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Ensilage and Bioconversion of Grape Pomace into Fuel Ethanol

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ABSTRACT: Two types of grape pomace were ensiled with eight strains of lactic acid bacteria (LAB). Both fresh grape pomace (FrGP) and fermented grape pomace (FeGP) were preserved through alcoholic fermentation but not malolactic conversion. Water leaching prior to storage was used to reduce water-soluble carbohydrates and ethanol from FrGP and FeGP, respectively, to increase malolactic conversion. Leached FeGP had spoilage after 28 days of ensilage, whereas FrGP was preserved. Dilute acid pretreatment was examined for increasing the conversion of pomace to ethanol via *Escherichia coli* KO11 fermentation. Dilute acid pretreatment doubled the ethanol yield from FeGP, but it did not improve the ethanol yield from FrGP. The ethanol yields from raw pomace were nearly double the yields from the ensiled pomace. For this reason, the recovery of ethanol produced during winemaking from FeGP and ethanol produced during storage of FrGP is critical for the economical conversion of grape pomace to biofuel.

KEYWORDS: *bioethanol, dilute acid pretreatment, ensilage, grape pomace*

INTRODUCTION

Grape pomace is the solid residue that remains after grape processing to juice and wine. It contains skin, pulp, seeds, and stems. The wine industry produces large quantities of pomace; 122 000 tons of dry pomace are produced per year in California.[1] Management strategies are needed that yield valuable products from residual pomace while accommodating compositional variation. Typically, pomace is composted as fertilizer,[2,3] processed into animal feed,[4,5] or extracted for grape seed oil and polyphenols.[6] However, these applications have limited markets and can absorb only a small portion of the waste generated.[7] As a result, alternative uses are needed to add value to grape pomace. Conversion of grape pomace into biofuels, such as fuel ethanol and biogas, is a promising possibility.

One of the challenges facing utilization of pomace is that it is produced seasonally. Despite the importance of pomace storage, little research has been conducted in this area. Drying is a common way to facilitate storage of biomass, but adds to the cost of storage, especially in temperate climates.[8] Furthermore, dry storage presents the risk of accidental or spontaneous combustion.[9,10] Moreover, drying may be unnecessary for some applications, such as conversion into biofuels and biobased products through anaerobic digestion or microbial fermentation, as these processes occur under aqueous conditions. Ensilage, the storage of wet or partially dry biomass, is an alternative to dry storage.[5,11] Ensilage aims to prevent deterioration, conserve biomass, and minimize carbohydrate degradation by facilitating lactic acid fermentation under moist, anaerobic conditions. Biomass can be preserved for years under these conditions.[8] Ensilage is a common approach to preserve forage (alfalfa, corn stover, grape pomace, etc.) for animals in agriculture and has been used to improve the nutrient availability of grape pomace used for ruminant feed.[4,5] Moreover, ensilage can be used to both preserve and pretreat biomass feedstocks for downstream conversion processes. For instance, ensilage improved the quality of particleboard derived from corn stover[12] and enhanced ethanol yield from fermented wheat grain compared to dry grain.[13]

In this study, ensilage was investigated as a means to preserve and enhance the digestibility of fresh and fermented grape pomace for conversion to biofuels. Particular attention was paid to pomace composition. Pomace from red wine production contains residual yeast biomass and ethanol in addition to fermented grape material. Alternately, pomace from white wine production contains higher levels of water-soluble carbohydrates (WSC) and less ethanol. High levels of WSC and ethanol in biomass, as are likely present in grape pomace, may create initial ensilage conditions that do not promote lactic acid bacteria (LAB) dominance and traditional ensilage via lactic acid fermentation. In response to this possibility, leaching of grape pomace in water was studied as a means to decrease potentially disruptive WSC and ethanol levels prior to ensilage. Additionally, acidic conditions are critical for preventing the growth of undesirable spoilage microorganisms and promoting fermentation of biomass by acidophilic bacteria during ensilage. Ideally, the pH should decrease to 4 for ensilage of biomass with high moisture content or 5 for biomass with moisture content below 50%. To promote a rapid decrease in pH, chemical additives (e.g., formic acid, acidic acid, and sulfuric acid)[14] or lactic acid bacteria inoculants have been added to

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biomass at the onset of ensilage. In addition to accelerating acid production to inhibit growth of detrimental microorganisms, inoculation of biomass with LAB may further control the microbial population by conferring antimicrobial properties to silage[15] and outcompeting other microorganisms for free sugars.[16] In light of these observations, pH and fermentation product concentrations were studied in response to LAB inoculation during grape pomace ensilage.

In addition to pomace storage, fermentation of ensiled pomace into ethanol was investigated as a potential avenue for adding value to grape pomace. Like many lignocellulosic biomass feedstocks, grape pomace may require pretreatment prior to enzymatic hydrolysis and fermentation for ethanol production. Dilute acid is a common pretreatment method and is effective for feedstocks ranging from hardwoods and softwoods to grasses, agricultural residues, municipal solid wastes, and animal wastes.[17] Dilute acid pretreatment was studied as a possible method for enhancing grape pomace conversion to ethanol. In addition, little information is available regarding simultaneous saccharification and fermentation (SSF) of grape pomace for ethanol production, and essentially no research has been done concerning the conversion of ensiled grape pomace into ethanol. As a result, SSF was performed using both raw and ensiled grape pomace, and final ethanol yields were compared.

MATERIALS AND METHODS

Grape Pomace. Grape pomace was collected from Sutter Home Winery (St. Helena, Napa, CA, USA) in October 2007 and frozen upon collection until use. Grape pomace used for 1200 L ensilage was obtained in August 2009. Fresh grape pomace (FrGP) and fermented grape pomace (FeGP) were obtained from white and red wine production, respectively. FrGP contains stems, seeds, and grape skins, as only juice is fermented to produce white wine. In contrast, grape skins and other solids are present during fermentation in the production of red wine, resulting in FeGP. FeGP had a lower WSC content compared to FrGP, as shown in Table 1 (for GP samples collected in 2007).

Table 1. Chemical Composition of Grape Pomace[a]

chemical component	FeGP (wt %, dry basis)	FrGP (wt %, dry basis)
cellulose	14.5	9.2
hemicellulose	10.3	4.0
pectin	5.4	5.70
lignin	17.2	11.6
protein	14.5	7.0
WSC	2.7	49.1
ash	8.0	12.6
total C	48.2	44.3
total N	2.5	1.2

[a]Both FeGP and FrGP were collected in 2007 and the as-received moisture contents were 66.4% and 65.5%, respectively.

Lactic Acid Bacteria Preparation. Eight LAB strains, including three heterofermentative and five homofermentative strains (Table 2), were examined for ensilage of FrGP and FeGP. LAB strains were provided by the Department of Viticulture and Enology at the University of California, Davis. As-received LAB cultures were transferred from plates to 5 mL of *Lactobacillus* deMan Rogosa Sharpe (MRS) medium and incubated overnight at 28 °C with a shaking speed of 140 rpm; 2.5 mL of LAB culture was then transferred to 50 mL of MRS medium under the same cultivation conditions. LAB stock cultures were created by mixing culture broth [optical density (OD) = 0.5 at 590 nm] and glycerol [15% (v/v) final concentration] and were stored at −80 °C. To prepare LAB inocula for ensilage, bacteria were grown in MRS using the aforementioned culture conditions until the OD at 590 nm reached 0.5. Cells were harvested at 4 °C by centrifugation at 11000*g* for 5 min (IEC MultiRF centrifuge, model 120, Thermo Electron Corp., Milford, MA, USA). The cell pellet was washed twice in 0.1 M sodium phosphate buffer (pH 7.0) to remove residual medium. Washed cell pellets were resuspended in sterilized deionized (DI) water, adjusted to an OD at 590 nm of 0.5, and held on ice until use.

Table 2. Lactic Acid Bacteria Investigated for Ensiling Grape Pomace[a]

lactic acid bacteria	source	type of fermentation
Lactobacillus plantarum B38 (LAB 1)	unknown	homofermentative
Lactobacillus plantarum L11a1 (LAB 2)	unknown	homofermentative
Lactobacillus plantarum 8014 (LAB 3)	unknown	homofermentative
Lactobacillus brevis NRRL B-1836 (LAB 120)	olives, fermenting	heterofermentative
Lactobacillus buchneri NRRL B-1837 (LAB 123)	tomato pulp	heterofermentative
Lactobacillus fermentum NRRL B-4524 (LAB 137)	beets, fermented	heterofermentative
Pediococcus pentosaceus (LAB 222)	plants	homofermentative
Lactobacillus gramminis B-14857 (LAB 247)	silage, grass	homofermentative

[a]Homofermentation ferments hexoses predominately to lactic acid; heterofermentation ferments hexoses to lactic acid and other products such as ethanol and acetic acid.

Leaching of Grape Pomace. FeGP and FrGP were leached in water to remove ethanol and WSC, respectively, prior to ensilage. Leaching was conducted with a solid-to-liquid ratio of 1:20 (g/g) at 25 °C for 2 h with an agitation speed of 150 rpm. The leached pomace was recovered by filtration using a 100 mesh screen, squeezed by hand to remove residual water, and stored in a refrigerator at 4 °C until use.

Bench-Scale Ensilage of Grape Pomace for LAB Screening. LAB strains (Table 2) were inoculated onto nonleached grape pomace by spraying resuspended LAB onto pomace at a rate of 10^6 CFU/g dry matter (DM). Grape pomace was inoculated with sterile DI water for negative controls. The final moisture content for each inoculated pomace was approximately 70%. Inoculated pomace was mixed in sealed bags, and then 6 g (dry weight) was packed into 50 mL tubes at a packing density of 0.75 g/mL. Each tube contained 10 mL of headspace. Tubes were capped and incubated at 26 °C for 28 days. Tubes were sampled at 0, 3, 7, 14, and 28 days for pH, organic acids, WSC, and ethanol measurements.

For grape pomace to be ensiled following leaching, pomace was inoculated with *Lactobacillus brevis* NRRL B-1836 (120), *Lactobacillus buchneri* NRRL B-1837 (123), and *Lactobacillus fermentum* NRRL B-4524 (137) using conditions similar to nonleached pomace.

1200 L Scale Ensilage of Grape Pomace. FeGP and FrGP in these experiments were collected in 2009 and directly packed into plastic bags in the field without LAB inoculation. Bags were sealed and stored in 1800 L plastic containers outside in a covered area in Davis, CA, for 1 year. Samples were withdrawn from containers at 0, 30, and 365 days for measurement of pH, organic acids, ethanol, and WSC. The ensiled samples were directly fermented into ethanol with *Escherichia coli* KO11 using the procedures described under Dilute Acid Pretreatment and Fermentation of Grape Pomace to Ethanol.

Dilute Acid Pretreatment and Fermentation of Grape Pomace to Ethanol. *Pretreatment.* Raw FrGP and FeGP (nonleached and nonensiled pomace collected in 2007) were pretreated in a 1 L benchtop Parr reactor (model 4525, Parr Instrument Co., Moline, IL, USA). The pretreatment mixture was composed of 10 wt % grape pomace (dry weight basis) and 1 wt % H_2SO_4. Pretreatment was performed at 120 °C for 5 min. After

Table 3. Average Ethanol Concentrations, Lactic Acid Concentrations, and pH Values during FeGP Ensiling[a]

	ethanol (mg/g DM)				lactic acid (mg/g DM)				pH			
lactic acid bacteria	0 days	7 days	14 days	28 days	0 days	7 days	14 days	28 days	0 days	7 days	14 days	28 days
Lactobacillus plantarum B38 (LAB 1)	132.22	122.91	124.61	101.06B	0.031	0.037	0.039	0.047A	3.56	3.53	3.49	3.49A
Lactobacillus plantarum L11a1 (LAB 2)	147.45	144.37	167.04	143.61AB	0.034	0.036	0.033	0.039A	3.47	3.49	3.50	3.50A
Lactobacillus plantarum 8014 (LAB 3)	149.52	130.06	130.58	120.41AB	0.045	0.031	0.043	0.035A	3.54	3.48	3.48	3.48A
Lactobacillus brevis NRRL B-1836 (LAB 120)	168.22	152.99	143.55	160.72A	0.011	0.017	0.028	0.030A	3.56	3.51	3.49	3.47A
Lactobacillus buchneri NRRL B-1837 (LAB 123)	133.39	139.61	130.70	127.46AB	0.025	0.038	0.026	0.043A	3.57	3.55	3.54	3.56A
Lactobacillus fermentum NRRL B-4524 (LAB 137)	138.81	140.57	133.34	147.91A	0.025	0.034	0.026	0.034A	3.58	3.47	3.53	3.51A
Pediococcus pentosaceus (LAB 222)	147.38	140.52	130.63	129.40AB	0.019	0.031	0.055	0.038A	3.52	3.57	3.56	3.52A
Lactobacillus gramminis B-14857 (LAB 247)	149.23	129.03	142.15	118.12AB	0.022	0.020	0.035	0.038A	3.52	3.55	3.54	3.47A
control	154.00	155.41	151.90	138.55AB	0.030	0.023	0.035	0.033A	3.51	3.50	3.57	3.59A

[a]The same uppercase letters in the same column of 28 days indicate no significant difference among treatments ($\alpha = 0.05$). Statistical analysis results for 0 days, 7 days, and 14 days are not shown.

pretreatment, a portion of pretreated pomace was washed with distilled water using a Buchner funnel until the pH of the filtrate reached 5−6. The pretreated and washed pomace was stored in a refrigerator at 4 °C, whereas the remaining slurry was directly stored in a refrigerator without washing or solid−liquid separation. The slurry and pretreated/washed pomace were used in fermentation for ethanol production.

Microorganism and Inoculum Preparation. The ethanologenic strain *E. coli* KO11 was used in all fermentation experiments. *E. coli* KO11 carries the genes *pdc* and *adhB* from *Zymmonas mobilis* integrated into the chromosome. Additional genetic changes have been made to minimize byproduct formation. *E. coli* KO11 was purchased from the American Type Culture Collection ATCC 55124. The culture stock was stored in 15% glycerol at −80 °C.

Inoculum was prepared as a mixture of Luria−Bertani (LB) (10 g/L tryptone, 5 g/L of yeast extract, and 5 g/L NaCl) broth and 5% (w/w) glucose solution in a 1:1 (v/v) ratio. *E. coli* KO11 was inoculated (1:50, v/v) into the mixture of LB broth and glucose solution and incubated at 37 °C for 12−16 h until the final OD at 590 nm of the culture reached 1.5. Cells were then harvested by centrifugation and washed in sterile DI water three times. The cell pellet was resuspended to original concentration in sterilized DI water until use.

Enzyme Preparation. Cellulase, β-glucosidase, and pectinase were used for saccharification. Cellulase (Cellulclast 1.5L) and β-glucosidase (Novozyme 188) were obtained as gifts from Novozymes Inc. (Davis, CA, USA). Pectinase (Pectinex 3XL) was purchased from Sigma Inc. (St. Louis, MO, USA). Enzyme stock solutions were sterilized by filtration before use.

Fermentation. Samples for fermentation included (1) ensiled FrGP from the 1200 L ensilage experiment, (2) pretreated and washed FrGP solids, (3) pretreated and washed FeGP solids, (4) pretreated FrGP slurry (pomace solids and acid hydrolysate), (5) pretreated FeGP slurry, (6) raw FrGP, and (7) raw FeGP. Grape pomace samples for fermentation (4 wt %, dry basis) and LB medium without glucose were neutralized to pH 7 using 10 M NaOH solution and then autoclaved at 121 °C and 15 psi for 20 min. Filter-sterilized cellulase, β-glucosidase, and pectinase were added to achieve 15 FPU/g solid, 15 CBU/g solid, and 60 PGU/g solid, respectively. *E. coli* KO11 inoculum was added at a rate of 0.5 mg cells/g dry pomace into 250 mL fermenters. The fermentation pH was buffered to 7.0 using 1 M sodium phosphate. The final working weight was 50 g, including pomace, buffer, LB medium, enzymes, and *E. coli* KO11 inoculum. Fermentation occurred at 37 °C with 140 rpm agitation. Aliquots of 1.5 mL were withdrawn from the liquid phase of the fermentation periodically over 72 or 168 h. Aliquots were centrifuged at 11000*g* for 10 min, supernatants were filtered through 0.2 μm syringe filters, and filtrates were used for analysis.

Analytical Methods. Ensiled pomace samples were analyzed for dry weight, pH, lactic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, ethanol, ammonia, and WSC. Dry matter was measured by drying 1 g of sample at 103 °C in a convection oven for 24 h.[18] The ash of samples was determined by igniting the samples in a muffle furnace at 550 ± 25 °C for 3 h.[19] Nitrogen content was measured according to the Kjeldahl method,[20] and crude protein was calculated as N × 6.25. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined according to the procedures of Vogel et al.[21] Hemicellulose and cellulose were calculated as (NDF − ADF) and (ADF − ADL), respectively. Pectin content was measured according to the methods developed by Ahmed and Labavitch[22] and Melton and Smith[23] using galacturonic acid as a standard.

Ensiled pomace was extracted in DI water for 30 min using a water-to-solid ratio of 10:1 (w/w). Extracts were centrifuged at 11000*g* for 10 min, and supernatants were filtered through 0.22 μm PTFE filter paper. Filtrates were used to measure pH, organic acids, ammonia, ethanol, and WSC. Organic acids and ethanol were measured using high-performance liquid chromatography (HPLC) (Shimadzu, Columbia, MD, USA). An ion exchange analytical column (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm) was used for separation. A refractive index detector (RID-10A, Shimadzu) and a photodiode array detector (SPD-M20A, Shimadzu) were used for identifying ethanol and organic acids, respectively. Before HPLC measurement, filtrates were acidified with 0.5 μL of 1 M sulfuric acid. 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. Ammonia was quantified using an ion-selective electrode (Accumet ammonia electrode, model 95-12, Fisher Scientific, Pittsburgh, PA, USA). The pH of extract filtrates was adjusted to 13 by adding 10 M sodium hydroxide prior to ammonia measurement.[24] WSC was analyzed by following the phenol−sulfuric acid method using glucose as a WSC standard.[25]

The activities of cellulase and β-glucosidase were quantified as FPU and CBU, respectively.[26] Pectinase activity was analyzed following the methods developed by Bailey and Pessa[27] and Dalal et al.[28]

Data Analysis. Treatment effects were analyzed using analysis of variance (ANOVA) and least significant difference (LSD) ($\alpha = 0.05$ and $p_{critical} = 0.05$) methods. JMP 8.0 software (version 8.0; SAS Institute, Raleigh, NC, USA) was used to perform statistical analyses. All treatments were performed in triplicate in this study unless specified, otherwise.

RESULTS AND DISCUSSION

LAB Screening Studies for Ensilage of Fermented and Fresh Grape Pomace. pH, ethanol, lactic acid, and volatile

Table 4. Average Ethanol Concentrations, Lactic Acid Concentrations, and pH Values during FrGP Ensiling[a]

	ethanol (mg/g DM)				lactic acid (mg/g DM)				pH			
lactic acid bacteria	0 days	7 days	14 days	28 days	0 days	7 days	14 days	28 days	0 days	7 days	14 days	28 days
Lactobacillus plantarum B38 (LAB 1)	8.66	160.67	131.37	99.21B	0.089	0.837	2.679	2.567AB	3.77	3.79	3.84	3.82AB
Lactobacillus plantarum L11a1 (LAB 2)	8.79	138.96	122.52	115.81AB	0.060	0.444	2.612	2.895AB	3.75	3.71	3.72	3.70B
Lactobacillus plantarum 8014 (LAB 3)	5.61	119.63	111.34	137.61AB	0.053	0.371	2.594	3.246AB	3.78	3.83	3.89	3.82AB
Lactobacillus brevis NRRL B-1836 (LAB 120)	7.11	153.46	149.22	161.11A	0.083	0.457	2.598	3.475A	3.86	3.80	3.83	3.87A
Lactobacillus buchneri NRRL B-1837 (LAB 123)	4.67	124.67	126.95	111.50AB	0.074	0.463	3.709	3.210AB	3.84	3.81	3.83	3.83AB
Lactobacillus fermentum NRRL B-4524 (LAB 137)	4.20	149.00	148.27	136.67AB	0.062	0.553	3.219	3.340A	3.80	3.80	3.91	3.85A
Pediococcus pentosaceus (LAB 222)	2.26	119.19	96.09	99.87B	0.086	0.321	1.479	2.513AB	3.83	3.85	3.82	3.81AB
Lactobacillus gramminis B-14857 (LAB 247)	4.91	110.07	115.53	124.99AB	0.076	0.583	2.900	3.304A	3.84	3.78	3.81	3.80AB
control	5.58	134.70	132.75	110.68AB	0.035	0.188	1.667	2.153B	3.90	3.78	3.78	3.77AB

[a]The same uppercase letters in the same column of 28 days indicate no significant difference among treatments ($\alpha = 0.05$). Statistical analysis results for 0 days, 7 days, and 14 days are not shown.

fatty acids (acetic acid, propionic acid, isobutyric acid, and butyric acid) concentrations were measured over 28 days during FeGP ensilage (Table 3). Both butyric/isobutyric acid and ammonia were used to indicate the activity of undesirable microorganisms such as enterobacteria and *Clostridium* spp., which can consume carbohydrates and lactic acid and negatively affect silage quality.[29−31] FeGP silage exhibited high initial ethanol concentrations of approximately 140 mg/g DM, and these levels did not significantly change during ensilage. Similarly, lactic acid, pH (Table 3), and volatile fatty acid (VFA) concentrations (data not shown) remained constant throughout ensilage. Organic acid concentrations were low, and neither isobutyric acid nor butyric acid was detected. Final pH, ethanol, lactic acid, and VFA concentrations were not significantly affected by inoculation using any of the LAB strains tested (Table 3). The high ethanol concentration and/or phenolic compounds in FeGP may have prevented the growth of lactic acid bacteria. Although FeGP was not preserved by ensilage, the ethanol already present in the raw material and low pH were sufficient to inhibit microbial growth during the 28 day screening study. Therefore, FeGP could be directly sealed for long-term storage without any pretreatment. FeGP could also be used as an antibacterial reagent in the costorage with other perishable biomass materials. For example, FeGP could be stored with FrGP so that the WSC in FrGP could be rapidly fermented into ethanol by the yeast (from wine fermenters) in the FeGP. For biofuel production, the native ethanol in FeGP could be recovered prior to the treatment of residue, which can be used for cellulosic ethanol production. However, ethanol-fermenting microbes should be selected carefully when FeGP is used to produce cellulosic ethanol because it has antibacterial properties.[32]

Ethanol and lactic acid concentrations rose during the first 7−14 days of FrGP ensilage for both control and LAB-inoculated treatments. For all treatments, ethanol production occurred during the first 7 days of ensilage, with initial levels of approximately 5 mg/g DM elevating to steady-state levels between 110 and 160 mg/g DM (Table 4). Lactic acid production occurred over the first 14 days of ensilage. The largest increase in lactic acid concentration was from 0.08 to 3.5 mg/g DM for FrGP silage treated with *L. brevis* NRRL B-1836. However, statistically equivalent increases were observed for other LAB treatments and the control. WSC decreased from 450 mg/g DM to approximately 50 mg/g DM in all FrGP silages (data not shown). The pH of all FrGP silages did not change significantly during ensilage, remaining at the initial level of approximately 3.9 (Table 4), which is sufficient to inhibit most microbial activity. Low levels of isobutyric acid and butyric acid were detected, indicating that *Clostridium* and enterobacteria, which are common spoilage organisms of forage silage, were successfully inhibited.[16]

The presence of specific epiphytic flora, high initial WSC, and low pH of FrGP likely facilitated ethanolic fermentation over lactic acid fermentation in FrGP silages. For example, high initial WSC could have promoted yeast growth over LAB growth as yeast can more rapidly utilize WSC for fermentation compared to LAB.[33] The native grape microflora consists of many yeast strains (e.g., *Torulaspora delbrueckii* and *Debaryomyces hansenii*) that exhibit limited growth and are eventually replaced by *Saccharomyces cerevisiae* during fermentation.[34−36] *S. cerevisiae* has greater ethanol tolerance relative to many types of yeast and has been suggested to completely suppress and outcompete indigenous yeast flora during ensilage.[7,34] *S. cerevisiae* is usually used under pH 3.5 in industrial ethanol fermentation and may adapt better to the low pH environment in silage compared to LAB.[37] Further studies are needed to measure the natural microflora succession during FrGP ensilage.

On the basis of the metrics measured, the control ensilage lacking exogenous LAB was equivalent to other LAB-inoculated treatments. Conventional forage silage relies on lactic acid fermentation conducted by epiphytic or inoculated LAB under anaerobic conditions. In the case of FrGP silage, ethanol as well as lactic and/or VFA could have affected the microbial flora and the stability of silage. For biofuel production, the ethanol produced during ensilage could be recovered by fractionation and the residue used for conversion to cellulosic ethanol.

Effect of Leaching on the Ensilage of Grape Pomace. On the basis of the ensilage results, the initial high concentration of WSC and ethanol in FrGP and FeGP, respectively, may have prevented conventional forage-type silage. Water leaching was examined previously to remove WSC and ethanol from FrGP and FeGP, respectively, to improve ensilage of pomace.[38] The results from the prior study demonstrated that ethanol present in FeGP and WSC present

in FrGP could be effectively removed and recovered using a water leaching process.[38]

To examine whether leaching improved storage of pomace, leached FeGP was ensiled for 28 days using LABs 120, 123, and 137. Unleached and leached FeGP without LAB inoculation were also tested as controls. Spoilage due to mold growth was observed in all ensiled FeGP samples except for unleached FeGP. One possible reason for leached FeGP spoilage was that the concentration of WSC was too low (<0.5 mg/g DM) to support the growth of LAB. In turn, undesirable microorganisms were able to dominate because of removal of ethanol. Therefore, although the leaching process allowed for recovery of ethanol, it was not compatible with ensilage of FeGP.

Leached FrGP was ensiled for 28 days following inoculation with LABs 120, 123, and 137. Unleached and leached FrGP without LAB inoculation were also tested as controls. Unlike ensilage of leached FeGP, little visible mold growth was observed during ensilage of leached FrGP. The pH of ensiled unleached FrGP remained constant at approximately 4.0 over the 28 day period of ensilage, whereas the pH dropped during ensilage of leached pomace regardless of LAB inoculation (Table 5). Initial WSC levels were much greater in unleached FrGP relative to leached FrGP with and without LAB inoculation. However, at the conclusion of ensilage, WSC concentration in leached FrGP dropped to a level similar to the unleached treatments. Unleached FrGP contained lower levels of organic acids and ammonia following ensilage compared to leached FrGP. However, ethanol concentration in ensiled unleached FrGP was >4 times greater than that observed in any leached treatment (Table 6).

Table 5. pH of Leached FrGP during Ensilage

	pH		
treatment	0 days	3 days	28 days
unleached	3.97	3.96	4.05
leached without LAB inoculation	4.14	4.03	3.91
leached with LAB 120 inoculation	4.14	4.07	3.85
leached with LAB 123 inoculation	4.14	4.03	3.89
leached with LAB 137 inoculation	4.14	4.08	3.81

The leaching process removed approximately 75% of the WSC, which could significantly benefit lactic acid production. Lactic acid concentration was 16 times higher in leached FrGP compared to nonleached FrGP. Without LAB inoculation, butyric acid concentration in leached FrGP silage was higher than that of other FrGP silages. As a result, LAB inoculation of FrGP may be necessary to prevent growth of spoilage microbes such as *Clostridium* spp. and achieve optimal storage.

1200 L Scale Ensilage of Grape Pomace and Conversion of Silage to Ethanol. For 1200 L scale ensilages of both FrGP and FeGP, the pH remained stable and relatively small increases in lactic acid were observed after 1 year of storage (Table 7). Ensiled FrGP and FeGP differed with respect to several analyte concentrations. Acetic acid accumulated to low levels in FrGP compared to FeGP. Butyric acid was undetectable throughout ensilage of FrGP, but was observed to decrease slightly in FeGP over the course of ensilage. Initial WSC levels were much greater in FrGP compared to FeGP. However, WSC declined to undetectable levels for both by the end of ensilage. Ethanol concentration rose over the course of ensilage for FrGP. However, higher levels were observed after 30 days of ensilage compared to 365 days, suggesting that ethanol concentration peaked at some point prior to the conclusion of storage. Alternately, the initial ethanol concentration of FeGP was relatively high, comparable to that of ensiled FrGP after 1 year of ensilage, but, unlike FrGP, ethanol levels decreased during ensilage. The possible reason for the reduction of ethanol concentration could be ethanol evaporation from incompletely sealed bags in relatively hot weather. Ethanol, whether present from yeast fermentation during ensilage or as wine process residue, was effective in protecting FrGP and FeGP from spoiling. Ethanol could be recovered from FrGP and FeGP either before or after storage. However, ethanol loss through evaporation prevented efficient storage of pomace and recovery of ethanol.

Raw FrGP and FeGP and ensiled FrGP underwent SSF using *E. coli* KO11 for ethanol production. For all grape pomace samples, the ethanol concentration peaked at 24 h of SSF and remained constant thereafter (Figure 1). Raw FrGP had the highest ethanol concentration at approximately 0.39 g/g DM, whereas fermentation of ensiled FrGP produced <0.02 g/gDM ethanol. Similar to FrGP, raw FeGP yielded much higher ethanol concentration, approximately 0.12 g/g DM, compared to the ensiled FeGP, which resulted in negligible ethanol yield during SSF (data not shown). Acetic acid and lactic acid levels in all treatments did not change significantly throughout SSF (data not shown). The ethanol yield from ensiled FrGP was much lower than raw FrGP. It is possible that the immediately fermentable WSCs within the pomace were completely consumed during ensilage, making them unavailable for later fermentation into ethanol. In light of this possible explanation, pretreatment of ensiled grape pomace may be required to improve its biodegradability for ethanol production.

Table 6. Concentrations of Organic Acids, Ethanol, Ammonia, and WSC of Leached FrGP at the 28th Day of Ensilage

						WSC (mg/g DM)	
treatment	lactic acid (mg/g DM)	acetic acid (mg/g DM)	butyric acid (mg/g DM)	ethanol (mg/g DM)	ammonia (mg/g DM)	initial	final
unleached	0.80	2.63	1.52	138.95	0.08	491.52	14.07
leached without LAB inoculation	13.58	14.63	8.06	32.60	0.46	101.21	9.08
leached with LAB 120 inoculation	13.50	14.55	3.79	33.17	0.24	101.21	11.17
leached with LAB 123 inoculation	13.67	15.08	5.58	32.64	0.32	101.21	10.81
leached with LAB 137 inoculation	12.36	15.85	2.00	31.66	0.21	101.21	10.93

Table 7. Analyte Concentrations for 1200 L Scale Ensilage of Grape Pomace

feedstock	time (days)	pH	lactic acid (mg/g DM)	acetic acid (mg/g DM)	butyric acid (mg/g DM)	ethanol (mg/g DM)	WSC (mg/g DM)
FrGP	0	3.84	0.38	ND[a]	ND	35.8	155.6
	30	3.64	1.21	3.60	ND	120.2	2.20
	365	3.67	2.31	3.00	ND	94.10	ND
FeGP	0	3.48	ND	ND	3.50	94.44	8.10
	30	3.48	ND	0.95	0.40	96.10	2.12
	365	3.44	5.51	41.46	2.25	56.35	ND

[a]ND, not detectable.

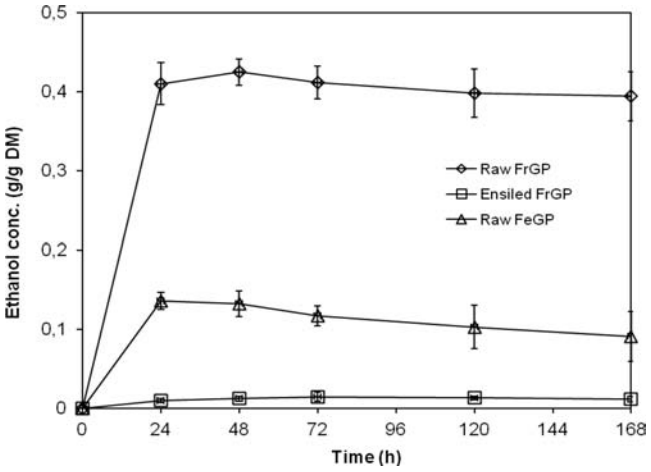

Figure 1. SSF of ensiled grape pomace (1 year of ensilage at 1200 L scale) for the production of ethanol.

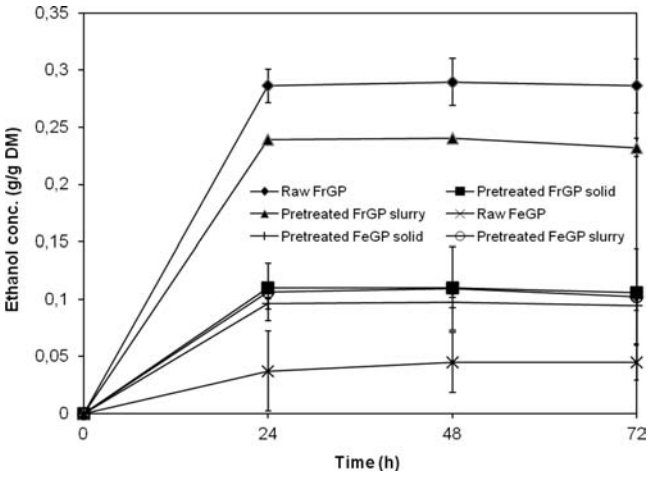

Figure 2. Ethanol concentrations from SSF of grape pomace.

Dilute Acid Pretreatment and Fermentation of Grape Pomace for Ethanol Production. Pretreated FrGP and FeGP were examined for ethanol production. Ethanol concentrations peaked and remained constant after 24 h for all treatments (Figure 2). FrGP had a much higher ethanol yield than FeGP under the same conditions. For FrGP, the highest ethanol yield was 0.29 g/g DM with fermentation of raw FrGP, followed by FrGP slurry (0.24 g/g DM) and pretreated washed FrGP solid (0.11 g/g DM). The ethanol yield of raw FrGP in this section is slightly different from that presented under 1200 L Scale Ensilage of Grape Pomace and Conversion of Silage to Ethanol. Differing collection times for raw pomace samples may have contributed to observed differences. Similar differences were observed for raw FeGP.

FrGP is the residue of white wine production in which the grape is squeezed and only the juice is used for the fermentation process. The residual sugars, 49.1% of the total FrGP solid (Table 1), were readily fermented by *E. coli* KO11. In addition, the pectin in raw FrGP, which would be hydrolyzed during acid pretreatment, could also contribute to ethanol production through the fermentation of galacturonic acid by *E. coli* KO11. Pretreatment may have been too harsh for FrGP resulting in the degradation of sugars. This might explain why raw FrGP had a higher ethanol yield than the treated FrGP slurry. The pretreated and washed FrGP solids likely consisted of cellulose for fermentation with few soluble sugars, which may be why this treatment achieved the lowest ethanol yield. Considering the previous results of FrGP ensilage, which demonstrated very high ethanol production during ensilage, the biofuel production strategy for FrGP could be solid (ensilage) or liquid fermentation of raw fresh FrGP and recovery of the ethanol, followed by utilization of fermented FrGP solids for other purposes.

Raw FeGP had the lowest ethanol yield at 0.05 g/g DM. With dilute acid pretreatment, the ethanol yields of FeGP were increased to 0.11 g/g DM for FeGP slurry and 0.10 g/g DM for pretreated and washed FeGP. Considering the fact that the as-received ethanol concentration of FeGP is about 0.16 g/g DM, the utilization of FeGP could start from recovery of ethanol trapped in FeGP prior to downstream pretreatment for additional ethanol production.

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