Applied Energy 93 (2012) 168–175

Contents lists available at SciVerse ScienceDirect

Applied Energy

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

Integrating sugar beet pulp storage, hydrolysis and fermentation for fuel ethanol production

Yi Zheng [1], Chaowei Yu, Yu-Shen Cheng, Christopher Lee [2], Christopher W. Simmons, Todd M. Dooley [3], Ruihong Zhang, Bryan M. Jenkins, Jean S. VanderGheynst *

Biological and Agricultural Engineering, University of California, One Shields Avenue, Davis, CA 95616, United States

ARTICLE INFO

Article history:
Received 9 October 2011
Received in revised form 20 December 2011
Accepted 27 December 2011
Available online 31 January 2012

Keywords:
Lignocellulosic biomass
Biofuel
Sugar beet pulp
Fuel ethanol
Ensilage

ABSTRACT

Sugar beet pulp (SBP) as received has a fairly high moisture content of 75–85%, which makes SBP storage a challenge. Ensilage was studied over 90 days and was found to effectively preserve SBP without lactic acid bacterium inoculation. Higher packing density yielded a slightly better silage quality. Ensilage improved sugar yield upon enzymatic hydrolysis of ensiled SBP washed with water. However, neither washing nor sterilization improved ethanol production from ensiled SBP using *Escherichia coli* KO11, suggesting ensiled SBP could be used directly in fermentation. The ethanol yield from ensiled SBP was nearly 50% higher than raw SBP. Fed-batch fermentation obtained approximately 30% higher ethanol yield than batch. Fed-batch could also be carried out at 12% solid loading with a 50% lower enzyme dosage compared to batch at the same solid loading, indicating opportunities to improve the economics of SBP conversion into liquid fuels.

1. Introduction

Rapid industrialization and population growth require environmentally sustainable energy sources. Bioethanol derived from plant biomass can contribute to a cleaner environment and help reduce US dependency on liquid fossil fuels. With the advancement of improved economics, rigorous sustainability analysis of lignocellulosic feedstocks and innovations in processing science, lignocellulosic bioethanol has become a more attractive fuel choice than earlier grain/starch based fuel ethanol [1]. Current commercial bioethanol production using crops such as sugar cane and corn as feedstocks are well-established. However, agriculture and fuel production compete for use of these crops [2]. As a result, utilization of more abundant, renewable, and inexpensive feedstocks such as lignocellulosic biomass could make bioethanol a more competitive alternative to fossil fuels [3]. Although lignocellulosic biomass constitutes the majority of renewable feedstocks, the complex structure of the cell wall makes degradation and subsequent processing of carbohydrates difficult.

Sugar beet pulp (SBP) is a valuable by-product from the manufacturing of beet sugar. Its carbohydrate (cellulose, hemicellulose, pectin, and others) contents have been reported to be as high as 85% (w/w, dry basis) and its lignin content as low as 1–2% (w/w, dry basis) [4,5]. It also contains 10–15% protein (w/w, dry basis). The US planted 1.2 million-acres of sugar beet crops and produced 29.5 million tons of sugar beet in 2009 [6], which resulted in more than 1.6 million dry tons of SBP after sucrose extraction [7]. Conventionally, SBP is dehydrated, pelletized and sold as a relatively low-value animal feed. The profitability of selling SBP as animal feed depends greatly on the economics of the energy and feed industries since SBP processing, including drying, pelletizing, and transporting, is energy-intensive [8]. In many parts of the world, utilization of SBP is an economically marginal part of beet sugar processing due to the low feed value and high drying cost [7]. In certain areas, dehydrating and pelletizing SBP contribute 30–40% of the overall energy cost of sugar beet processing [9]. Therefore, the beet sugar industry seeks to add value to SBP via a process that does not require drying. In light of this, converting SBP into fuel ethanol through biological pathways, including hydrolysis and fermentation, is an attractive option.

Storage is a major challenge in utilizing SBP for fuel ethanol production. Drying of SBP is common as it avoids carbohydrate loss due to microbial activity. However, in most countries this method is too expensive [10]. Furthermore, dry storage may not be advantageous when SBP is intended for conversion to biofuels and bio-based products since anaerobic digestion and fermentation are typically aqueous processes. For these reasons, it is worth investigating wet storage methods that minimize SBP carbohydrate loss while maintaining moisture content after sugar extraction.

* Corresponding author. Tel.: +1 530 752 0989; fax: +1 530 752 2640.
E-mail address: jsvander@ucdavis.edu (J.S. VanderGheynst).
[1] Present address: Novozymes Inc., Denmark.
[2] Present address: Department of Chemical Engineering, Pennsylvania State University, State College, PA, United States.
[3] Present address: EdeniQ, Inc., Visalia, CA, United States.

0306-2619/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.apenergy.2011.12.084

Ensilage is a storage technology applied to wet or partially dry biomass [11,12]. During ensilage, water soluble carbohydrates (WSCs) are rapidly fermented under anaerobic conditions into various organic acids, preferably lactic acid, to quickly and substantially lower the pH. Lactic acid bacterium (LAB) inoculants have often been used to control the fermentation pattern to avoid undesirable growth of spoilage microorganisms such as butyric acid-producing clostridia and enterobacteria [13–15]. The addition of molasses benefited the growth of LAB and improved the SBP silage quality with low dry matter loss [10]. Both the addition of acids and a pressing process were used to preserve SBP in ensilage to achieve high quality SBP with high lactic acid yield and low dry matter loss [16]. Ensilage has been used to preserve animal feed and may be applicable to preserve lignocellulosic biomass such as SBP for biofuel production. It was found that ensiling storage of maize improved biogas yield by 15% in anaerobic digestion compared to non-ensiled maize [17]. Moreover, Passoth et al. [18] reported that the ethanol yield from moist wheat grain was increased by 14% through airtight storage (ensilage), compared with the control obtained from traditionally dried stored grain. Previous research showed that the ensilage process significantly improved enzymatic hydrolysis of SBP for reducing sugar production [19]. However, little research has been done to examine post-ensiling treatments such as washing, sterilization, and chemical pretreatment prior to enzymatic hydrolysis and ethanol fermentation.

Both enzymatic hydrolysis and fermentation are critical processes for bioconversion of SBP into fuel ethanol. Costly thermochemical pretreatment might not be needed for effective bioconversion of SBP due to the low lignin and high pectin contents. Pectin removal by pectinase hydrolysis improved cellulose hydrolysis [20,21]. Therefore, pectinase is usually used in addition to cellulase/β-glucosidase to hydrolyze SBP into monosaccharides and galacturonic acid for fermentation into fuel ethanol. However, conventional ethanol-fermenting yeasts and native strains such as *Saccharomyces cerevisiae* cannot metabolize both arabinose and galacturonic acid into ethanol [22]. Genetically engineered bacteria including *Escherichia coli* KO11, *Klebsiella oxytoca* P2 and *Erwinia chrysanthemi* EC 16 have been used to ferment hexoses, pentoses and galacturonic acid into ethanol [7]. *E. coli* KO11 was the most efficient at fermenting arabinose and galacturonic acid and yielded the highest ethanol concentration of 25.5 g/L followed by *K. oxytocaa* P2 and *E. chrysanthemi* EC 16. Rorick et al. [8] used both *E. coli* KO11 and *S. cerevisiae* (Type II -YSC2) in parallel and serial fermentation processes to convert SBP solids into ethanol. The highest ethanol yields for *E. coli* KO11 (0.144 g ethanol/g-dry SBP) were much higher than those for *S. cerevisiae* (0.092 g ethanol/g-dry SBP).

In this paper, ensilage was studied to stabilize SBP in 20-L containers. The effects of both packing density and LAB inoculation level (*Lactobacillus fermentum* NRRL B-4524) on the silage quality were investigated. Washing and sterilization of ensiled SBP were examined to determine if they improved or deteriorated reducing sugar yield upon enzymatic hydrolysis and ethanol yield from *E. coli* KO11 fermentation. In addition, size reduction, gas purging, solid loading level, and operation mode (fed-batch and batch) were studied to determine their effects on ethanol yield from SBP using *E. coli* KO11.

2. Materials and methods

2.1. Materials

SBP was obtained from Spreckels Sugar Company in Mendota, CA in 2007. The moisture content as-received was about 78% (wet basis). Fresh SBP was stored at −20 °C until use. *L. fermentum* NRRL B-4524 (LAB 137) was offered by the Department of Viticulture and Enology at University of California, Davis. The ethanologenic *E. coli* strain KO11 was purchased from the American Type Culture Collection (ATCC 55124).

2.2. Preparation of lactic acid bacterium inoculum and SBP ensilage set-up

Ensilage was performed at the 20-L scale. *L. fermentum* NRRL B-4524 was identified as the best LAB strain for SBP silage quality in previous studies at 50 and 1000 mL scales [19] and was further examined here. Ensilage of SBP without LAB inoculation was conducted as a negative control. The packing density of silage was selected as a variable in this study as it was found to significantly affect the ensilage quality in a previous study [23]. Three packing density levels, 0.48, 0.72, and 0.96 g/cm^3, were used. Three replicates were performed for each packing density for both LAB-inoculated silage and the control.

The LAB inoculum for ensiling SBP were prepared by thawing frozen *L. fermentum* stock and establishing a seed culture by adding 100 uL stock to 5 mL Lactobacillus deMan Rogosa Sharpe (MRS) medium. The seed culture was grown overnight at 28 °C with an agitation of 140 rpm. A 2.5 mL aliquot of seed culture was transferred to 100 mL fresh MRS medium in a 250-mL Erlenmeyer flask, which was incubated at 28 °C with 140 rpm agitation. Cells were harvested at an optical density (OD) (590 nm) of 0.5 by centrifuging the culture at 7700 g for 5 min at 4 °C. The cell pellet was washed twice in 1 M sodium phosphate buffer (pH = 7.0) to remove residual media. Washed cell pellets were resuspended in sterilized deionized (DI) water, adjusted to an OD value of 0.5, and kept cool on ice until used.

SBP was treated with either prepared LAB inoculum or sterilized DI water (for the control) using 1-L sprayers. The LAB inoculation level was 10^6 CFU/g-dry matter (DM). SBP was thoroughly mixed while spraying inoculum or water to achieve uniform inoculation. The final moisture content of inoculated SBP was 80%. The inoculated SBP (8 wet kg, equal to 1.6 dry kg) was packed into 30-L polyethylene bags with different density-specific working volumes. The bags were sealed using a thermal sealer and hung on a steel rack at ambient temperature (ca. 22 °C) for 90 days. Each ensilage bag was equipped with a one-way gas outlet valve, through which the produced gas within the bag was released to the ambient environment. After 90 days of ensilage, SBP silage was harvested and pH, organic acids, water soluble carbohydrates, ammonia, and ethanol levels were measured.

2.3. Effect of water washing on the enzymatic digestibility of ensiled SBP

Water washing was conducted by mixing deionized water with SBP silage to achieve liquid-to-solid ratios of 5:1, 10:1, 15:1, 20:1, 30:1, and 50:1 (g:g wet SBP silage). The mixtures were stirred with stir bars at 200 rpm for 1 h at ambient temperature, then filtered through glass fiber filter paper (Grade 934-AH, Whatman) with a Büchner funnel. The washed SBP silage was collected and stored in a refrigerator for enzymatic hydrolysis experiments. The unwashed SBP silage was also hydrolyzed as a control. Enzymatic hydrolysis was conducted with 6% solid loading at a 100-mL working volume in 250-mL flasks for 168 h. Hydrolyzates were withdrawn periodically for reducing sugar measurement.

2.4. Enzymatic hydrolysis

SBP was hydrolyzed using an enzyme mixture containing cellulase (Celluclast 1.5 L), β-glucosidase (Novozymes 188) and pectinase (Pectinex® Ultra SPL). All enzymes were purchased from

Sigma-Aldrich Corp (St. Louis, MO, USA). The enzyme loading was 15 filter paper units (FPUs)/g-cellulose supplemented with 15 cellobiase units (CBUs)/g-cellulose and 60 polygalacturonase units (PGUs)/g-pectin unless specified otherwise. The enzymes were sterilized by filtration through 0.2 μm filters before used. Sodium citrate buffer (0.05 M) was used to maintain a pH of 4.8 during enzymatic hydrolysis. The mixture of SBP and buffer were autoclaved at 120 °C and 15 psi for 20 min and cooled to approx. 50 °C prior to the addition of enzymes. The total working volume was 100 mL in 250-mL bottles which were incubated in a shaking incubator at 50 °C and 140 rpm for 72 or 168 h. Hydrolyzates of 1 mL were periodically withdrawn, cooked in boiling water for 15 min to denature the enzymes, and then centrifuged at 11,000 g for 10 min. The supernatants were used for reducing sugar measurements.

2.5. Fermentation of raw and/or ensiled SBP for ethanol production by *E. coli* KO11

2.5.1. *E. coli* KO11 inoculum preparation

E. coli KO11 was used in all fermentation experiments for ethanol production in this paper. Chloramphenicol acyl transferase (*cat*) and the *Zymmonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) for ethanol production are integrated into the chromosome of this strain [24]. This strain also carries additional genetic modifications to minimize fermentation by-product formation. The culture stock was stored in 15% glycerol at −80 °C until used.

E. coli KO11 was grown in a 1:1 (v/v) mixture of Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L of yeast extract, and 5 g/L NaCl in DI water) and 5% (w/w) glucose solution. *E. coli* KO11 culture stock was transferred into the mixture (1:50, v/v) of LB medium and glucose, and incubated at 37 °C for 12–16 h until the OD of the culture reached 1.5 at 590 nm. Cells were harvested by centrifugation at 7700 g for 5 min at 4 °C, washed in sterilized DI water three times, and resuspended in sterilized DI water with an adjusted OD of 1.5 at 590 nm. 5-mL resuspended cell "solution" was used in fermentation at an initial cell loading of 0.5 mg cells per gram dry SBP.

2.5.2. General fermentation procedures

In *E. coli* KO11 fermentation, hydrolytic enzymes, including cellulase, pectinase and β-glucosidase were required. The fermentation scheme used in this paper was simultaneous saccharification and fermentation (SSF) unless specified otherwise. Cellulase and pectinase concentrations were 15 FPU/g-cellulose and 60 PGU/g-pectin, respectively, supplemented with β-glucosidase of 15 CBU/g-cellulose. Enzymes were sterilized by filtration through 0.2 μm filters and mixed with autoclaved LB medium which was made in 1 M sodium phosphate buffer with a pH at 6.0. The mixture was added to the autoclaved sugar beet pulp in 250-mL medium bottles (prewarmed to 37 °C) with a 100-mL working volume. *E. coli* KO11 inoculum prepared in Section 2.5.1 was then added into the 250-mL medium bottles which were incubated at 37 °C and 140 rpm for 7 days. 1.5-mL fermentation broths were sampled periodically for measurement of fermentation products, including ethanol, lactic acid and acetic acid. The broths were centrifuged at 11,000 g for 10 min. The supernatants were filtered through 0.2 μm syringe filters to 2-mL vials prior to high-performance liquid chromatography (HPLC) measurements.

2.5.3. Effects of gas purging, particle size and solid loading on ethanol production from raw SBP

Two purging gases, CO_2 and He, were used to purge the cultures for 5 min prior to fermentation to drive trapped air (oxygen) out of the cultures. Fermentation without purging was also conducted as a control. Raw SBP was ground at 10,000 rpm for 5 min in a blender and pressed through a 20-mesh screen. The fermentations of both ground and unprocessed SBP were compared to test the effect of size reduction on the ethanol yield. In the tests of purging gas and particle size, solid loading was controlled at 4% (w/w, dry basis). SBP solid loading within the range of 2–12% (w/w, dry basis) were tested in this study to determine the proper solid loading level for *E. coli* KO11 fermentation. Since the type of purging gas and size reduction were found to have no significant effect on the SBP fermentation, neither purging nor size reduction was used in the test of solid loading. Other fermentation conditions were described in Section 2.5.2.

2.5.4. SSF vs. separated hydrolysis and fermentation (SHF) and fed-batch vs. batch on fermentation of raw SBP for ethanol production

In SHF (when indicated), enzymatic hydrolysis was performed with 12% autoclaved SBP solid (w/w, dry basis) in the first 24 h at 50 °C and pH = 5.0 (1 M HCl and NaOH were used if necessary) without the addition of *E. coli* KO11 inoculum. Other hydrolysis procedures and conditions were described in the Section 2.4. Before inoculation (*E. coli* KO11 inoculum was prepared by using the procedure described in Section 2.5.1), the hydrolyzates were cooled to 37 °C, and the pH of the hydrolyzate was adjusted to 6.0 using 1 M sterilized sodium phosphate buffer and/or 10 M NaOH. In SSF, the same levels of enzymes, SBP solid loading, and *E. coli* KO11 inoculum were used as in SHF, except that the hydrolysis and fermentation occurred simultaneously in SSF. The detailed procedures and conditions of SSF were described in Section 2.5.2.

Both fed-batch and batch were conducted in the SSF process for comparison. Fed-batch was started from 6% (w/w, dry basis) solid loading; enzymes were loaded based on the 6% solid loading (not 12% as batch); and *E. coli* KO11 inoculum was loaded based on the final target solid loading of 12% (w/w, dry basis). 7.5 g wet SBP in 1 M sodium phosphate buffer was added every 6 h during the first 24 h to bring the final solid loading to 12%. Neither fresh enzymes nor *E. coli* KO11 inoculum was added with the fresh SBP during fed-batch. In the batch process, the 12% solid loading (w/w, dry basis) was loaded with corresponding enzymes and *E. coli* KO11 inoculum at the beginning of fermentation, not loaded stepwise like fed-batch. Other fermentation procedures and conditions were described in Section 2.5.2.

2.5.5. Fermentation of ensiled SBP for ethanol production

SBP silage obtained from the 20-L ensilage experiment with a packing density of 0.96 g/cm^3 and without LAB inoculum was used in this study to determine if certain treatments (washing, sterilization, etc.) are needed prior to ethanol fermentation using *E. coli* KO11. Ensiled samples were treated using four different methods, including (1) washing followed by sterilization, (2) washing without sterilization, (3) non-washing with sterilization, and (4) non-washing and non-sterilization. Fermentations of raw SBP with and without sterilization were also conducted as controls. In the washing process, the ensiled samples were mixed with DI water using a stir bar at room temperature for 30 min with the solid-to-water ratio of 1:10. Washing was stopped by filtering the samples through a glass filter paper on a Büchner funnel. The sterilization of samples was performed by autoclaving at 121 °C for 20 min. The SBP samples prepared through different methods were fermented using *E. coli* KO11 with 4% (w/w) solid loading. Other fermentation conditions are described in the Section 2.5.2.

2.5.6. Plate count analysis during *E. coli* KO11 fermentation of ensiled SBP

Standard plate counts (colony forming unit, CFU) were performed in duplicate by withdrawing samples from fermenters at 24-h intervals during the fermentation of ensiled SBP as described

in Section 2.5.5. Cultures were prepared in serial dilutions and aliquots were plated on LB medium containing 20 g/L glucose solidified with 1.5% (w/v) agar with and without chloramphenicol. The amount of chloramphenicol in plates was 40 mg/L.

2.6. Analytical methods

Dry matter was determined by drying 1 g samples at 103 °C in a convection oven for 24 h. Dried samples were ignited in a muffle furnace at 550 °C for 3 h for ash content measurement [25]. Nitrogen content was measured by following the Kjeldahl method, and crude protein was calculated as N × 6.25 [26]. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined by the procedures developed by Goering and Van Soest [27]. Hemicellulose and cellulose were calculated as (NDF-ADF) and (ADF-ADL). The pectin content was determined as galacturonic acid by the protocol proposed by Ahmed and Labavitch [28] and Melton and Smith [29].

Ensiled samples were diluted 1/10 (w/w) in water and stirred for 30 min prior to measurement. The mixtures were centrifuged at 7700 g for 10 min, and then the supernatant was filtered through 0.22 μm polytetrafluoroethylene (PTFE) filter paper. The resulting filtrates were used to measure pH, organic acids, ammonia, ethanol, and WSC. The pH was measured using a pH electrode (Accumet Model 20, Fishersci, Pittsburgh, PA). Organic acids, glucose, cellobiose and ethanol were measured using a HPLC (Shimadzu, Columbia, MD) equipped with Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm, Hercules, CA). A refractive index detector (RID-10A, Shimadzu, Columbia, MD) was used to identify sugars and ethanol, and a UV detector (SPD-20A Prominence, Shimadzu, Columbia, MD) was used to identify organic acids. The mobile phase was 5 mM sulfuric acid. The separation temperature and mobile phase flow rate were 60 °C and 0.6 mL/min, respectively. A wavelength of 215 nm was used on the UV detector. Ammonia was determined using an ion-selective electrode (Accumet ammonia electrode, model 95-12, Fisher Scientific, Pittsburgh, PA). The pH of extract filtrates was adjusted to 13 by adding 10 M sodium hydroxide before ammonia measurement [30]. WSC levels were determined as glucose by the phenol-sulfuric acid method developed by Dubois et al. [31].

The activities of cellulase and β-glucosidase were quantified based on FPU and CBU, respectively [32]. Pectinase acitivity, PGU, was analyzed by the methods developed by Dalal et al. [33] and Bailey and Pessa [34].

2.7. Data analysis

Data analysis was performed using JMP 7.0 (SAS Institute Inc., Raleigh, NC, 2008). The significance levels of different treatments were determined by analysis of variance (ANOVA) and least significant difference (LSD) ($\alpha = 0.05$).

3. Results and discussion

3.1. SBP storage by 20-L ensilage

Ensiled samples were removed from reactors, mixed thoroughly, and analyzed for dry matter, pH, organic acids (lactic acid, acetic acid, butyric acid, and iso-butyric acid), ethanol, ammonia, and WSC. Butyric acid, iso-butyric acid and ammonia were used to indicate the activity of undesirable microorganisms such as enterobacteria and *Clostridium* spp. [35–37].

Both LAB 137 and the control were able to decrease pH below 4.5 during ensilage (Table 1). Acidic conditions benefit the inhibition of undesirable *Clostridium* spp., enterobacteria and/or yeast fermentations which usually lead to the degradation of carbohydrates and lactic acid and deteriorate silage quality [35–38]. The concentrations of acetic acid, ammonia, ethanol, and WSC are not significantly different among different treatments. The control and LAB 137 have similar results for the concentration of lactic acid that silage with packing density of 0.96 g/cm^3 has numerically, but not statistically higher lactic acid concentration than the other packing densities. Silage quality under the control treatment without LAB inoculation was not significantly different from that inoculated with LAB, indicating that SBP can be ensiled without LAB inoculation. Little butyric and iso-butyric acids were detected, suggesting that undesirable organisms, such as enterobacteria and *Clostridium* spp. were inhibited. In addition, silage quality was homogeneous throughout each bag in the vertical direction (data not shown), indicating consistent ensiling occurred within the 20-L bags.

3.2. Enzymatic hydrolysis of ensiled SBP

While ensilage is a promising storage technology for SBP, it is unknown whether low pH and the presence of compounds that accumulate during ensilage would positively or negatively affect enzymatic activity during hydrolysis. Water washing studies were

Table 1
Effects of inoculum and packing density on SBP silage quality at 20-L scale. [a]

Inoculum	Packing density (g/cm^3)	pH	Amount change during ensilage (mg/g initial dry SBP)[b]						
			Lactic acid	Acetic acid	Propionic acid	Total organic acid	Ammonia	Ethanol	WSC reduction[c]
Control (no LAB inoculation)	0.48	3.93 AB (0.00)	28.44 AB (1.17)	37.20 A (0.35)	9.76 A (0.29)	75.40 AB (1.70)	1.617 A (0.208)	16.16 A (0.09)	11.71 A (1.83)
	0.72	4.03 A (0.05)	28.70 AB (0.99)	37.60 A (1.41)	9.95 A (0.10)	76.25 AB (0.61)	1.453 A (0.078)	16.22 A (0.67)	10.39 A (0.86)
	0.96	3.91 B (0.04)	33.00 A (2.02)	34.69 A (1.11)	9.99 A (0.23)	77.68 A (1.09)	1.896 A (0.255)	15.81 A (0.62)	11.82 A (2.38)
LAB 137	0.48	3.94 AB (0.03)	27.51 B (0.61)	37.87 A (0.40)	9.66 AB (0.11)	75.03 AB (0.56)	1.933 A (0.489)	17.08 A (0.26)	11.77 A (2.54)
	0.72	3.94 AB (0.04)	24.43 B (3.53)	37.74 A (3.87)	9.00 B (0.40)	71.16 C (1.59)	1.542 A (0.201)	16.11 A (1.20)	13.11 A (1.87)
	0.96	3.95 AB (0.03)	28.92 AB (0.21)	35.07 A (0.89)	9.59 AB (0.19)	73.58 BC (1.20)	1.945 A (0.287)	17.45 A (1.29)	12.45 A (0.89)

[a] All data but pH were corrected by the initial values; the numbers in parentheses are standard deviation; values not connected by the same letter (A, B and C) are significantly different ($p \leqslant 0.05$).

[b] Amount change (mg/g initial dry SBP) = [final concentration (mg/g final dry SBP) × final dry SBP (g)-initial concentration (mg/g initial dry SBP) × initial dry SBP (g)]/initial dry SBP (g).

[c] WSC is consumed during ensilage, therefore, the amount change in WSC represents reduction.

designed to determine if washing improved hydrolysis rates and sugar yields of ensiled SBP upon enzymatic hydrolysis.

Washing with higher water-to-solid ratios resulted in slightly increased reducing sugar concentration (Fig. 1). After 168 h of hydrolysis, the reducing sugar concentration for non-washed SBP silage was 30 mg/mL, which was not significantly different from that observed in SBP silage washed using a water-to-solid ratio of 5:1. Although SBP silage washed using a 50:1 ratio had the highest final reducing sugar concentration of 38 mg/mL, it did not achieve a significantly higher hydrolysis yield compared with SBP silage washed with ratios of 15:1, 20:1, and 30:1. The difference (1 mg/mL) between the 1:10 ratio and the 1:15 ratio is numerically small. Therefore, the ratio of 10:1 would likely be sufficient and practical to remove the inhibitors in the SBP silage and improve the enzymatic hydrolysis of ensiled SBP.

3.3. Effect of gas purging and particle size reduction on ethanol production

The presence of LB medium in batch fermentation significantly increased ethanol concentration by 6 mg/mL as shown in Section 3.5. For this reason LB medium was used for all fermentation experiments in this study. As shown in Fig. 2a and b, no significant difference was observed among the tested purging gases on the yield of ethanol and lactic acid. CO_2 purge led to the highest acetic acid concentration after 72 h fermentation (Fig. 2c). SBP size reduction did not result in a significant difference in the yields of ethanol, acetic acid or lactic acid. As a result, gas purging and size reductions were omitted from additional fermentation studies. SBP fermentations using *E. coli* KO11 exhibited rapid fermentation; the 24-h ethanol concentration reached over 90% of the final concentration of 7 mg/mL. After 72 h, negligible ethanol was produced although the concentration of lactic and acetic acids still increased until 120 h. The final ethanol yield reached 0.2 g ethanol/g-dry SBP. The ratio between ethanol, lactic acid and acetic acid under conditions leading to maximum ethanol yield at 72 h was approximately 3:1:1.

3.4. Effect of SBP solid loading on ethanol production

Ethanol concentration increased from 3 to 14 mg/mL as SBP solid loading was changed from 2% to 10%, while ethanol concentration dropped to 9 mg/mL at 12% solid loading (Fig. 3a). The decrease in ethanol production with 12% solid loading may have been due to glucose inhibition of *E. coli* KO11 [39]. For all solid loading levels, the ethanol concentration leveled off after 72 h of fermentation. Lactic acid and acetic acid concentrations increased slightly with elevated SBP solid loading (Fig. 3b and c). However, 12% solid loading achieved the highest acetic acid and lactic acid concentration after 168 h. *E. coli* KO11 is capable of fermenting galacturonic acid in SBP to acetic acid [22]. It is likely that the increasing levels of SBP resulted in an increase in fermentation of galacturonic acid and accumulation of acetic acid.

There is no significant difference in the ethanol yield among the different solid loadings. The final ethanol yield was 0.2 g ethanol/g-dry SBP. In addition, the ratio between ethanol, lactic acid and acetic acid (in that order) increased with the increase in solid loading. With 10% solid loading, the ratio between ethanol, lactic acid and acetic acid was 1:0.03:0.13 while this ratio became 1:0.07:0.5 when solid loading decreased to 2%. Therefore, 10% SBP solid loading was used for further fermentation studies.

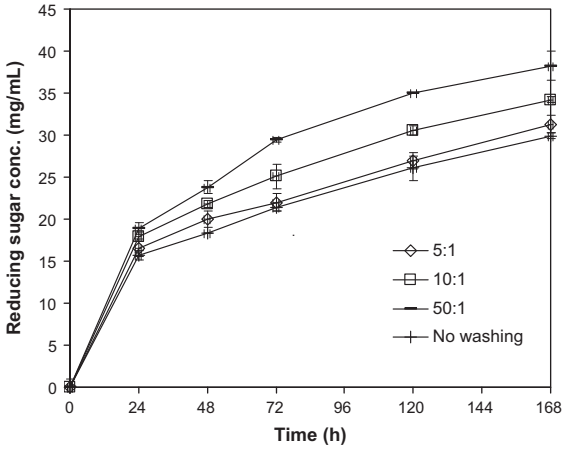

Fig. 1. Effect of water washing on enzymatic hydrolysis of ensiled SBP (For clearer view, the data for 1:15, 1:20 and 1:30 were not shown).

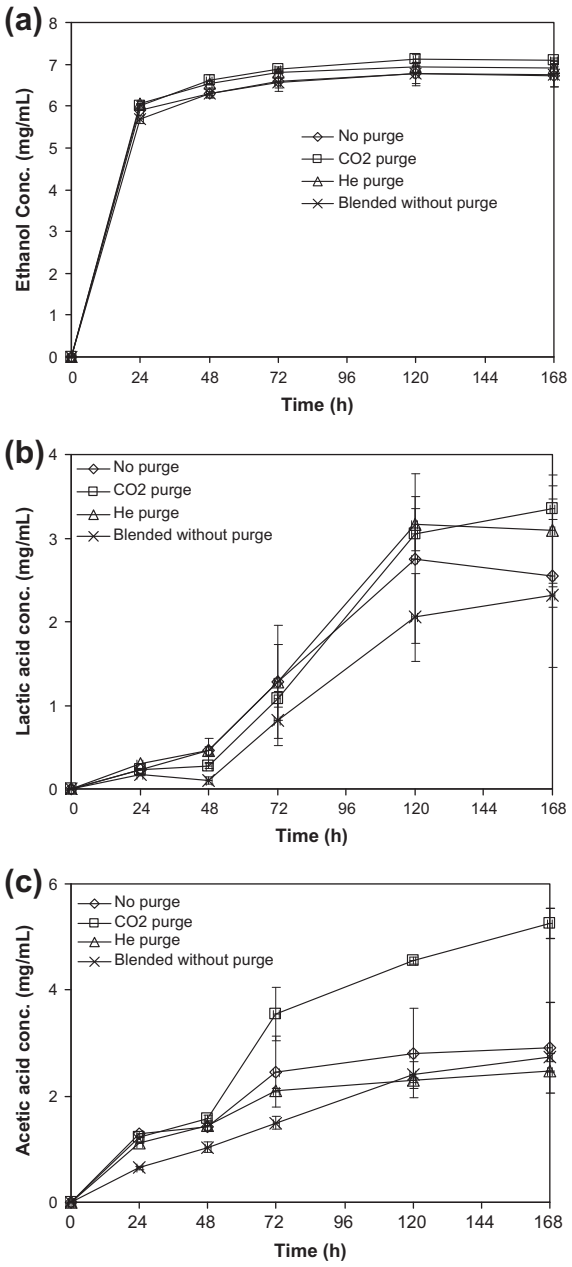

Fig. 2. Effect of gas purging and SBP size reduction on *E. coli* KO11 fermentation.

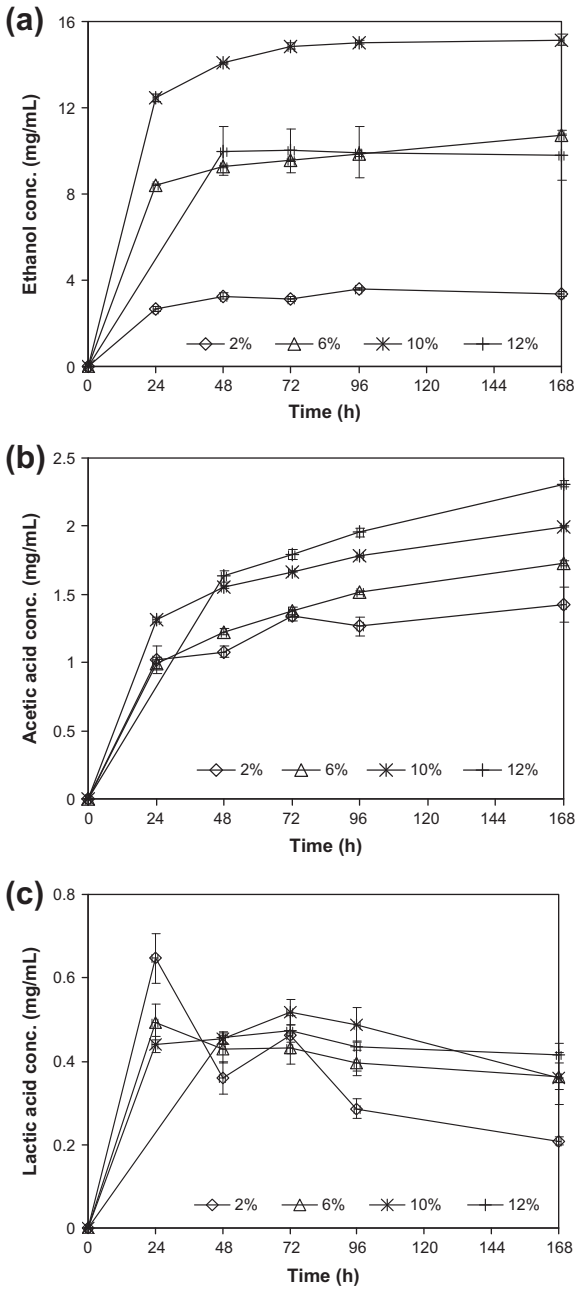

Fig. 3. Effect of solid loading on the SBP fermentation by *E. coli* KO11 (For clearer view, the data for 4% and 8% solid loading were not shown).

3.5. Effect of fermentation mode on ethanol production from SBP

High-solid loading is more desirable than low-solid loading during either enzymatic hydrolysis or fermentation because of higher sugar and ethanol concentrations and lower product recovery and equipment costs. However, high-solid loading has inherent problems such as mixing and sugar inhibition. To overcome these problems, fed-batch SSF was used to conduct SBP fermentation for ethanol production with *E. coli* KO11. Our previous results showed that fed-batch operation reduced the requirements for enzyme dosage while maintaining similar sugar yield (unpublished data). Therefore, ethanol yields from fed-batch fermentation of SBP were compared with those from batch fermentation.

SBP enzymatic hydrolysis prior to *E. coli* KO11 fermentation in SHF facilitated mixing. However, it increased the maximum

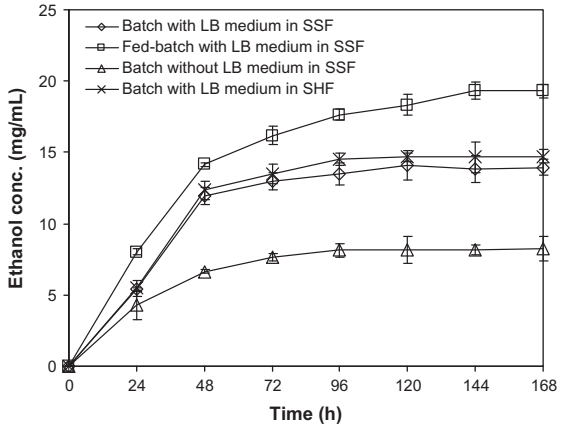

Fig. 4. Effect of fermentation scheme on ethanol production by *E. coli* KO11.

ethanol yield by only 1 mg/mL compared with SSF (Fig. 4). Additionally, fed-batch fermentation achieved higher ethanol yield (5-6 mg/mL more ethanol) and required half as much enzyme in *E. coli* KO11 fermentation of SBP compared to batch fermentation. Using fed-batch SSF instead of SHF to mitigate problems with mixing and product inhibition of enzymes during fermentation could potentially reduce equipment and overall process costs.

3.6. Fermentation of ensiled SBP for ethanol production by E. coli KO11

The necessity of various treatment processes for ensiled SBP prior to down-stream conversion was investigated. SBP samples were ensiled in 20-L bags for 90 days. Biochemical conversion of ensiled SBP into ethanol was performed using *E. coli* KO11 fermentation with enzyme supplementation. In fermentation of ensiled SBP, sterilization did not affect ethanol yield. However, sterilization did enhance ethanol yield for raw SBP. Washing significantly decreased the maximum ethanol concentration for ensiled SBP (Fig. 5). The results demonstrate that the ensiling process can significantly improve ethanol yield and that washing and sterilization of ensiled SBP are unnecessary with respect to ethanol production. To better understand the effects of sterilization and washing, further research is needed to study microorganism populations in ensiled SBP.

E. coli KO11 density was not necessarily related to ethanol yield. For example, in fermentation of ensiled non-washed sterilized SBP,

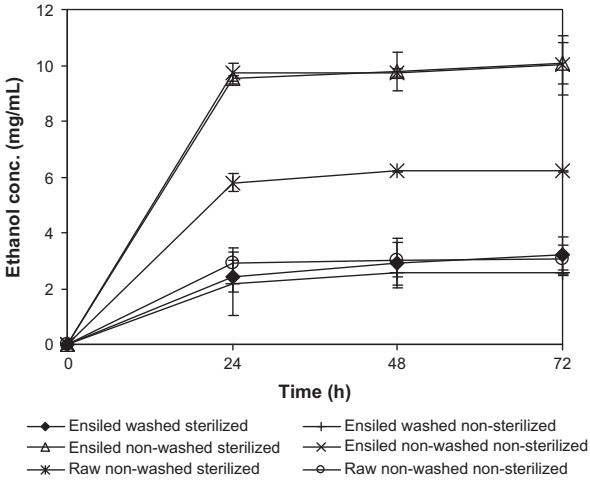

Fig. 5. Ensiled SBP fermentation by *E. coli* KO11 for ethanol production.

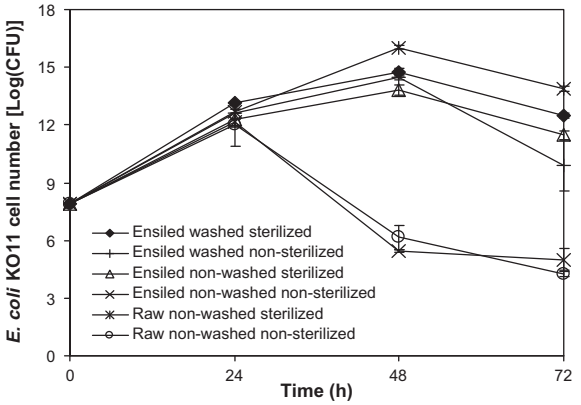

Fig. 6. Plate count of *E. coli* KO11 colony forming units per mL of culture broth during fermentation of ensiled SBP.

the *E. coli* KO11 population increased during the first 48 h, but ethanol yield leveled off after 24 h (Fig. 6). For the fermentation of ensiled non-washed non-sterilized SBP, the *E. coli* KO11 population decreased after 24 h while achieving similar ethanol yield to the ensiled non-washed sterilized SBP. One possible explanation is that enzymes were inhibited after 24 h of fermentation, resulting in little additional sugar release from enzymatic hydrolysis after this point. More research is needed to determine the reasons underlying these results.

4. Conclusions

LAB inoculation is unnecessary for obtaining good SBP silage quality. Higher packing density yields slightly better silage quality. Neither gas purging nor size reduction is needed for *E. coli* KO11 fermentation. Increasing SBP solid loading from 2% to 10% results in increased ethanol yield and decreased yields of by-products. Fed-batch achieves much higher ethanol yield than batch at 50% lower enzyme dosage. Ensiled SBP has higher reducing sugar yield than raw SBP upon enzymatic hydrolysis, but needs water washing. In fermentation of ensiled SBP using *E. coli* KO11, sterilization is unnecessary and washing significantly decreases ethanol yield of ensiled SBP. Ensilage could be used to not only store biomass, but also pretreat biomass to enhance biofuel yield. Based on ethanol yields presented here (0.2 g ethanol/g SBP) and SBP yields of 1.6 million dry tons per year in the US [7], SBP could provide 411,900 m^3 (108 million US gallons) of ethanol per year. The trade off between using SBP for liquid fuel production rather than animal feed could be a potential drawback and would need to be considered in an overall economic analysis of biofuel production from SBP.

Acknowledgements

The funding for this research was provided by Chevron Technology Ventures. The authors also wish to thank the Department of Viticulture and Enology at University of California, Davis for providing lactic acid bacteria for this research, Burt Vannuchi for construction of ensilage racks, and Kameron Chun for assistance with HPLC maintenance.

References

[1] Hoover FA, Abraham J. A comparison of corn-based ethanol with cellulosic ethanol as replacements for petroleum-based fuels: a review. Int J Sust Energy 2009;28:171–82.
[2] Cherubini F, Jungmeier G. LCA of a biorefinery concept producing bioethanol, bioenergy, and chemicals from switchgrass. Int J Life Cycle Assess 2010;15:53–66.
[3] Zaldivar J, Nielsen J, Olsson L. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 2001;56:17–34.
[4] Micard V, Renard CM, Thibault JF. Enzymatic saccharification of sugar-beet pulp. Enzyme Microb Technol 1996;19:162–70.
[5] Michel F, Thibault JF, Barry JL. Preparation and characterisation of dietary fibre from sugar beet pulp. J Sci Food Agric 1988;42:77–85.
[6] Haley S. Sugar and sweeteners outlook. <http://www.ers.usda.gov/publications/sss/2010/Feb/SSSM258.pdf> [accessed September 2010].
[7] Doran JB, Caripe J, Sutton M, Foster B. Fermentations of pectin-rich biomass with recombinant bacteria to produce fuel ethanol. Appl Biochem Biotechnol 2000;84–86:141–52.
[8] Rorick RE, Nabar N, Pryor S. Enzymatic hydrolysis and fermentation of sugar beet pulp. Reno, Nevada, 2009; paper No. 096193.
[9] Coons FF. Dried pulp. In: McGinnis RA, editor. Beet–Sugar Technology. Denver, CO: Beet Sugar Development Foundation; 1982. p. 587–99.
[10] Olsen E. The fermentation of sugar beet pulp silage. J Gen Microbiol 1951;5:817–29.
[11] Pirmohammadi R, Rouzbehan Y, Rezayazdi K, Zahedifar M. Chemical composition, digestibility and in situ degradability of dried and ensiled apple pomace and maize silage. Small Rum Res 2006;66:150–5.
[12] Pirmohammadi R, Golgasemgarebagh A, Azari AM. Effect of ensiling and drying of white grape pomace on chemicals composition, degradability and digestibility for ruminants. J Anim Vet Adv 2007;6:1079–82.
[13] Holzer M, Mayrhuber E, Danner H, Braun R. The role of *Lactobacillus buchneri* in forage preservation. Trends Biotechnol 2003;21:282–7.
[14] Megías MD, Hernández F, Cano J, Martinez-Teruel A, Gallego JA. Effects of different additives on the cell wall and mineral fractions of artichoke (*Cynara scolymus* L) and orange (*Citrus aurantium* L) by-product silage. J Sci Food Agric 1998;76:173–8.
[15] Murphy PT, Moore KJ, Richard TL, Bern CJ, Brumm TJ. Use of iodoform to improve lactic acid production in the biomass ensilage conversion system. OR: Portland. ASABE; 2006; paper No. 067004.
[16] Dolezal P, Pyrochta V, Dolezal J. Effects of chemical preservative and pressing of ensiled sugar-beet pulp on the quality of fermentation process. Czech J Anim Sci 2005;50:553–60.
[17] Vervaeren H, Hostyn K, Ghekiere G, Willems B. Biological ensilage additives as pretreatment for maize to increase the biogas production. Renewable Energy 2010;35:2089–93.
[18] Passoth V, Eriksson A, Sandgren M, Ståhlberg J, Piens K, Schnürer J. Airtight storage of moist wheat grain improves bioethanol yields. Biotechnol Biofuels 2009;2:16.
[19] Zheng Y, Yu CW, Cheng YS, Zhang RH, Jenkins MB, VanderGheynst J. Effects of ensilage on storage and enzymatic hydrolysis degradability of sugar beet pulp. Bioresour Technol 2010;102:1489–95.
[20] Micard V, Renard CMGC, Thibault JF. Influence of pretreatments on enzymic degradation of a cellulose-rich residue from sugar-beet pulp. LWT–Food Sci Technol 1997;30:284–91.
[21] Spagnuolo M, Crecchio C, Pizzigallo MDR, Ruggiero P. Synergistic effects of cellulolytic and pectinolytic enzymes in degrading sugar beet pulp. Bioresour Technol 1997;60:215–22.
[22] Grohmann K, Baldwin EA, Buslig BS, Ingram LO. Fermentation of galacturonic acid and other sugars in orange peel hydrolysates by the ethanologenic strain of Escherichia coli. Biotechnol Lett 1994;16:281–6.
[23] Ruppel KA. Effect of bunker silo management on hay crop nutrient management. M.S. Thesis, Cornell University, Ithaca, NY; 1992.
[24] Moniruzzaman M, York SW, Ingram LO. Effects of process errors on the production of ethanol by *Escherichia coli* KO11. J Ind Microbiol Biot 1998;20:281–6.
[25] American Society of Testing and Materials, D4444-92. Standard test method for direct moisture content measurement of wood and wood-base materials. Designation: ASTM D4444-92 (Reapproved 1997). ASTM, West Conshohocken, PA; 1997. p. 517–21.
[26] AOAC (Association of Official Analytical Chemist). Official methods of analysis, association of official analytical chemists. 15th ed. Gaithersburg, USA: AOAC Press; 1990.
[27] Goering HK, Van Soest PJ. Forage fiber analyses (apparatus, reagents, procedures and some applications). Agriculture Handbook No. 379. USA: USDA-ARS, Washington, DC; 1970.
[28] Ahmed AER, Labavitch JM. A simplified method for accurate determination of cell wall uronide content. J Food Biochem 1997;1:361–5.
[29] Melton LD, Smith BG. Determination of the uronic acid content of plant cell walls using a colorimetric assay. Curr Protoc Food Anal Chem 2001:E3.3.1–4.
[30] APHA. Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, DC; 1998.
[31] Dubois M, Gilles KA, Hamilton JK, Roberts DA, Smith F. Colorimetric method for the determination of sugars and other related substances. Anal Chem 1956;28:350–6.
[32] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987;59:257–68.
[33] Dalal S, Sharma A, Gupta MN. A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities. Chem Cent J 2007;1:1–6.

[34] Bailey MJ, Pessa E. Strain and process for production of polygalacturonase. Enzyme Microb Technol 1990;12:266–71.
[35] Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH, Spoelstra SF. Microbiology of ensiling. In: Buxton DR, Muck RE, Harrison JH, editors. Silage science and technology. Madison, WI: American Society of Agronomy; 2003. p. 31–93.
[36] Saarisalo E, Skytta E, Haikara A, Jalava T, Jaakkola S. Screening and selection of lactic acid bacteria strains suitable for ensiling grass. J Appl Microbiol 2007;102:327–36.
[37] Woolford MK. The Silage Fermentation. New York: Marcel Dekker, Inc.; 1984.
[38] Richard TL, Proulx S, Moore KJ, Shouse S. Ensilage technology for biomass pretreatment and storage. CA: Sacramento. ASABE; 2001. paper No. 01-6019.
[39] Olsson L, Hahn-Hägerdal B, Zacchi G. Kinetics of ethanol production by recombinant *Escherichia coli* KO11. Biotech Bioeng 1994;45:356–65.