: 14,240 U pectinase with no other enzymes, 11.0 ± 0.3, all with SD error bars.

11.5 FPU Spezyme CP, 13 U β-glucosidase, and 2850 U pectinase were used per g cellulose with *S. cerevisiae* D_5A. After thirteen days, duplicate fermentations produced 31.9 ± 0.15 (flask A) and 31.2 ± 0.3 (flask B) g/L ethanol. The enzymatic saccharification assay determined that fermentation flask A had 5.3% ± 0.01% residual glucose and flask B had 6.7% ± 1.2% residual glucose compared to starting SBH of 34.9% ± 0.7% glucose. Based on this, the degree of conversion of cellulose was calculated to be 84.9% of theoretical for flask A and 80.9% for flask B, indicating extensive hydrolysis and fermentation of the cellulose in the SBH substrate.

3.8. Fate of protein during fermentation

Soybean hulls contain 9–14% protein, and by eliminating the pretreatment process, any protein in the fermentation residues may be minimally impacted by *S. cerevisiae* D_5A in the SSF process. To examine this, the level of protein remaining in fermentation solid residues was determined. Examination of the residues from the larger-scale fermentation determined that flask A contained 25.6% ± 0.15% protein, while flask B had 27.1% ± 0.39% compared to the starting SBH feedstock which containing 10.7% protein, yielding about a 2.5× increase in protein content in the fermentation residue on a dry basis. The highest concentrations were recorded to be 30.5% ± 0.6% protein for biomass loading experiments with a 5% biomass loading test (Fig. 4). Protein concentrations in the fermentation residue of eight duplicate samples

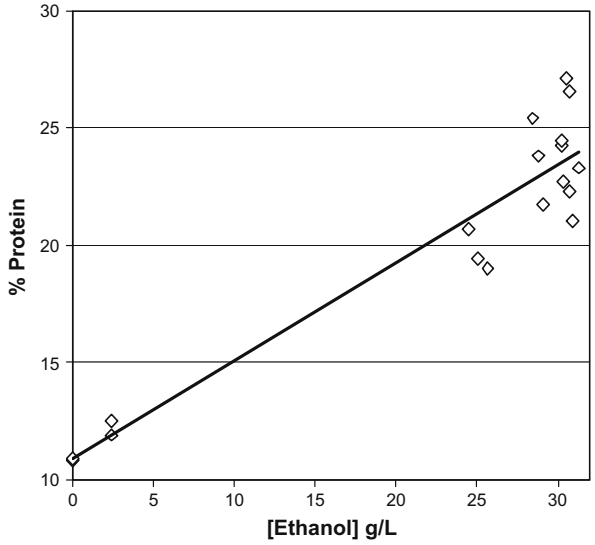

Fig. 8. Comparison of SSF ethanol concentration and fermentation residue protein levels for 16 fermentations compared to SBH starting protein concentration of 10.7% with linear calculated fit line.

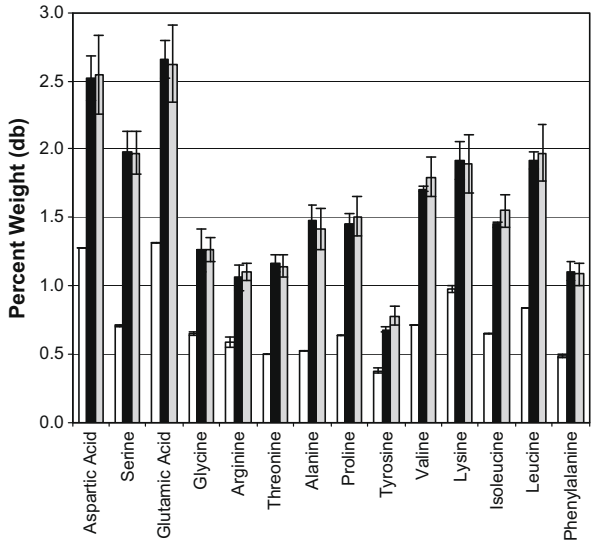

Fig. 9. Comparison of fourteen amino acids from fermentation substrate, SBH (white bar), and duplicate fermentation residues from SSF flasks A (black bars) and B (gray bars) with SD error bars. Percent of material weight on a dry basis.

were compared to ethanol concentrations relative to the starting SBH substrate, and the data shown in Fig. 8 is consistent the protein concentration increasing as the ethanol concentration increases. These data were obtained by combustion and analysis of nitrogen content with protein levels obtained using the 6.25 N to protein factor (Delorme et al., 1981). The fermentation residues were also assayed for amino acid content, with Fig. 9 showing the concentration of fourteen amino acids determined for the starting SBH and compared to each of the larger-scale fermentations, A and B. The data is presented as percent by weight with the difference between SBH and the fermentation residues representing the concentrating effect detected by nitrogen combustion analysis shown previously. The data in this Fig. demonstrates a 2.2-fold increase in concentration of the amino acids for the average of fermentation samples A and B, suggesting little if any loss of amino acids.

4. Discussion

Soybean hulls contain a unique mixture of carbohydrates, significant levels of protein, and only a little lignin (Mullin and Weili, 2001) compared to other typical plant or biomass materials. While soybean hull's susceptibility to enzymes had been shown previously in a biodegradability study (Enayati and Parulekar, 1995), the use of soybean hulls for fermentation and ethanol or chemical production has received little attention, in part due to the existing market for the hulls as bovine feed. Recently Schirmer-Michel demonstrated fermentation of a soybean hull acid hydrolysate after detoxifications to produce almost 6 g/L ethanol or xylitol with *C. guilliermondii* yeast (Schirmer-Michel et al., 2008). Similarly, Corredor recently demonstrated a high degree of enzymatic hydrolysis of soybean hulls by dilute acid and modified steam-explosion pretreatment liberating up to 72% of available hexose sugars in soybean hulls (Corredor et al., 2008) and proposed the resulting hydrolysate would be suitable for ethanol fermentation. By contrast, as shown here, soybean hulls are unique among various agricultural residues in that they do not require the extensive/expensive thermochemical pretreatment described in these publications to make polymeric carbohydrates accessible to cellulase and other enzymes that depolymerize their complex carbohydrates to fermentable sugars. Only cellulases and β-glucosidase were required for significant depolymerization of the complex carbohydrates to permit *S. cerevisiae* D_5A to ferment ethanol in the SSF-mode with enzyme dosages at less that fifteen Spezyme CP® and β-glucosidase enzyme units per gram cellulose in SBH. The difference in susceptibility of soybean hulls compared to two agricultural residues, corn stover and wheat straw, and a dedicated biomass source, switchgrass, verifies the lack of a need for pretreatment to obtain high ethanol concentrations for the SBH used. Furthermore, ethanol concentrations from SBH are 3–4-fold higher than from these other biomass sources, even with increased enzyme dosages. Addition of pectinase is beneficial due to the high levels of pectin in the hulls, especially for accelerating the depolymerization/fermentation process making the cellulose more accessible, although the benefit is indirect since *S. cerevisiae* D_5A cannot metabolize pectin enzyme hydrolysis products (J. Doran-Peterson, personal communication). While 15% biomass loadings were routinely used, tests with thick biomass concentrations of 20% as well as 5% and 10% loading demonstrated SSF could produce essentially proportional ethanol concentrations over the whole range of feedstock levels, permitting significant flexibility in fermentation. However, biomass loadings above 15% suffer from mixing difficulties initially but change in the subsequent 24–48 h due to thinning of the solids by cellulase action. Thus, use of fed batch or continuous systems could overcome the solids loading limitations.

Successful fermentation of SBH to ethanol was not restricted to *S. cerevisiae* as two genetically engineered bacteria were also effective in fermenting SBH to ethanol. In fact, *E. coli* KO11 produced the highest concentration of ethanol in part because it can utilize xylose, pectin, and uronic acid (Doran et al., 2000). Under the conditions tested, the benefits of genetic modification of *Z. mobilis* 8b (Mohagheghi et al., 2004), which can ferment pentose sugars to ethanol, were not evident as it produced the lowest concentration of ethanol. The non-xylose utilizing *S. cerevisiae* produced more ethanol than *Zymomonas*, but conditions were not optimized for each microorganism, which included a higher initial pH for *Zymomonas* than used at NREL (Mohagheghi et al., 2004), so better performance is possible. Further work was not undertaken with either bacterial ethanologen since they currently are not accepted in animal feed, an important goal of this work. It is interesting to note that both weight loss data (Fig. 5a) and fermentation results (Fig. 5b) showed that vial weight loss is effective for tracking

ethanol yields, especially at high biomass loadings, avoiding disrupting the fermentation by sampling.

Analysis of SBH harvested in two different years showed they contained about 10.7% protein. With the discovery that SSF conditions did not require pretreatment for production of high titers of ethanol in the broth with 81–85% cellulose conversion, it was realized that the protein, which is currently used in bovine feed (Blasi et al., 2000), could emerge from the SSF process largely intact. Analysis of multiple fermentation residues demonstrated that production of high levels of ethanol significantly increased protein levels in the fermentation residues as shown in Fig. 8. Larger-scale fermentation of SBH also demonstrated production of over 3% ethanol concentrations was accompanied by increased protein levels to over 25%, thus verifying *S. cerevisiae* does not hydrolyze and consume significant amounts of the SBH protein.

Importantly, the increase in protein concentration during fermentation was due to a reduction in the residual glucan levels to an average of 6% due to SSF removal of the cellulose fiber and other complex carbohydrates. Analysis of the amino acids by an outside testing firm detected fourteen amino acids in both the SBH and duplicate fermentation residues, with an average of 2.2-fold increase in the amino acids present in these residues. After fermentation, lysine levels increased by 2-fold to about 1.9% while threonine increased about 2.3-fold to over 1.1%, showing that fermentation upgraded the protein concentration of these essential amino acids. Interestingly, it was observed that the fermentation residues was granular and flowable upon drying (data not shown), in spite of high-protein levels, a desirable attribute for handling protein feed additives on a large scale.

Elimination of pretreatment should significantly reduce the cost of ethanol derived from soybean hulls. For example, the cost of pretreatment and accompanying toxin removal has been estimated at 19% of the total cost of ethanol (Aden et al., 2002), or about 50¢ for cellulosic ethanol at $2.50/gallon. In addition, eliminating pretreatment avoids production of fermentation inhibitors (Klinke et al., 2004), such as furfurals and lignin monomers which are formed by an acidic pretreatment (Martinez et al., 2001). While the elimination of pretreatment itself amounts to a significant cost savings due to process simplification and avoiding pretreatment inhibitors, these benefits can be augmented because most pretreatment processes chemically degrade proteins found in biomass, eliminating its nutrient and commercial value for animal feed. It has been determined here that without pretreatment, soybean hulls retain much of their protein in the residues after fermentation, yielding both ethanol and a potentially valuable co-product of high-protein animal feed. Currently soybean hulls are used for feed due to their moderate level of protein and relatively high lysine content (Rackis et al., 1961) and therefore effective for supplementing low lysine corn feed. Yet the fastest growing feed market in the US is monogastric animals such as poultry and swine, but they cannot be fed high levels of soybean hulls due to the hull's high cellulose fiber content, relegating hulls to the beef and dairy cow market (Bernard and McNeill, 1991; Weidner and Grant, 1994). The results presented here show that the cellulose fiber in soybean hulls can be removed by hydrolysis and fermentation to ethanol, yielding a higher protein, lower fiber content residue potentially available for the whole animal feed market.

Fermentation results further show that with only enzymatic processing, up to 50 gallons of ethanol can be produced per ton of soybean hulls by *S. cerevisiae* assuming the 80% conversion of cellulose demonstrated in this work. With about 95 million tons of soybean produced in the US in 2006 (USDA: www.nass.usda.gov), and approximately 5–8 million tons of soybean hulls produced per year, this simplified process could produce as much as 300 million gallons of ethanol and provide up to 1.5–2 million tons of higher protein animal feed in the US. The soybean crop from Brazil plus Argentina exceeds the magnitude of the US crop (Goldemberg, 2008), and their ethanol production has reached approximately 5×10^9 gallons from sucrose from sugarcane. So, the US and these South American countries have the soybean hull supply, ethanol production technology, and infrastructure for use of ethanol for transportation fuel. Since the ethanol demand is growing while food availability is becoming an issue of concern, this process could be beneficial to fill the demand for both ethanol and a more versatile, higher-value animal feed.

Acknowledgements

The authors thank Sara All and Kara Podkaminer (Dartmouth), and Miguel Rodriguez (ORNL) for technical assistance, and Genencor/Danisco for providing samples of SpezymeCP® and Accellerase 1000® cellulases. This work was funded in part by the National Institute of Standards and Technology (NIST) through Grant Number #60NANB1D0064 awarded to Dartmouth College, where much of the early work was performed. Additional support was obtained from the Oak Ridge National Laboratory BioEnergy Science Center, a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the US Department of Energy Office of Science.

References

Aden, A.M., Ruth, M., Ibsen, K., Jechura, J., Neeves, K., Sheehan, J., Wallace, B., Montague, L., Slayton, A., Lukas, J., 2002. Lignocellulosic biomass to ethanol process design and economics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis of corn stover. NREL TP-510-32438.

Asghari, A., Bothast, R.J., Doran, J.B., Ingram, L.O., 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. J. Ind. Microbiol. 16, 42–47.

Bernard, J.K., McNeill, W.W., 1991. Effect of high fiber energy supplements on nutrient digestibility and milk production of lactating dairy cows. J. Dairy Sci. 74, 991–995.

Blasi, D.A., Drouillard, J., Titgemeyer, E.C., Paisley, S.I., Brouk, M.J.. 2000. Soybean hulls composition and feed value for beef and dairy cattle. Kansas State Univ Document 00-79-E.

Corredor, D., Sun, X., Salazar, J., Hohn, K., Wang, D., 2008. Enzymatic hydrolysis of soybean hulls using dilute acid and modified steam-explosion pretreatments. J. Biobased Mater. Bioenergy 2, 43–50.

Delorme, C.B., Wojcik, J., Gordon, C., 1981. Method of addition of cellulose to experimental diets and its effect on rat growth and protein utilization. J. Nutr. 111, 1522–1527.

Doran, J.B., Aldrich, H.C., Ingram, L.O., 1994. Saccharification and fermentation of sugar cane bagasse by *Klebsiella oxytoca* P2 containing chromosomally integrated genes encoding the *Zymomonas mobilis* ethanol pathway. Biotechnol. Bioeng. 44, 240–247.

Doran, J.B., Cripe, J., Sutton, M., Foster, B., 2000. Fermentation of pectin-rich biomass with recombinant bacteria to produce fuel ethanol. Appl. Microbiol. Biotechnol. 84–86, 141–152.

Ehrman, C., 1994. Method for determination of total solids in biomass. In: Laboratory Analytical Procedure No. 001. National Renewable Energy Laboratory, Golden, CO.

Enayati, N., Parulekar, S.J., 1995. Enzymatic saccharification of soybean hull-based materials. Biotechnol. Prog. 11, 708–711.

Gauss, W.F., Suzuki, S., Takagi, M., 1976. Manufacture of alcohol from cellulosic materials using plural ferments. United States Patent 3990944.

Goldemberg, J., 2008. The Brazilian biofuels industry. Biotechnol. Biofuels 1, 6. doi:10.1186/1754-6834-1-6.

Hsu, T., 1996. Pretreatment of biomass. In: Handbook on Bioethanol: Production and Utilization. Taylor and Francis, Washington. DC, pp. 179–212.

Ipharraguerre, I.R., Clark, J.H., 2003. Soyhulls as an alternative feed for lactating dairy cows: a review. J. Dairy Sci. 86, 1052–1073.

Karhumaa, K., Wiedemann, B., Hahn-Hägerdal, B., Boles, E., Gorwa-Grauslund, M., 2006. Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microbial Cell Factories 5 (18). doi:10.1186/1475-2859-5-18.

Klinke, H., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26.

Kuyper, M., Hartog, M., Toirkens, M., Almering, M., 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res. 5, 399–409.

Lee, Y.Y., Iver, P., Torget, R., 1999. Dilute-acid hydrolysis of lignocellulosic biomass. Adv. Biochem. Eng. Biotechnol. 65, 93–115.

Lee, D., Owens, V., Boe, A., Jeranyama, 2007. Composition of herbaceous biomass feedstocks. South Dakota State University Publication SGINC1-07.
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.
Mielenz, J.R., 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4, 324–329.
Mohagheghi, A., Dowe, N., Schell, D., Chou, Y-C., Eddy, C., Zhang, M., 2004. Performance of a newly developed integrant of *Zymomonas mobilis* for ethanol production on corn stover hydrolysate. Biotechnol. Lett. 26, 321–325.
Mosier, C.A., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol. 96, 673–686.
Mullin, W.J., Weili, X., 2001. Study of soybean seed coat components and their relationship to water absorption. J. Agric. Food Chem. 49, 5331–5335.
Olofsson, K., Bertilsson, M., Liden, G., 2008. A short review of SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 1, 7. doi:10.1186/1754-6834-1-7.
Philippidis, G.P., Smith, T.K., Wyman, C.E., 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol. Bioeng. 41, 846–853.
Rackis, J.J., Anderson, R.L., Sasame, H.A., Smith, A.K., van Etten, C.H., 1961. Amino acids in soybean hulls and oil meal fractions. J. Agric. Food Chem. 9, 409–412.
Schirmer-Michel, A.C., Flôres, S.H., Hertz, P.F., Matos, G.S., Ayub, M.A., 2008. Production of ethanol from soybean hull hydrolysate by osmotolerant *Candida guilliermondii* NRRL Y-2075. Bioresource Technol. 99, 2898–2904.
Sedlak, M., Ho, N., 2004. Production of ethanol from cellulosic biomass hydrolysates using genetically engineered saccharomyces yeast capable of cofermenting glucose and xylose. Appl. Biochem. Biotechnol. 114, 403–416.
Smith, D., Greenfield, S.B., 1979. Distribution of chemical constituents among shoot parts of Timothy and switchgrass at anthesis. J. Plant Nutr. 1, 81–99.
Strombaugh, S., Jung, H., Orf, J., Somers, D., 2000. Genotypic and environmental variation in soybean seed cell wall polysaccharide. Crop Sci. 40, 408–412.
Weidner, S.J., Grant, R.J., 1994. Soyhulls as a replacement for forage fiber in diets for lactating dairy cows. J Dairy Sci. 77, 513–521.
Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Coordinated development of leading biomass pretreatment technologies. Bioresource Technol. 96, 1959–1966.
Yang, B., Wyman, C.E., 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnol. Bioeng. 86, 88–98.
Zhang, Y-H., Himmel, M.E., Mielenz, J.R., 2006. Outlook for cellulase improvement: screening and selection strategies. Biotechnol. Adv. 24, 452–481.