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Synergistic Ball Milling–Enzymatic Pretreatment of Brewer's Spent Grains to Improve Volatile Fatty Acid Production through Thermophilic Anaerobic Fermentation

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Abstract: Brewer's spent grain (BSG) as the major byproduct in the brewing industry is a promising feedstock to produce value-added products such as volatile fatty acids (VFAs). Synergistic ball mill–enzymatic hydrolysis (BM-EH) process is an environmentally friendly pretreatment method for lignocellulosic materials before bioprocessing. This study investigated the potential of raw and BM-EH pretreated BSG feedstocks to produce VFAs through a direct thermophilic anaerobic fermentation process without introducing a methanogen inhibitor. The highest VFA concentration of over 30 g/L was achieved under the high-solid loading fermentation (HS) of raw BSG. The synergistic BM-EH pretreatment helps to increase the cellulose conversion to 70%. Under conventional low TS fermentation conditions, compared to the controlled sample, prolonged pretreatment of the BSG substrate resulted in increased VFA yields from 0.25 to 0.33 g/g_{VS}, and butyric acid became dominant instead of acetic acid.

Keywords: brewer's spent grain; ball mill–enzymatic hydrolysis pretreatment; thermophilic anaerobic fermentation; volatile fatty acid profile

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1. Introduction

Brewer's spent grain (BSG) is a major byproduct of the brewing industry, with 40 million wet tons produced annually worldwide [1]. Due to its abundance in lignocellulosic fibrous material and relatively high protein content, BSG is commonly used as animal feed for cattle or poultry [2]. However, fresh BSG has a high moisture content and can perish during storage and transportation, while drying the wet BSG can be energy-intensive and costly. Currently, despite being used as animal feed and a small portion for biogas production, over 20% of BSG is not well-utilized and is disposed of in landfills, releasing millions of tons of CO_2 greenhouse gas equivalent and posing a threat to the environment. In recent years, biorefinery has been investigated as an alternative valorization route to convert this cheap and easily accessible biomass into multiple value-added compounds such as volatile fatty acids (VFAs), amino acids, and second-generation biofuels [2].

In recent years, the biosynthesis of VFAs from biomass has gained much interest in the biorefinery area. Acidogenic fermentation, as a part of the anaerobic digestion (AD) process, can be readily accomplished in well-established AD facilities while yielding products with higher value than biogas [3]. VFAs produced from such mixed-culture systems consist mainly of acetic, propionic, isobutyric, butyric, and valeric acids, which are key platform chemicals widely used in various conventional industries and are currently being researched as a C-source for the synthesis of bioproducts such as lipids [4] and bioplastics [5], among others. It has been found that when food waste is used as feedstock in an acidogenesis process, adjusting the initial substrate/inoculum (S/I) ratio to over three

can suppress methane production and achieve the highest VFA yield of 0.8 g/g_{VS} under optimized conditions [6].

Thermophilic conditions (>50 °C) have been found to facilitate a stable system under higher organic loading, as it enables better mass and heat transfer [3]. Furthermore, a higher temperature could lead to the physicochemical solubilization of the feedstock at the beginning and help build a greater population of cellulolytic and xylanolytic microbes that would boost VFA production under short hydraulic retention time (HRT) [7]. Similar conclusions have been drawn in previous research that shortening the sludge retention time and increasing the temperature efficiently converts microbial communities in the AD system towards accumulating specific VFAs instead of producing methane [8].

In recent years, there have been several studies exploring the production of VFA from BSG using mesophilic acidogenic fermentation systems under various conditions [9–11]. The results indicate that raw BSG can be used as a feedstock in fed-batch AD systems to produce VFA, with the main components being propionic, acetic, and butyric acid. However, due to differences in the origin of the BSG samples and AD conditions, the concentration and composition of VFA produced vary. For example, Sarkar and coworkers studied batch acidogenic fermentation of raw BSG and achieved a VFA recovery of 8.9 g/L rich in acetic and butyric acids under alkaline conditions of pH = 9 [10]. To maximize the utilization of lignocellulosic feedstocks such as BSG, efficient pretreatment processes are needed to improve the hydrolysis and biodegradability and to facilitate the release of fermentable sugars [12,13]. For instance, Guarda et al. [9] used sulfuric acid (3%) at 121 °C to pretreat the BSG sample before feeding the resulting supernatant (neutralized with $Ca(OH)_2$) into an expanded AD granular sludge bed reactor, achieving higher volumetric VFA productivity with a lower HRT of 2.5 d compared to a previous study [11].

In addition to the aforementioned chemical pretreatment methods using acid or alkaline, ball milling (BM) is a physical pretreatment method to reduce the material size to micro or even nano scale. The reduced particle size provides a high specific surface area and easy access for enzymes and microorganisms, thus facilitating the bioconversion process [12,14,15]. Martin-Sampedro et al. demonstrated that smaller lignocellulosic nanofibrils undergo more rapid and complete hydrolysis when incubated in multicomponent enzyme systems [16]. Therefore, a process intensification approach was developed that integrates ball milling and enzymatic hydrolysis (BM-EH) in a one-pot process, providing an environmentally friendly pretreatment process [17]. This BM-EH process was proved to significantly simplify the pretreatment process and enhance the efficiencies for releasing monosaccharides and the fermentation of different feedstocks such as solid digestate, corn stover, switchgrass, and miscanthus [17].

In this research, the synergistic BM-EH method was optimized for BSG pretreatment and compared with EH pretreatment method alone, with respect to polysaccharides' conversion. The raw and pretreated BSG were subjected to a direct thermophilic anaerobic VFA fermentation process without inducing any methanogen inhibitor. Based on the resultant VFAs profiles and yields, a three-cycle fermentation process was proposed to exhaust the VFAs production potentials by replacing the supernatant during fermentation. This research is the first to explore the whole process to produce VFAs from BM-EH pretreated BSG feedstocks in terms of pretreatment efficiency and the effect of different fermentation strategies.

2. Materials and Methods

2.1. Materials

Wet distillery stillage was collected from Wilderness Trail Distillery (Danville, KY, USA), filtered, and dried at 105 °C to obtain the BSG sample. The BSG sample was then grounded using a 0.75 qt food grinder (WARING COMMERCIAL®, McConnellsburg, PA, USA) and passed through a No. 7 sieve (2.8 mm) before being stored in an airtight glass jar at room temperature. The enzyme used for enzymatic hydrolysis was a mixture of cellulase (CTec2, Cellic®) and hemicellulase (HTec2, Cellic®). Both enzymes were from

Novozymes North America (Franklinton, NC, USA). A planetary ball mill (MSK-SFM-1S model, MTI corporation, Richmond, CA, USA) was applied for the ball milling process. The enzymatic hydrolysis process took place in an Innova® 42 incubator. The activated sludge was the effluent from an anaerobic digester operated by Quasar Energy Group, LLC (Wooster, OH, USA) that was fed with biosolids (sludge from wastewater treatment plant) and food wastes. The activated sludge was stored at 4 °C upon receipt.

2.2. BSG Pretreatment

The BSG sample underwent various processing methods, including enzymatic hydrolysis only (EH), one cycle (BM-EH-1) or two cycles (BM-EH-2) of BM-EH pretreatment, and a control group treated with autoclaving alone (CTRL), as illustrated in Figure 1. In brief, the dried BSG sample was first prepared to 15% paste with citrate buffer (0.1 M, pH = 5.0) in the ball mill jars and added with milling balls to a balls/wet biomass ratio of around 2.25 (w/w). Then, the whole jars were autoclaved at 121 °C for 30 min and cooled to room temperature in an ice bucket before adding enzymes. The sealing ring gaskets used to seal the jars were sterilized by wiping with 75% ethanol. The enzymatic hydrolysis process was carried out under control conditions of 50 °C and 150 rpm shaking. An enzyme mixture consisting of CTec2 and HTec2 at a volume ratio of 1:1 (v/v) and a protein dosage of 20 mg/g dry biomass was used. In each BM-EH cycle, the BSG paste was ball milled at 600 rpm for 3 h [17]. To prevent overheating at the high ball milling speed, an optimized strategy was implemented where every after 1 min of continuous ball milling, the machine ceased for 1 min to cool down. As a result, each BM-EH cycle lasted for a total of 6 h. The optimized ball milling strategy and enzyme mixture composition was determined based on previous experiments. In these experiments, the temperature of the BSG paste was monitored under different BM-rest settings and a typical enzyme mixture of CTec2: HTec2 = 9:1 (v/v) [17] was compared for the EH pretreatment part.

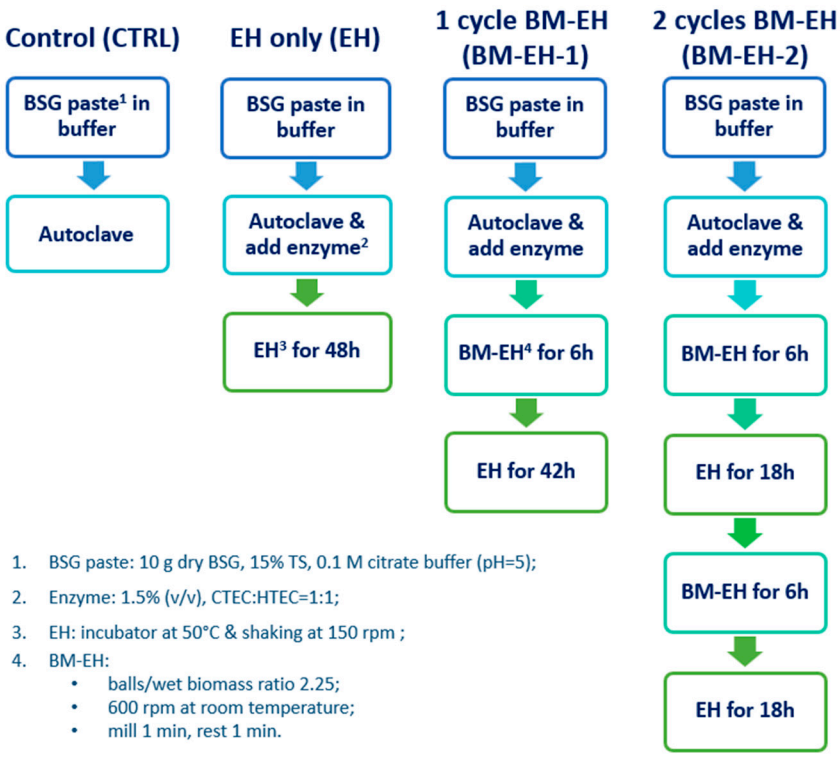

Figure 1. A flowchart of the experiments to pretreat brewer's spent grain (BSG) with an optimized ball milling–enzymatic pretreatment method (EH—enzymatic hydrolysis; BM—ball milling; BM-EH—synergistic ball milling and enzymatic hydrolysis for 1 or 2 cycles).

2.3. Characterization of the Raw and Pretreated BSG Samples

The total solid (TS) content (%) and volatile solid (VS) content (%) of the raw BSG, CTRL sample, and pretreated BSG samples were determined according to the standard methods examination of water and wastewater [18]. The CTRL and pretreated BSG samples were collected and weighed, then the TS weight (g) and VS weight (g) were calculated accordingly. To determine their compositions, a known weight (g) of each sample was centrifuged at 4500 rpm for 40 min, and the supernatant and precipitate were characterized separately. The supernatant was filtered through 0.2 µm nylon filter and the glucose, cellobiose, xylose, and arabinose concentrations were determined using a Dionex UltiMate 3000 HPLC (Dionex Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector and a Biorad Aminex HPX-87H column, applying 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min, and the column temperature was set to 50 °C. The collected precipitate was rinsed four times and dried in a freeze drier before weighing and other characterization. The rest of the samples were frozen at −20 °C for use in the following fermentation test.

2.3.1. Composition and Proximate and Ultimate Analysis

The structural carbohydrate composition—cellulose, hemicellulose, acid soluble lignin (ASL), and acid insoluble residue (AIR)—of the dried precipitates was measured according to an NREL laboratory analytical procedure [19]. Based on the weight of the wet samples and dried precipitates, the amount of each dissolved or precipitated carbohydrate component was calculated, and the percentage was calibrated accordingly based on raw BSG sample. The calibrated percentage of each component in all samples was calculated by the Equations (1)–(3), where P_i (%), P_j (%), and P_U (%) are the percentage of the precipitating component i (cellulose, hemicellulose, ASL, or AIR), the dissolved component j (glucose, cellobiose, xylose, or arabinose), and the undetected components U, respectively.

$$P_i = \frac{W_{d,s} \times C_{i,s}}{W_{d,r}} \times 100\% \quad (1)$$

$$P_j = \frac{(W_{w,s} - W_{d,s}) \times \frac{C_{j,s}}{F_j}}{W_{d,r}} \times 100\% \quad (2)$$

$$P_U = 1 - P_i - P_j. \quad (3)$$

The subscripts s and r represent the sample s (CTRL, EH, BM-EH-1, or BM-EH-2) and the raw BSG, respectively. W_w (g) is the weight of the wet sample s. W_d (g) is the weight of the freeze-dried sample s or r (raw BSG). C_i (%) is the percentage of component i in the precipitated sample s or r. C_j (g/mL) is the concentration of component j in the supernatant (assuming the density of supernatant to be 1000 kg/m^3). F_j is the stoichiometric conversion factor for specific monosaccharides such as glucose, cellobiose, and xylose/arabinose, which are 1.11, 1.055, and 1.14, respectively [17]. The conversion of cellulose and hemicellulose during pretreatment (CTRL, EH, BM-EH-1, or BM-EH-2) was calculated by Equations (4) and (5), respectively, with consistent parameter notation.

$$CNV_{cellulose,s} = \frac{(W_{w,s} - W_{d,s}) \times (C_{glucose,s}/F_{glucose} + C_{cellobiose,s}/F_{cellobiose})}{W_{d,r} \times C_{cellulose,r}} \times 100\%. \quad (4)$$

$$CNV_{hemicellulose,s} = \frac{(W_{w,s} - W_{d,s}) \times (C_{xylose,s} + C_{arabinose,s})/F_{xylose/arabinose}}{W_{d,r} \times C_{hemicellulose,r}} \times 100\%. \quad (5)$$

The content of hydrogen, carbon, and nitrogen in the freeze-dried samples, as well as the calibrated volatile matter, fixed carbon, and ash percentage on a dry weight basis, was

measured according to the standard proximate and ultimate analysis procedure (ASTM E870-82) [20].

2.3.2. Surface Morphology Analysis

The surface morphology of the BSG samples subjected to different pretreatment processes was assessed by comparing their respective Scanning Electron Microscopy (SEM) images. To investigate the effect of ball milling alone on the surface morphology, imaging was also performed on the BSG sample treated solely with ball milling. These samples underwent one or two cycles of ball milling, termed as BM-1 and BM-2, respectively (i.e., intermittently milled for 6 or 12 h at the optimized setting). The precipitate of the EH-induced samples (i.e., EH, BM-EH-1, and BM-EH-2) that were rinsed and freeze-dried, as well as the directly freeze-dried BSG samples (i.e., raw BSG, BM-1, and BM-2), was sputter-coated with an ultrathin gold layer and then analyzed using an FEI Quanta 20 FEG instrument at beam accelerating voltages of 5 kV. Furthermore, the surface area of the raw and the only BM-treated BSG samples was analyzed using the Brunauer–Emmett–Teller (BET) method based upon the N_2 adsorption–desorption isotherm. The analysis was conducted at 77 K using a Micrometrics ASAP2020 surface area and porosity analyzer. The raw BSG sample was further ground down to 0.5 mm before use in both morphology analyses to ensure accuracy.

2.3.3. Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Powder Diffraction (XRD)

The impact of ball milling alone and various pretreatment methods on the chemical fingerprinting of the BSG samples was determined using FTIR. Representative samples including the freeze-dried ground raw sample (<0.5 mm) and the sample BM-2, as well as the rinsed and freeze-dried sample CTRL, EH, and BM-EH-2, were selected. FTIR was conducted by a Thermo Nicolet Nexus 870 ATR-FTIR (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer, and the spectra were collected using an average of 32 scans over the wavenumber range between 650 and 4000 cm^{-1} with a spectral resolution of 1.93 cm^{-1}. Baseline correction was performed afterwards using the OMNIC 6.1a software. The powder X-ray diffraction patterns of samples of raw ground BSG (<0.5 mm), BM-1, and BM-2 were collected from 10 to 80° at 0.01°/step using Cu Kα X-ray energy produced from a Rigaku SmartLab system.

2.4. Thermophilic AD for VFA Production

The control and pretreated BSG samples were used in an anaerobic fermentation test to produce VFA. To eliminate the pH buffering effect induced by citrate buffer in the CTRL sample and evaluate the actual potential of raw BSG for VFA production, two additional conditions were included: a high-solid fermentation system using dried raw BSG (labeled as 'raw BSG (HS)') and a wet system using raw BSG slurry with the TS adjusted to be in the same range of the pretreated BSG samples (approximately 20% TS, labeled as 'raw BSG') using DI water. The TS and VS of the activated sludge (SLG) and all substrates, as well as the inoculated mixture before and the digestates after the fermentation experiments, were measured according to the standard methods for the examination of water and wastewater [18].

The substrate was inoculated with a fixed substrate/inoculum (S/I) ratio of 3.0 (on VS basis) for all conditions, following acclimation of the SLG inoculum at 55 °C for 1 day. For the raw BSG (HS) condition, 50 mL serum bottles were used, and each bottle was sealed with a rubber stopper and connected to a 1 L gas bag to collect any possibly produced biogas. Since a negligible amount of gas was collected during this test, the subsequent experiments were performed in disposable plastic centrifuge tubes with tightly screwed caps. All containers were placed in an incubator (435 model, Thermo Forma™, Thermo Scientific, Waltham, MA, USA) at 55 °C and were manually vortexed twice a day. This was necessary because the resulting TS values of the mixture after inoculated with sludge remained high, ranging from 12% to 14%, indicating an excessive thickness that impeded

proper flow and mixing under standard shaking conditions. Digestate samples were collected daily to monitor the TS, VS, pH, and VFA concentrations. The samples were centrifuged at 4500 rpm for 10 min, and the pH value and VFA concentrations in the supernatant were measured using the same method as described previously. The VFA concentration contributed by the activated sludge was deducted for all effluent samples.

In order to prevent acid accumulation in the system and enhance the VFA production, a three-cycle fermentation method was evaluated. After 2 days of fermentation and prior to acidification, the supernatant containing the VFAs was separated from the sedimented solids by centrifugation at 4500 rpm for 20 min. The VFA-rich supernatant was then collected, and the precipitate was washed with DI water and refilled with the same amount of DI water for the next two cycles of 2 days' fermentation (6 days in total). The supernatant from the original digestate and the washing effluent after each fermentation cycle were collected and analyzed for the VFA composition to calculate the overall VFA production.

2.5. Statistical Analysis

Statistical analysis was conducted using the data analysis tool in Microsoft Excel or in SigmaPlot 14.0. All experiments were run in replicate (n = 2 or 3). An analysis of variance (ANOVA) with a significance level of p-value less than 0.05 was conducted to compare the means.

3. Results and Discussion

3.1. Effects of the BM-EH Pretreatment on BSG to Liberate Monosaccharides

The BM treatment significantly increased the uniformity of the substrate slurry. The milling–rest cycle of either 1 min–1 min or 5 min–5 min strategy was proven to efficiently avoid overheating of the BSG paste, and the temperature remained below 35 °C under both conditions (Figure S1). Compared to the samples that underwent separate BM and EH pretreatment (BM + EH), the samples treated with the synergistic BM-EH process had a darker color and were more liquified. The conversion of cellulose was slightly higher when using the 1 min–1 min milling-rest settings compared to that of 5 min–5 min (Table S1). So, the 1 min–1 min BM strategy was selected for the following experiments.

It is worth noting that when the HTec to CTec ratio was (1:9), the conversion of hemicellulose was relatively low for all conditions (Table S1). Unlike most other raw lignocellulosic biomass or some food waste, the structural carbohydrate analysis of the dry BSG samples revealed that they had a higher hemicellulose content on a weight basis of dry matter (39.0 $\pm$ 0.5%) than cellulose (28.1% $\pm$ 0.5%). The measured values were consistent with the range of hemicellulose and cellulose content previously reported for BSG by Olszewski et al. [21], which was 21.8~40.2% for hemicellulose and 12.0~25.4% for cellulose. Similar findings were also reported by Zeraatkar Dehnavi [22], where the hemicellulose and cellulose content of the raw BSG samples were found to be in the range of 28.4~32.5% and 15.1~16.8%, respectively. Therefore, to enhance the effectiveness of the BM-EH method in breaking down the hemicellulose-rich BSG substrate, the HTec to CTec ratio was increased to 1:1.

The composition of the raw and pretreated BSG samples were calculated based on the initial total solids and are shown in Figure 2. The slight reduction in the cellulose, hemicellulose, and ASL found in the precipitated CTRL sample was consistent with the decrease in the fixed carbon percentage, which decreased from 16.1% to 9.0% in the precipitate (Table 1). The loss of fixed carbon, as well as the hydrogen, carbon, and nitrogen, contents in the precipitate may be attributed to the soaking process in pH = 5 buffer, which may have converted the mass to small amounts of soluble monosaccharides and other unknown compounds. The reduction in fixed carbon also contributed to the increase in the ash percentage in the precipitate, which was due to the calibration process. Overall, there were limited changes in the overall composition of BSG in the CTRL treatment considering both the solid and leachate.

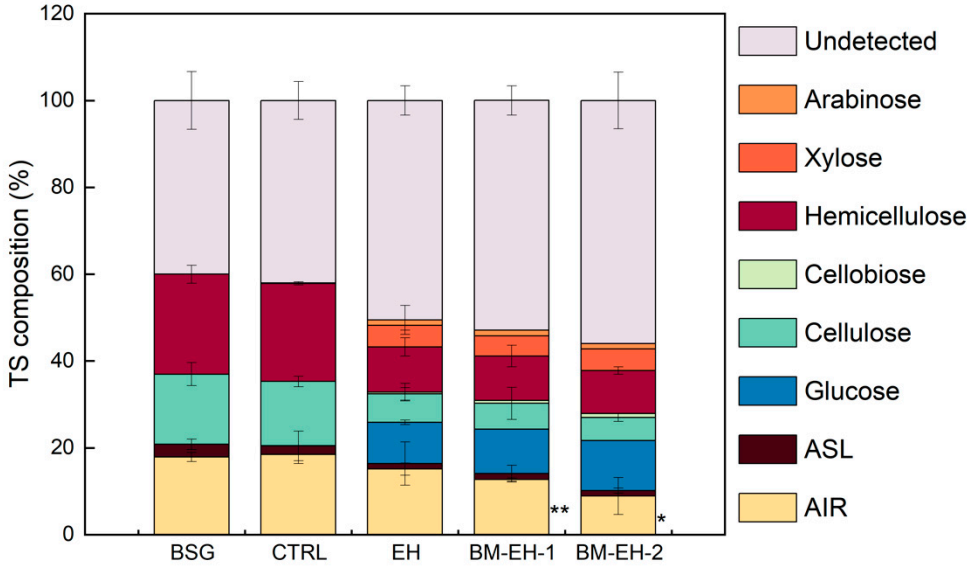

Figure 2. Composition of the BSG samples before and after different pretreatment processes (CTRL—control BSG sample subject with only autoclavation; ASL—acid soluble lignin; AIR—acid insoluble residue). Data are expressed as the mean ± SD of independent experiments. The statistical significance of the effects of the control and pretreatments on the AIR content was calculated vs. the raw BSG sample (* $p \leq 0.05$, ** $p \leq 0.01$).

Table 1. Characteristics of the raw, control, and pretreated BSG precipitates and the activated sludge (SLG) sample [1].

Component	BSG	CTRL	EH	BM-EH-1	BM-EH-2	SLG
Volatile Matter, % [2]	80.72 ± 1.18	83.60 ± 1.68	83.12 ± 0.81	81.84 ± 0.93	81.36 ± 0.70	57.89 ± 0.58
Fixed Carbon, % [2]	16.09 ± 0.19	8.99 ± 0.34	10.44 ± 0.16	11.42 ± 0.47	11.65 ± 0.13	1.65 ± 0.28
Ash, % [2]	3.18 ± 0.33	7.41 ± 0.49	6.44 ± 0.51	6.74 ± 0.09	6.99 ± 0.28	40.47 ± 0.48
Hydrogen, %	7.00 ± 0.10	6.64 ± 0.04	6.62 ± 0.17	6.63 ± 0.08	6.64 ± 0.06	5.30 ± 0.16
Carbon, %	47.35 ± 0.46	45.11 ± 0.25	44.61 ± 0.48	44.15 ± 0.13	43.95 ± 0.05	28.71 ± 0.07
Nitrogen, %	4.14 ± 0.10	3.82 ± 0.06	3.88 ± 0.07	3.78 ± 0.02	3.80 ± 0.04	4.03 ± 0.07

[1] Data shown are the average and standard deviation based on replicates. [2] All samples were freeze-dried before proximate and ultimate analysis, and the values were calibrated based on dry weight.

As indicated in Figure 3, the conversions of cellulose were 0.3%, 60.2%, 64.9%, and 70.3% for the CTRL, EH, BM-EH-1, and BM-EH-2 conditions, respectively. The cellulose conversion was increased when the synergistic BM-EH process was prolonged from 6 h to 12 h. However, the ball milling had an insignificant effect on the conversion of hemicellulose, as the hemicellulose conversion was around 37% regardless of whether BM was applied or not. The AIR content was lower in the EH sample compared to the CTRL and further decreased with a longer ball milling time. The AIR fraction consists of acid insoluble lignin and ash in BSG, as well as any added insoluble enzymes. The reduction in the AIR content may be attributed to the increased proportion of small-sized AIR particles (e.g., <1.5 μm) in the slurry after the more rigorous pretreatment, which could result in a smaller portion being retained on the glass fiber filter and detected.

3.2. Characterization of Materials

The SEM images with lower magnification visually confirmed that the BM-treated samples had significantly smaller particle sizes and decreased density compared to the raw BSG samples (Figure S2a–c). Correspondingly, the BET analysis indicated that the surface area of the samples increased after the BM-1 and BM-2 treatments compared to the raw BSG, with values of 0.714 and 0.994 m^2/g, respectively, compared to 0.541 m^2/g for the

raw BSG. Additionally, the pore volume at a relative pressure of 0.99 also increased from 7.75×10^{-4} cm^3/g for the raw BSG to 1.26×10^{-3} and 1.71×10^{-3} cm^3/g for the BM-1 and BM-2 treatments, respectively. The BET analysis results also showed that there was a gradual reduction in the BSG particle size as the BM treatment time increased. Nevertheless, it is important to acknowledge that prolonged BM pretreatment results in elevated energy consumption. Ball milling is characterized as an energy-intensive process with high capital investment in mechanical equipment. Therefore, optimizing the operational parameters such as milling time, mode, speed, and other factors becomes vital to minimize the overall costs, particularly in preparation for commercial application [17,23].

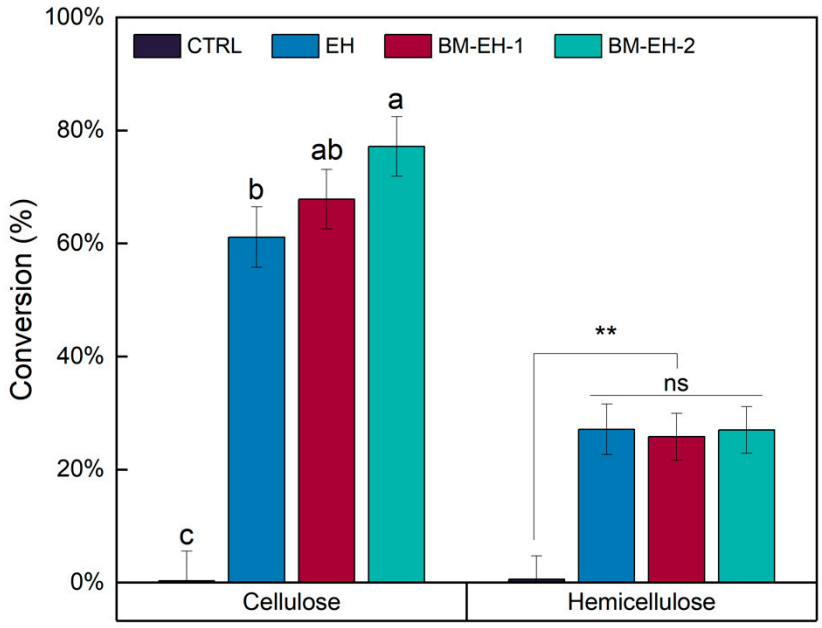

Figure 3. The conversion of cellulose and hemicellulose in the control BSG sample and BSG samples after different pretreatments. Data are expressed as the mean ± SD of two independent experiments (ns $p > 0.05$, ** $p \leq 0.01$). Different letters on the top of error bars designate significant differences ($p \leq 0.05$) between different pretreatments.

The surface morphology of the materials was more clearly illustrated in the SEM images at higher magnification, as shown in Figure 4. The bulky layer-like structure and rough surface of the raw BSG sample (Figure 4a) were disrupted after BM treatment, resulting in thinner chips with edges that were rich in irregular caves (Figure 4b,c). The small particles on the surface of the raw BSG were not observed in the images of the BM-EH samples, and the structure became less dense after prolonging the ball mill treatment. This finding was in accordance with the loss of the AIR portion, as discussed in the previous section. A simple comparison between the raw and enzymatically hydrolyzed BSG samples (Figure 4a,d) revealed an engraving process of the material surface with the enzymes that resulted in abundant caverns. In addition, when both BM and EH were applied simultaneously (Figure 4e,f), as the pretreatment duration prolonged, the uneven appearance of surface changed from large hollows to numerous tiny holes that formed a sieve-like structure. The change in the surface morphology was expected to provide easier access for acid-producing bacteria and potentially benefit the subsequent fermentation process.

No significant difference was observed in the FTIR spectra between the raw and pretreated BSG samples (Figure S3), suggesting that the chemical structure of the solid residues remained similar after the pretreatment. All the samples showed the characteristic functional group of lignin such as the ester bonds of carboxylic group around 1744 cm^{-1}, C=C bonds in aromatic rings around 1526 cm^{-1}, and aryl–alkyl ether bonds around 1244 cm^{-1} [24]. This observation was consistent with the results from the prox-

imate and ultimate analysis. This is because the lignin component was not specifically removed during the pretreatments. The peaks at 3300 cm^{-1}, 1645 cm^{-1}, and 1036 cm^{-1} in the FTIR spectra are related to polysaccharides, water, and cellulose, respectively, according to previous research [25].

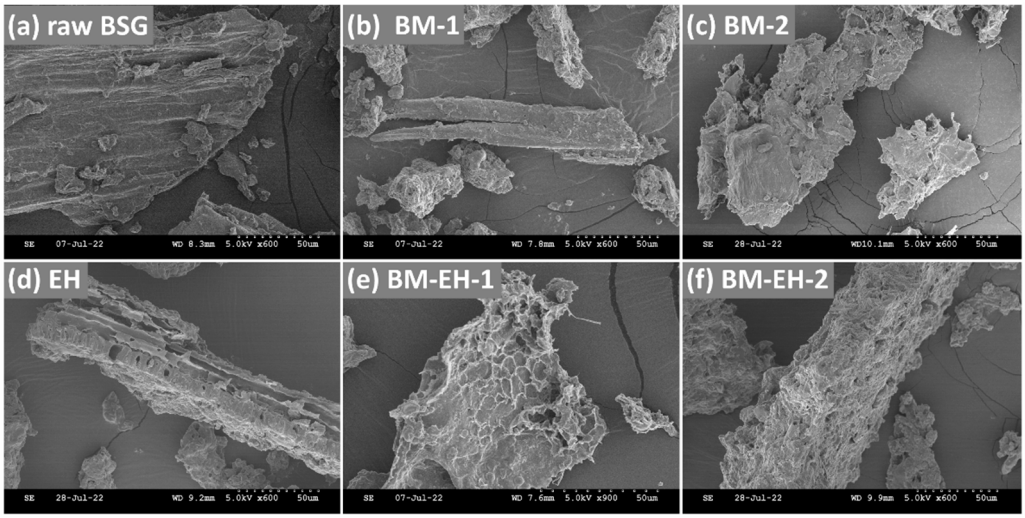

Figure 4. Scanning electron microscopic images at higher magnification for raw BSG and the BSG samples after different pretreatments. BM-1 and BM-2 represent BM for 1 and 2 cycles, respectively.

The XRD spectra of all the unpretreated and pretreated BSG samples showed no distinct amorphous regions (Figure S4). This result can be explained by the presence of abundant hemicellulose and lignin in the BSG samples, which are amorphous in nature. The crystallinity of plant biomass is primarily contributed by cellulose and is typically determined based on the intensity of the peaks at $2\theta \approx 18^\circ$ and 22° (cellulose crystallinity index (CCI) = $(1 - (I_{18}/I_{22}))$ [26]. However, in the present study, the combined peaks at around 20° were observed in all samples, which made the conventional calculation method for CCI invalid. Despite this, a reduction in the cellulose crystallinity after BM treatment was still demonstrated by the blunting of the curve of the combined peak for the BM treated BSG samples compared to the raw BSG. This is because the blunting of peaks may be attributed to either the loss of the cellulose partition or the reduction in the cellulose crystallinity, and no cellulose removal process was applied in the tests [27]. This result was consistent with previous studies, which revealed a reduction in the CCI in lignocellulosic biomass and a higher recovery of sugars in the following enzymatic hydrolysis process [28,29].

3.3. VFA Fermentation

The fermentation of the raw BSG (HS) lasted for 10 days, during which a negligible amount of biogas was collected, indicating the successful suppression of methanogenesis. The highest total VFA concentration was obtained within 4 days, reaching a value of 33.7 g/L (i.e., 0.26 g VFA/g substrate VS). The rapid accumulation of VFAs and the dramatic drop in the pH from 7.7 to 6.5 indicated an acidification of the system, which was assumed to have inhibited the acidogenic fermentation process. The low pH and increased ionic strength in the system can suppress the dissociation of VFAs, and the un-dissociated species would penetrate into microbial cell and inhibit the metabolic activity [10]. The fermentation process in the other systems also ceased after 4 days, and the change in the pH and VFA composition was determined (Figure 5) to compare the microbial communities' tolerance for VFA accumulation in the acidogenic fermentation systems using the differently pretreated substrates.

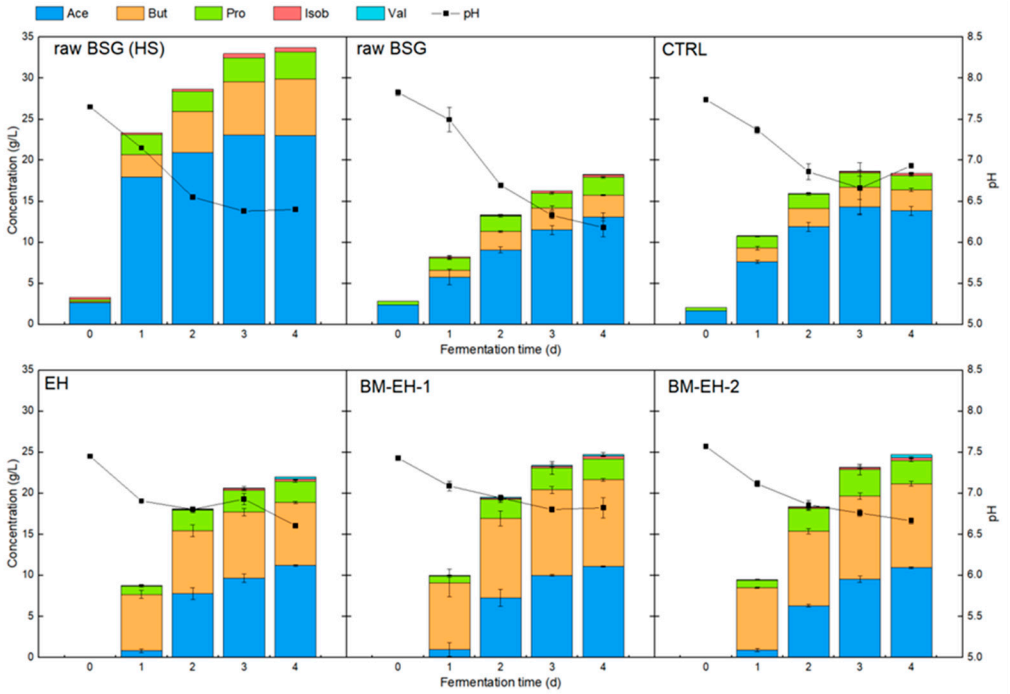

Figure 5. Volatile fatty acid (VFA) composition and pH change in 4 days of thermophilic anaerobic fermentation using raw and various pretreated BSG samples as substrate (raw BSG (HS)—dried raw BSG sample and in high solid fermentation; raw BSG—wet BSG paste (20%) prepared with DI water).

The comparison between the fermentation performance of the raw BSG (HS) and the raw BSG conditions showed that the TS had a significant effect. The fermentation process with a higher TS of 19.5% resulted in a higher maximum overall VFA yield (0.27 g/g VS, Figure 6) than the conventional conditions with a lower TS, demonstrating the acclimation of acid-producing bacteria to higher VFA concentrations under the HS condition. This finding agreed with the suggestion that a high solid condition could enhance the conversion of lignocellulose to VFA [30]. Among the wet systems with a TS of 12~14%, the raw BSG condition had the lowest VFA yield and showed a rapid drop in the pH, which was attributed to the absence of citrate buffer in the system. The citrate buffer induced in the CTRL and pretreated BSG conditions to facilitate the EH process remained in the fermentation system and contributed to maintaining a pH level above 6.5 throughout the fermentation experiment.

The fermentation system utilizing the EH-pretreated BSG substrates (EH, BM-EH-1, and BM-EH-2) outperformed the CTRL system, exhibiting higher VFA concentration and yield. It is noteworthy that all the EH pretreated substrates were converted to a VFA mixture richer in butyric acid than acetic acid, which was the dominant VFA species for the raw BSG and CTRL systems. This result was consistent with a previous study [9] and can be attributed to the elevated concentration of xylose in the hydrolysates [31]. Furthermore, the improved cellulose conversion achieved through the BM-EH pretreatment may have also contributed to the alteration of the VFA profile. The BM-EH-1 and BM-EH-2 conditions achieved VFA concentrations of around 25 g/L and a maximum yield as high as 0.33 g/gVS, which were higher than that obtained with EH pretreatment alone. It is worth noting that the observed increase in the total VFA production was mainly attributed to the increase in the butyric acid concentration. There was no significant difference observed in terms of the VFA concentration and composition between the BM-EH-1 and BM-EH-2 conditions. In all the fermentation systems, the butyric acid was observed to accumulate rapidly within the first day of the experiment, while the production of acetic acid continued and reached its maximum concentration on the third or fourth day.

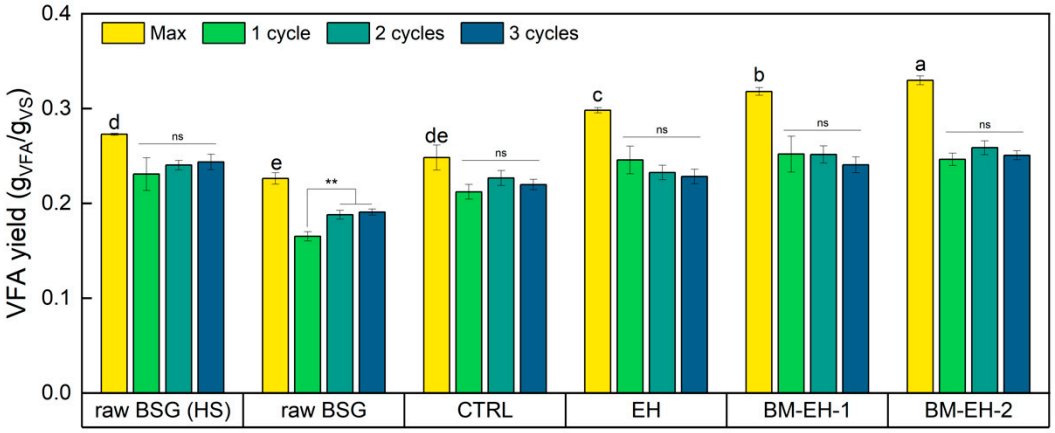

Figure 6. The maximum overall VFA production rate and production rate after 1, 2, and 3 (2d) cycles of fermentation in the thermophilic anaerobic fermentation using raw and various pretreated BSG samples as the substrate. Data are expressed as the mean ± SD of three independent experiments. The maximum VFA production was compared among different substrates, and the histograms bearing different letters are significantly different ($p \leq 0.05$). The production after 1, 2, and 3 (2d) cycles of fermentation were analyzed for each substrate (ns $p > 0.05$, ** $p \leq 0.01$).

As discussed previously, the accumulation of undissociated acids was considered the primary inhibitor of microbial activity in the systems. Propionic acid, in particular, was identified to have a stronger inhibitory effect at a lower tolerance limit of 1.0~3.2 g/L [32]. The maximum concentration of propionic acid detected in the current research all fell in the same range. Previous research has demonstrated that fermentative microbes can be protected by extracting propionic acid from the digester [33]. So, in the current study, a three-cycle thermophilic anaerobic fermentation method was implemented to remove VFA from the system, aiming to further release the fermentation potential of the organic residues remaining in the precipitate. However, it was found that there was no significant increase in the cumulative VFA yield, as shown in Figure 6. On the contrary, the fermentation process appeared to be interrupted by the direct collecting and washing process. Several reasons may have contributed to this result. Firstly, the removal of alkalinity in the supernatant along with VFA may have led to a reduction in the buffering capacity of the system, which could have contributed to the decrease in acidogenesis activity [34]. Secondly, the collecting and washing process may have broken the anaerobic condition, and the centrifugation step at 4500 rpm and 20 min may also have had a deactivating effect on the microorganisms. Thirdly, the maximum VFA yield was also limited by the nature of the substrate. In comparison to food waste, which can reach a maximum VFA yield of 0.8 g/g_{VS}, BSG is not as easily converted to VFA.

4. Conclusions

BSG was demonstrated to be a suitable substrate to produce short-chain VFAs through anaerobic acid fermentation. Under high solid fermentation condition, the acid-producing bacteria could be acclimated to a higher VFA concentration of over 30 g/L. EH pretreatment helped liberate polysaccharides in the BSG samples, leading to an increased VFA concentration in the fermentation effluent. Furthermore, butyric acid rapidly accumulated and dominated the VFA profile, instead of acetic acid. Simultaneous application of BM and EH resulted in a higher cellulose conversion compared to the EH pretreatment alone, and the conversion increased with the prolonged BM-EH processing time. As a result, the BM-EH pretreatment approach outperformed the EH pretreatment alone and achieved the highest VFA yield of 0.33 g/g_{VS}. Further evaluation is needed to assess the costs and benefits of implementing this approach on a commercial scale, considering the high energy intensity and expenses of BM and EH pretreatments. A direct replacement of the

supernatant containing the accumulated VFAs was found to be an ineffective approach in alleviating VFA inhibition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11061648/s1, Figure S1: Temperature change of brewer's spent grain (BSG) paste during ball milling with different milling–rest strategies; Figure S2: Scanning electron microscopic images of the raw BSG at lower magnification; Figure S3: Fourier transform infrared spectroscopy spectra of the raw and pretreated BSG samples; Figure S4: X-ray powder diffraction spectra of the raw and ball milled BSG samples; Table S1: BSG composition before and after different pretreatments.

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Abbreviations

EH—enzymatic hydrolysis; BM—ball milling; BM-EH—ball milling and enzymatic hydrolysis; CTRL—control; ASL—acid soluble lignin; AIR—acid insoluble residue; HS—high solid.

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