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Combination Strategy of Bioenzymes and Sophorolipid Pretreatments Enhance Volatile Fatty Acid Production Based on Co-Fermentation of Waste Activated Sludge and Rubberwood Hydrolysates

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Abstract

In this study, we developed a combination strategy of bioenzymes and sophorolipid (SL) co-pretreatment to enhance volatile fatty acids (VFAs) in co-fermentation of waste activated sludge (WAS) and rubberwood hydrolysates (RWHs). Among all the pretreatments, SL and laccase co-pretreatment markedly increased soluble bioavailable substrates (carbohydrates and proteins) by inducing EPS catabolism and WAS disintegration, and obtained the highest VFAs yield of 7049.43 mg/L. The proportion of VFA composition can be controlled by modifying the types and amounts of added bioenzymes. Under SL and laccase co-pretreatment conditions, RWHs were more efficiently converted into VFAs due to the higher activity of WAS, resulting in lower cellulose (3.41%) and lignin (0.66%) content in the fermentation broth. Compared with other pretreatments, SL and laccase co-pretreatment enhanced the enrichment of the functional microorganisms, including anaerobic fermentation bacteria (*Firmicutes*, *Bacteroidota*, and *Proteobacteria*) and reducing bacteria (*Acinerobacter* and *Ahniella*). Therefore, the combination pretreatments might be a promising solution for strengthening VFA accumulation in the WAS and RWH co-fermentation.

Keywords: volatile fatty acids; anaerobic fermentation; bioenzymes; rubberwood hydrolysates; waste activated sludge

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1. Introduction

With global resource scarcity and environmental degradation, circular economy has become an important development direction [1]. Waste activated sludge (WAS), a significant secondary output from sewage processing facilities, represents a valuable but underutilized resource that can play a crucial role in promoting circular economy. Due to the high content of protein and polysaccharides in WAS [2], high-value metabolic products (volatile fatty acids), which serve as an efficient carbon source for nitrogen and phosphorus removal in wastewater as well as a precursor for synthesizing biodegradable plastics such as polyhydroxyalkanoates [3], can be extracted from WAS through anaerobic fermentation.

This dual potential of WAS—mitigating environmental risks while generating valuable resources—has garnered widespread attention.

Anaerobic fermentation is divided into hydrolysis, acid production, and methane production stages. The hydrolysis stage proved to be the rate-limiting step for volatile fatty acid (VFA) production because of the obstruction caused by extracellular polymers and semi-rigid cell walls. In addition, the consumption of VFAs by methanogenic bacteria and the imbalance of carbon to nitrogen concentration ratio (C/N) in substrate can lead to low VFA yield. To address these issues, multiple strategies have been devised to enhance the hydrolysis of WAS for metabolite extraction, encompassing physical [4,5], chemical, and biological techniques [6–9]. Nonetheless, severe reaction conditions, elevated expenses, and secondary pollution issues have obstructed their widespread use.

Recently, co-fermentation and pretreatment technology have attracted more and more attention of researchers for improving the production of VFAs in anaerobic fermentation [10,11]. Rubberwood, an inexpensive and plentiful biomass resource, comprises roughly 8% free sugars and starch. Furthermore, it comprises up to 54% cellulose and 23% hemicellulose, while the lignin content is relatively low at just 13% [12,13]. Consequently, rubberwood can be processed into C6 and C5 sugars through a series of chemical and biological methods, making it suitable for use in fermentation technology. At the same time, rubberwood can be used as a carbon source to ensure the balance of C/N ratio in fermentation and can also be used for secondary fermentation to increase the yield of VFAs. To improve the hydrolysis rate of WAS, various pretreatment methods have been studied. The combined pretreatment methods are a promising manner to promote the decomposition of sludge and the production of VFAs. Table 1 shows the VFA yield under different combined pretreatment methods. Although these combined pretreatments increase the production of VFAs to some extent, the use of physical or chemical combined methods leads to increased costs, such as high energy consumption and pollution. Therefore, the combined method, with environmentally friendly and low-cost, is a promising strategy.

Table 1. Comparison of VFA production via different methods.

Pretreatment Methods	Pretreatment Conditions	VFA Yield	Ref.
Ultrasonic-alkali	6 W/mL, 120 min	392.5 mg COD/g VSS	[14]
Citric acid-potassium ferrate	35 °C, 60 min	4751.6 mg COD/L	[15]
Sodium citrate-heat	120 °C, 30 min	354.5 mg COD/g VS	[16]
Rhamnolipid-alkali	70 °C, 60 min	1584.89 mg/L	[17]
Rhamnolipid-alkali	90 °C, 60 min	1500 mg/L	[18]

Biosurfactant has been proved to be used for the production of VFAs due to their simple operation, environmental friendliness, and minimal corrosion to anaerobic systems [19]. Sophorolipid (SL), as a common biosurfactant generated by non-pathogenic yeasts, is noted for its high efficacy, environmental safety, and excellent biodegradability across various temperatures and pH levels. SL can promote the dissolution of exopolysaccharides (EPSs), and inhibit methane production to some extent, which effectively promote the production of VFAs in anaerobic fermentation [20]. However, due to the lower degree of decomposition and hydrolysis ability, the increase of VFA products from WAS pretreated with SL alone is not significant. As a biological pretreatment method, bioenzymes can significantly enhance the solubilization of organic materials and the breakdown of macromolecules in WAS, which can be utilized by bacteria for the production of VFAs [21,22]. Consequently, the combined pretreatment using SL and bioenzymes will serve as a method for enhancing VFA production during anaerobic fermentation.

Herein, we proposed using a combination of bioenzymes and sophorolipid (SL) co-pretreatment to boost VFA generation during the co-fermentation of activated sludge with rubberwood hydrolysates (RWHs). Based on the total amount and composition of VFAs, bioenzymes and sophorolipid (SL) co-pretreatment conditions were optimized. In addition, the dissolution efficiency of organic matter and changes in organic matter composition were analyzed in co-fermentation. Furthermore, the effects of pretreatments on the conversion of RWHs into VFAs and the change of cellulose, hemicellulose and lignin content in the fermentation broth were discussed, and the microbial community succession under the different bioenzymes and sophorolipid pretreatments was also analyzed in detail.

2. Materials and Methods

2.1. Sludge Source and Rubber Wood Characteristics

WAS was collected from the secondary sedimentation tank of a wastewater treatment plant located in Hebei, China. The basic WAS characteristics are as follows: total suspended solids 301 ± 5.6 mg/L, total carbohydrates 1.22 ± 0.3 mg COD/L, total proteins 2.11 ± 0.5 mg COD/L, and pH 6.8 ± 0.2.

Rubberwood was purchased from Hainan, China. Before use, rubberwood was air-dried, ground, and screened to obtain wood powder (40–80 meshes) for use in the experiments.

2.2. Rubberwood Feedstock Hydrolysis-Enzymatic Hydrolysis

The mixture of 15 g of rubberwood powder and 0.45 g of Tween 40 as well as 120 mL of deionized water, loading in the reaction kettle, was reacted at 180 °C for 30 min. The resulting residue was transferred to a 1 L beaker, mixed with deionized water to reach 500 mL, and the pH was adjusted to 4.8. Subsequently, 1.5 mL of cellulase (enzyme mixture) was added and reacted at 45 °C for 72 min under magnetic stirring to obtain the hydrolysate mixture. Then, the mixture was kept in a water bath at 90 ° C for 20 min. After filtering and separating, the obtained hydrolysate (RWH) was stored at −4 °C for use.

2.3. Anaerobic Fermentation

A total of 0.6 g/g TSS of SL and 15% (*w*/*w*, enzyme weight/TSS weigh) of bioenzymes (laccase (LA), lysozyme (LY), alpha amylase (AA), acid protease (AP)) were added to a 500 mL fermenter containing 200 mL WAS and were vibrated for 1 h. Subsequently, 100 mL of RWH containing 15 g/L reducing sugar was added. Then, pH of the fermentation system was set to 5 using 1 M NaOH or HCl. All the fermenters were sealed after purging with nitrogen for 5 min to remove oxygen. Finally, the fermentation system was incubated at 35 °C for 144 h to obtain volatile fatty acids. Four pretreated fermenters were denoted as SL+ LA, SL + LY, SL+ AA, and SL+ AP, respectively. The fermenter pretreated with SL was used as the control.

2.4. Detection Methods

After centrifugation of the fermentation broth, the supernatant was collected by filtration through a 0.45 μm vacuum membrane. The SCOD was assessed following the standard procedure outlined by APHA [23]. To measure the content of reducing sugar, the dinitrosalicylic acid (DNS) method was employed. Cellulose, hemicellulose, and lignin levels were analyzed using the Van Soest method, as referenced in previous research [24]. Glucose and xylose concentrations were assessed according to the previous literature [25]. Total VFAs were assessed using a colorimetric technique. For the analysis of individual VFA components, a gas chromatograph (model 7890A, Agilent, Santa Clara, CA, USA) was employed, featuring an HP-Innowax capillary column alongside a flame ionization

detector. An injection volume of 0.5 µL and 50 mL/min nitrogen flow rate were used in testing. The temperatures of the injection port and flame ionization detector were precisely adjusted to 250 °C and 200 °C, respectively.

2.5. Analysis of the Sludge Organic Composition

The properties of sludge-derived dissolved organic matter (DOM) were examined using an F-7100 Aqualog Synchronous absorption 3D-EEM fluorescence spectrometer manufactured by Hitachi in Tokyo, Japan, in order to explore the fluorescence characteristics. Emission spectra were captured across a wavelength range from 200 to 550 nm, while excitation spectra were measured across a range of 200 to 400 nm, with data points collected at 5 nm intervals [26]. The scanning rate was established at 1200 nm/min, and the photomultiplier voltage was set to 600 V. The fluorescence response percentage (Pi, n) for the DOM was calculated following the methodology put forth by Wang et al. Before proceeding with the analysis, the samples underwent filtration using a 0.45 µm membrane, were centrifuged at 4500 rpm for a duration of 10 min, and then diluted 50-fold with ultrapure water.

2.6. Microbial Community Analysis

The changes in microbial communities were investigated by Suzhou Jinweizhi Biotechnology Co., Ltd. (Suzhou, China) Adhering to the manufacturer's guidelines, genomic DNA was extracted from the samples, and its concentration was assessed using the Qubit® dsDNA HS Assay Kit. The company then created next-generation sequencing libraries with the help of the MetaVX Library Preparation Kit before proceeding with Illumina sequencing. Specifically, they amplified the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene using 20 to 50 ng of DNA. The forward primer utilized was 'CCTACGGRRBGCASCAGKVRVGAAT,' while the reverse primers contained the sequence 'GGACTACNVGGGTWTCTAATCC.' Fragment sizes were validated by 1.5% agarose gel electrophoresis to ensure a target size of approximately 600 bp.

3. Results and Discussion

3.1. Impact of Pretreatment Methods on VFA Generation

Figure 1 depicts the variation in total VFAs generated from waste activated sludge (WAS) under different pretreatment methods. Figure 1a illustrates the notable increase in maximum VFA accumulation, rising from 3974.12 mg/L in the control to 5112.23 mg/L in the SL + LY group. The incorporation of SL resulted in a reduction of surface tension between the hydrophobic organic materials and the fermentation broth, thereby facilitating the degradation of WAS and improving the biodegradability of the organic compounds released. Lysozyme, a key hydrolytic enzyme, effectively disrupts microbial cell walls by cleaving the β-1, 4 glycosidic bonds, facilitating the release of intracellular compounds [27]. The combination of SL and LA significantly enhanced VFA production, achieving the highest yield of 7049.43 mg/L. SL and LA promoted the release of intracellular proteins and carbohydrates, providing additional substrates for acidogenic bacteria. This process resulted in a swift increase in VFAs during the initial 48 h of anaerobic fermentation. Similarly, the SL + AA group demonstrated high acidification efficiency, yielding 3989.33 mg/L of VFAs. In contrast, the SL + AP group produced a maximum VFA yield of 3120.45 mg/L, which was lower than the control group. While SL and AA break down proteins and carbohydrates into simpler molecules (e.g., peptides, amino acids, and simple sugars) [28,29], the combination of SL and AP may have inhibited the growth of hydrolytic acidification bacteria, reducing its effectiveness. However, it was also found that there were two distinct peaks of VFA concentration in all fermenters before and after 72 h of fermentation. The slight decrease of VFAs in the fore stage of fermentation was related to substrate consump-

tion, while the increase of VFAs in the later stage of fermentation was due to the release of a large amount of organic matter from the pretreated sludge during the fermentation process.

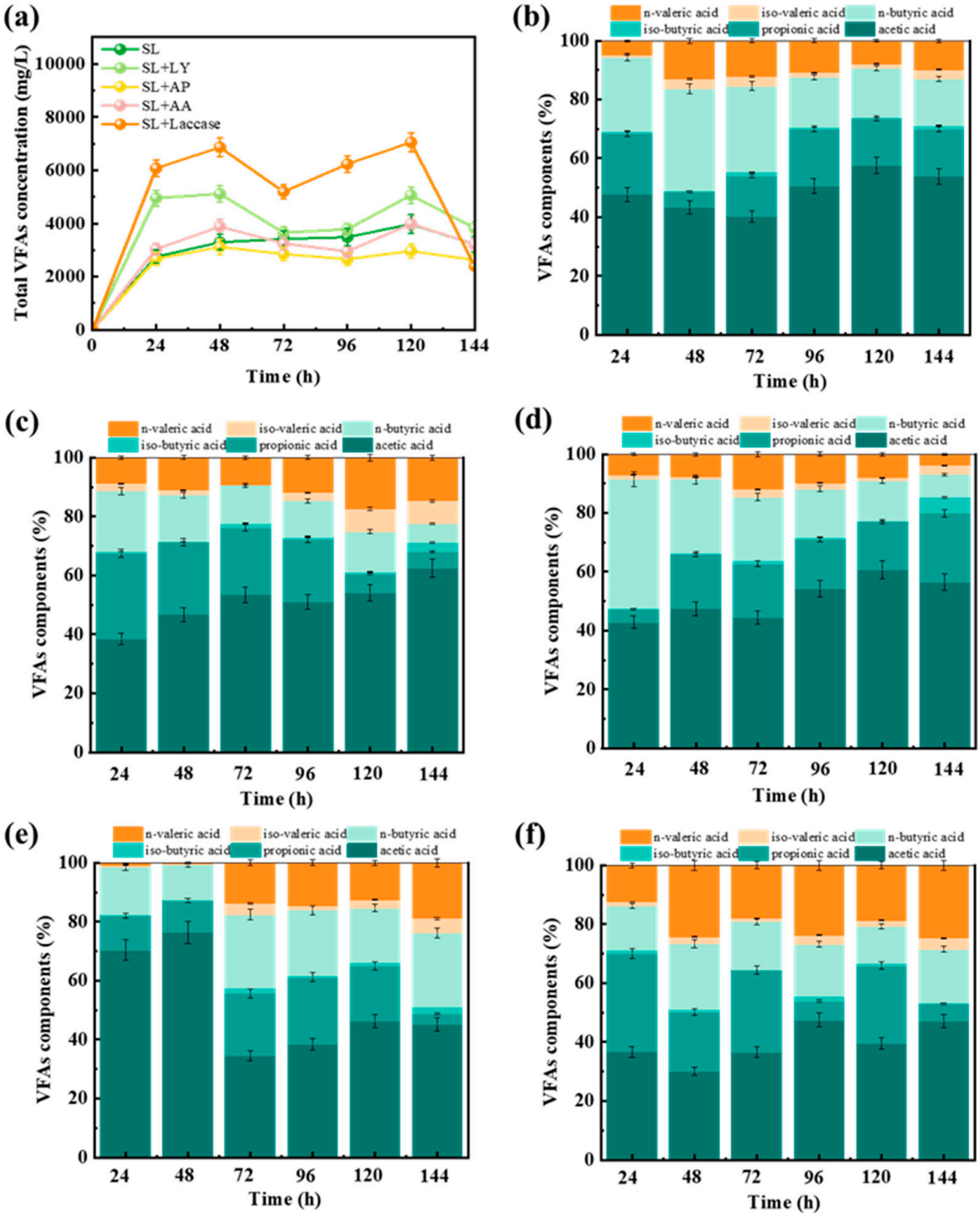


Figure 1. (**a**) Total VFA concentration and (**b**–**f**) the fraction of individual VFAs from the different pretreatment groups: (**b**) SL; (**c**) SL + LY; (**d**) SL + AA; (**e**) SL + AP; and (**f**) SL + LA.

Furthermore, VFAs were composed of six individual kinds of acids. As shown in Figure 1b–f, the results indicated that acetic acid was the predominant organic compound generated during the fermentation of SL, SL + LY, SL + AA, SL + AP, and SL + LA, with peak percentages of 57.59%, 62.46%, 60.68%, 76.36%, and 47.50%, respectively. With the increase of fermentation time, except for the SL + AP group, the amount of acetic acid in the other groups rose. Acetate emerged as a favored carbon source, and the substantial production

of acetic acid proved advantageous for its subsequent use in wastewater treatment facilities in refineries [30]. As the fermentation time increases, the proportion of propionic acid in SL + LY, SL + AP, and SL + LA decreased gradually, while the proportion of propionic acid in the SL and SL + AA groups increased gradually. It was found that the species of acid-producing bacteria in different pretreatment groups were quite different, inferring the enrichment of acetic acid-producing bacteria in SL + LY, SL + AP, and SL + LA and the increased activity of some key propionic acid synthase in the SL + AA group. In summary, the findings indicate that various pretreatments may affect microbial growth and metabolic pathways, leading to variations in VFA composition.

3.2. Enhancement of Bioavailable Substrates Facilitated by Various Pretreatment Methods

SCOD serves as a key measure to assess the degree of sludge dissolution and hydrolysis. Figure 2 shows that SL + LY, SL + AA, SL + AP, and SL + LA co-pretreatment enhanced the dissolved organic content of WAS. Compared with the control, the SCOD of the SL + LY, SL + AA, SL + AP, and SL + LA groups increased by 196.38%, 149.54%, 149.79%, and 261.78%, respectively, indicating the high effectiveness of bioenzymes coupled with sophorolipid in enhancing sludge solubilization (Figure 2a). Among them, SL + LA achieved the highest SCOD (2348.30 mg/L), followed by the SL + LY group (2061.672 mg/L). It was worth noting that the change of SCOD in the SL + LA group exhibited an increasing trend, while lysozyme showed a downward trend. SL + LA could effectively promote organic matter leaching, thus providing more organic matter for microorganisms to utilize to produce VFAs. In particular, WAS had been shown to contain cellulose, which could be decomposed into small molecules by LA for microbial utilization [31]. The alterations observed during fermentation with LY were primarily due to the action of lysozyme, which disrupted microbial cell walls during the hydrolysis of WAS [32]. Nonetheless, when WAS underwent pretreatment with lysozyme, the enzyme's effectiveness in the sludge system diminished progressively as the treatment duration was extended. Additionally, the gel-like network formed by EPSs in WAS encapsulated the β-1, 4 glycosidic bonds on cell walls, limiting LY's access and catalytic efficiency. As a result, LY was more effective during the initial stages of WAS hydrolysis but showed reduced efficacy over time.

As shown in Figure 2b–d, the SL + LA group exhibited the highest soluble polysaccharide concentration, with the SL + LY group ranking second. The contents of protein and carbohydrate in the SL + LA and SL + LY groups showed significantly higher levels compared to other groups, demonstrating that this pretreatment method enhanced the release of organic matter and supplied abundant substrates for VFA production. At the same time, the content of ammonia nitrogen in the two groups was also relatively high and showed an upward trend, demonstrating that protein was used to convert more acid. The findings clarified why the two groups achieved the highest VFA production. Additionally, it was also found that the sludge solubility of the SL + AA and SL + AP groups was relatively poor. It was well known that AA could decompose starch into small molecules, and AP could decompose proteins into polypeptides, which had the effect of promoting more powerful growth of the bacteria and achieved the effect of improving the ability of saccharification and fermentation [28,29]. However, the lower VFAs from these two pretreatment groups were due to the inability to effectively crack sludge, resulting in limited organic matter for microbial synthesis of VFAs.

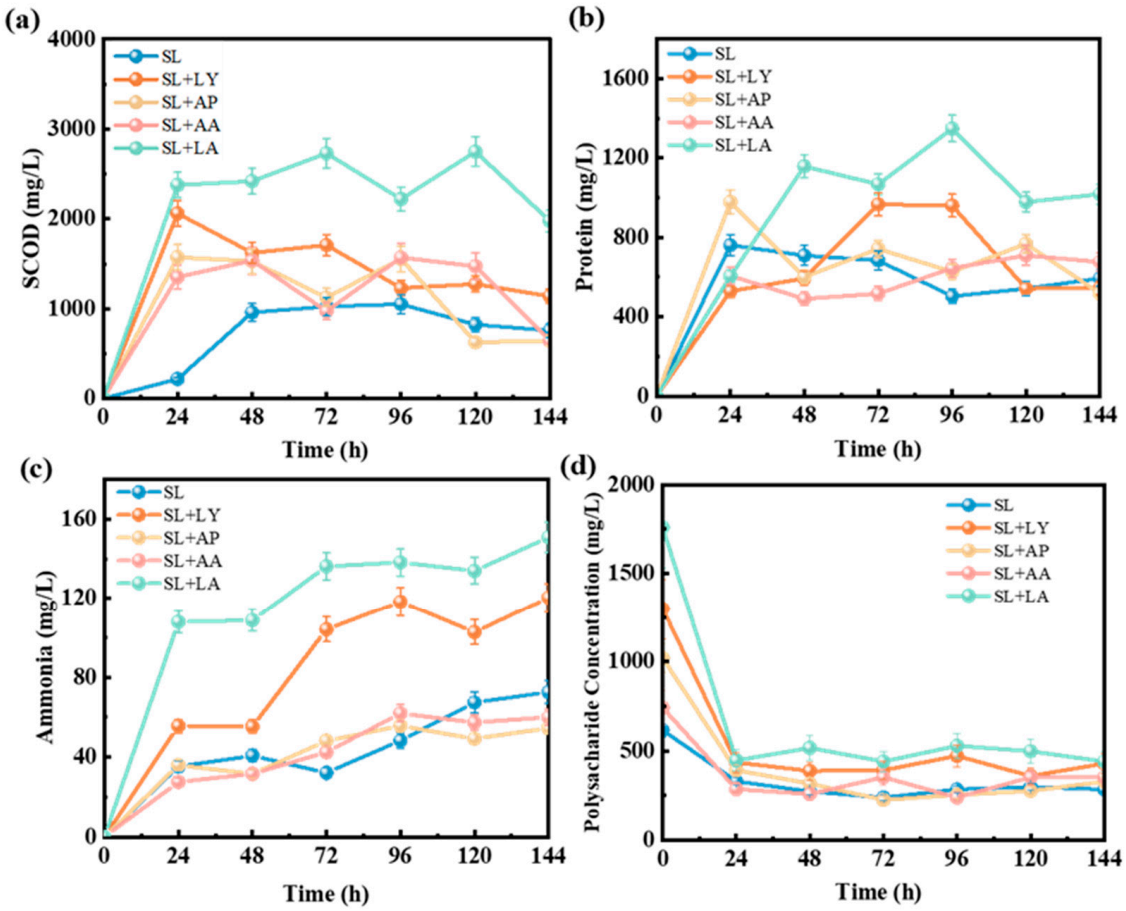


Figure 2. Variations of SCOD (**a**), protein (**b**), ammonia (**c**), and polysaccharide concentration (**d**) during the fermentation process.

3.3. The Conversion of Rubberwood Hydrolysates into VFAs

As illustrated in Figure 3a, Under the pretreatment conditions of SL + LA, the maximum VFA content of 15 g/L RWH was 7049.43 mg/L, while that without RWH was 2378.12 mg/L. This indicated that excluding the factor of WAS, the VFAs produced by RWH as a carbon source are 4671.31 mg/L, demonstrating that the RWH served as the primary substrate for VFA generation during co-fermentation. The production of VFAs in the control group was 3974.12 mg/L, implying that the higher activity of WAS leaded to the conversion of RWH into more VFAs under SL + LA pretreatment condition.

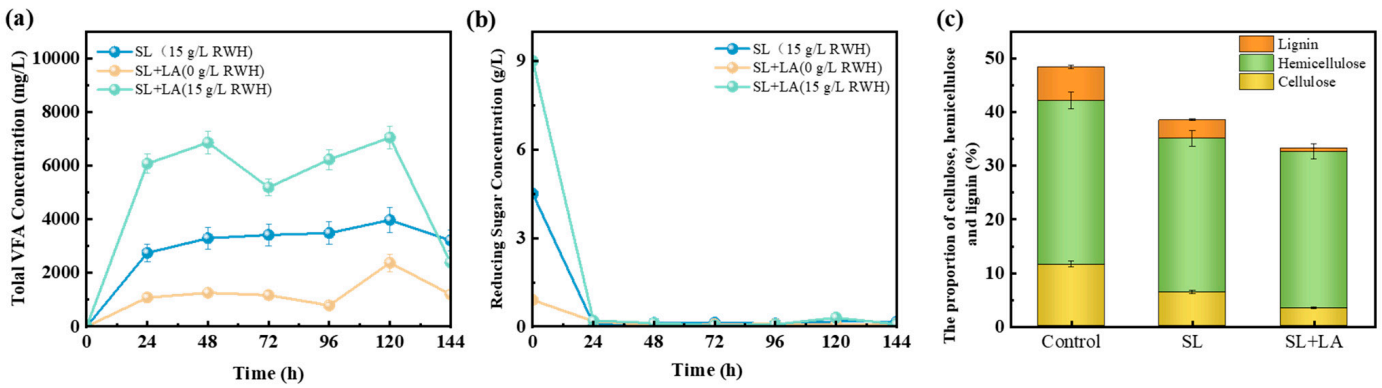


Figure 3. The yield of VFAs (**a**) and the change of reducing sugar (**b**) with or without hydrolysate under different pretreatment methods. The distribution of cellulose, hemicellulose, and lignin (**c**) in anaerobic fermentation.

Figure 3b is the change of reducing sugar under different pretreatment methods. The content of reducing sugar in the SL + LA group was the highest (9.01 g/L), while that in the control group was only 4.50 g / L. This showed that in the early stage of pretreatment, SL + LA could make a large amount of organic matter dissolved and converted into reducing sugar. The concentration of reducing sugar was only 0.91 g / L without adding hydrolysate. It could be seen that the reducing sugar of all groups decreased rapidly in the first 24 h, and the SL + LA group decreased the fastest, indicating that the acid-producing bacteria were the most in this stage. Meanwhile, the additional hydrolysate and the reducing sugars decomposed by SL + LA from the sludge were mostly converted into VFAs by microorganisms, which also explained the reason for the rapid increase of VFA production within 24 h in the SL + LA group.

At the same time, the introduction of LA brought about a significant change in the composition of cellulose, hemicellulose, and lignin across the reactors. As shown in Figure 3c, there was a marked reduction in the levels of both cellulose and lignin following the addition of LA. In the control group, cellulose and lignin accounted for 11.53% and 6.29%, respectively. However, cellulose and lignin contents reduced to 6.33% and 3.47% in the SL group and further dropped to 3.41% and 0.66% in the SL + LA group. The results indicated that LA could degrade lignin, which led to the easier utilization of cellulose and hemicellulose for converting into VFAs. In addition, it was also found that the addition of SL and LA pretreatment had little effect on the hemicellulose content. Generally, the evident decrease of cellulose and lignin indicated that the addition of SL and LA could increase the bioavailable substrates, which could enhance VFA production during anaerobic fermentation.

3.4. Organic Matter Compositions in Fermented Liquids Across Various Pretreatment Groups

The organic content in sludge plays a crucial role in determining its biodegradability and shaping the microbial community structure [33]. The DOM composition of fermentation broth from anaerobic fermentation of WAS/RWH under different pretreatment methods was analyzed using 3D-EEM (Figure 4). The EEM spectrum is commonly categorized into five sections: Region I (aromatic protein-like, EX 200–250 nm, EM 200–330 nm), Region II (tryptophan protein-like, EX 200–250 nm, EM 330–380 nm), Region III (fulvic acid-like, EX 200–250 nm, EM 380–550 nm), Region IV (soluble microbial products, EX 250–400 nm, EM 200–380 nm), and Region V (humic acid-like, EX 250–400 nm, EM 380–550 nm). In addition to boosting the levels of organic matter released from sludge flocs and cells, the various pretreatment methods also improved their biodegradability, which in turn facilitated the production of VFAs. Soluble microbial metabolites, consisting of straightforward organic compounds, can be readily used by microorganisms to fuel their metabolic processes. As shown in Figure 4, the control group exhibited weaker fluorescence intensity, while combined pretreatments (except SL + AP) improved organic matter dissolution. The SL + LA group displayed the highest fluorescence intensity, indicating significant production of soluble microbial byproducts. The most significant transformations were observed in Regions II and IV with the combination of SL and LA, indicating that its key elements were proteins abundant in tryptophan and soluble microbial substances, both of which are easily transformed into VFAs [34]. These results indicated that the collaborative effect of SL and LA enhanced the accessibility of dissolved organic matter in the fermentation medium. In contrast, the SL + LY group's lower VFA production was attributed to humic acid interference with anaerobic fermentation [35]. Similarly, the SL + AA and SL + AP groups produced fewer VFAs due to the limited dissolved organic matter, resulting in insufficient substrate for VFA generation.

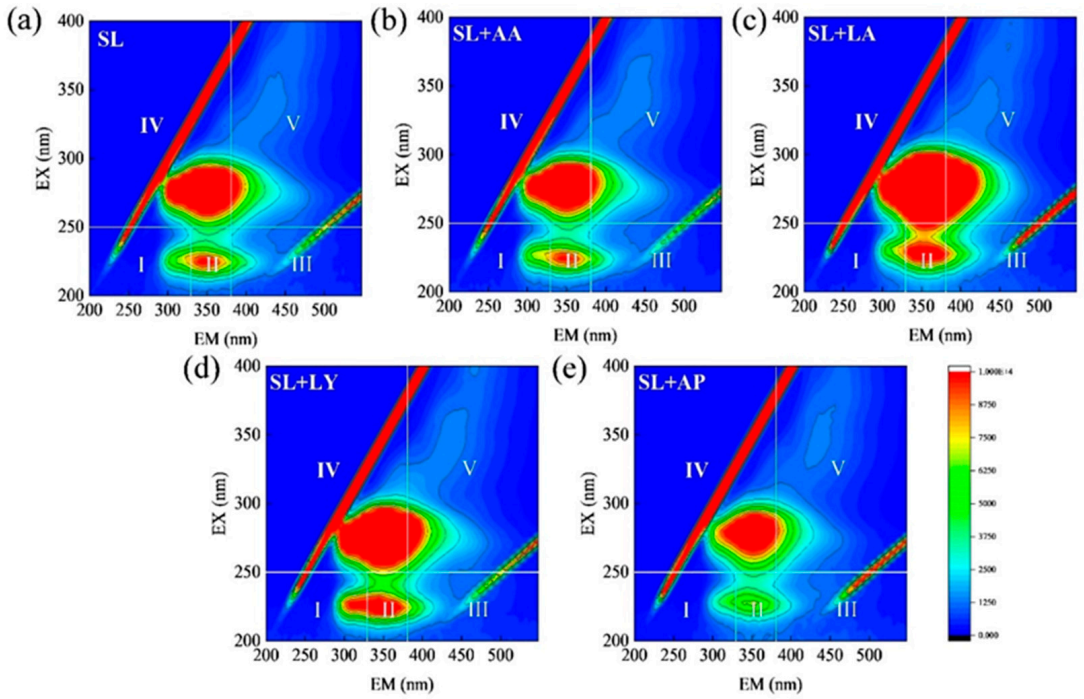


Figure 4. Three-dimensional excitation–emission matrix (3D-EEM) spectra of the fermentation broth on day 6 in various experimental groups: (**a**) SL; (**b**) SL + AA; (**c**) SL + LA; (**d**) SL + LY; (**e**) SL + AP.

3.5. Impact of Pretreatment Techniques on Microbial Communities During Anaerobic Fermentation

Microbial communities in various groups were examined through high-throughput sequencing methods. As shown in Figure 5a, bioenzymes reduced the diversity of the anaerobic microbial community. Among them, SL + LA pretreatment had the lowest species abundance and homogeneity, which might be due to the enrichment of functional hydrolysis and acid-producing bacteria. As shown in Figure 5b, PCoA analysis further exhibited that compared with the SL group, SL + LA pretreatment showed the greatest variability in microbial species, while SL + AA possessed the least variability, indicating that different bioenzymes could significantly influence microbial community structures [36].

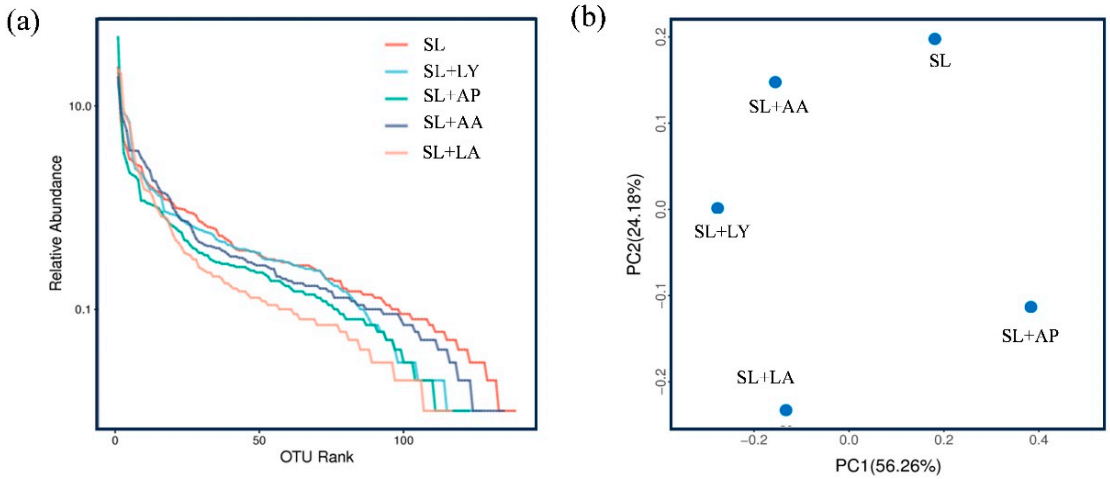


Figure 5. Relative abundance (**a**) and principal coordinate analysis (PCoA) based on OUT level (**b**) of all pretreatment groups.

The phylum-level distribution of microbial communities is depicted in Figure 6a. Clearly, *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Chloroflexi*, *Aciobacteria*, *Synergisteres*, *Actinobacteria*, and *Patescibacteria* were identified as one of the top eight predominant bacterial

groups during anaerobic fermentation. It was obvious from Figure 6a that the predominant bacterial groups in all fermenters were basically the same, which was consistent with previous literature research results [36]. The predominant bacteria in the control reactor were *Firmicutes* (49%), followed by *Bacteroidetes* (18%) and *Proteobacteria* (8%). However, the relative abundance of *Firmicutes* increased significantly to 83% (SL + LA), 60% (SL + AA), 61% (SL + AP), and 65% (SL + LY). In the reactor, *Firmicutes* were recognized as common anaerobic microorganisms that can break down a range of organic substances, especially proteins and carbohydrates. Additionally, the SL + LA group had a more pronounced impact on the microbial community in WAS compared to the SL group. The dominant microbial communities observed during the hydrolysis stage of anaerobic fermentation were *Firmicutes*. Consequently, the pretreatment using SL and LA improved the ratio of active microorganisms that play a role in hydrolysis and the accumulation of VFAs. There was an increase in the population of *Firmicutes*, alongside a decrease in the microbial abundance within the SL + LA reactor condition. This suggests that while functional microorganisms thrive, other microbial species diminish, which positively influences the hydrolysis of WAS and the acidification process. This pattern closely corresponds to the higher VFA production detected in the related fermentation reactors.

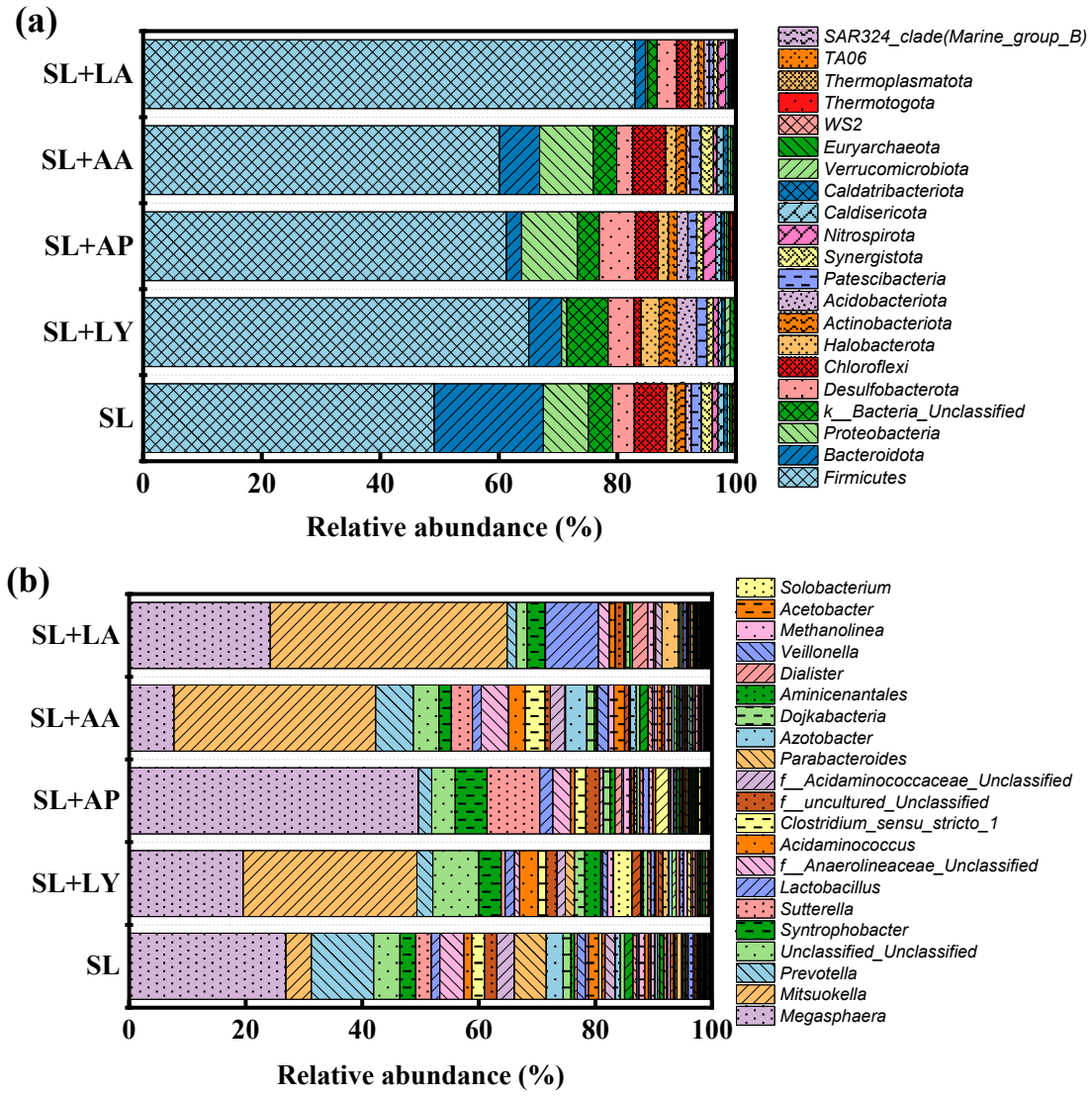


Figure 6. The microbial communities at the (**a**) phylum and (**b**) genus levels in various reactors during the co-fermentation of WAS and RWH.

At the genus level, Figure 6b illustrates that diverse functional bacteria participating in VFA production were detected in various fermenters, albeit with noticeably different abundances. In the control reactor, the primary functional bacteria were *Mitsuokella* and *Prevotella*. It can be clearly seen that *Megasphaera* in the SL + LA group was 0.88 times higher than the control group, while *Mitsuokella* was 10.25 times higher than the control group. The high ability of *Mitsuokella* to ferment carbohydrates was good evidence that this pretreatment could effectively induce the conversion of carbohydrates into VFAs. In addition, *Mitsuokella* content in the SL + AP group was the lowest, and *Megasphaera* was 1.85 times higher than that of the control, which explained the inability of SL + AP pretreatment to promote the production of VFAs. The dominant genera in the SL + LY group were *Mitsuokella* (30%), *Prevotella* (3%), and *Syntrophobacter* (4%), while the dominant genera in the SL + AA group were *Mitsuokella* (35%) and *Prevotella* (6%), which were hydrolyzed acid-producing bacteria.

In summary, various pretreatments significantly influenced the microbial community in WAS. Initially, these pretreatments reduced the relative abundance of the microbial community. Additionally, their effects varied across different phyla or species. For example, SL + LA increased the percentage of microorganisms participating in hydrolysis and VFA accumulation while reducing the proportion of bacteria unrelated to VFA production. The addition of SL + LY shifted the overall microbial community structure toward hydrolytic and acidogenic functional bacteria.

4. Conclusions

Waste activated sludge and rubberwood hydrolysates for anaerobic co-fermentation were pretreated using a combination of bioenzymes and sophorolipid co-pretreatment. Among them, SL and laccase co-pretreatment had become the most effective method for enhancing volatile fatty acid production, because of increased soluble bioavailable substrates and WAS destruction. Meanwhile, VFAs reached a peak of 7049.43 mg/L at 120 h, higher than the control group and other pretreatment groups. The combined pretreatment provided rich substrates (carbohydrates and proteins) for the anaerobic VFA-producing microorganisms (such as *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Ahniella*). Therefore, the co-pretreatment was an inexpensive and effective method to improve VFA accumulation in the sludge and biomass carbohydrate co-fermentation.

Author Contributions: F.Y.: Methodology, investigation, writing—original draft. W.B.: Methodology, investigation, and data curation. X.M.: Conceptualization, writing—review and editing. J.L.: Investigation. Y.Z.: Data curation. D.L.: Investigation. All authors have read and agreed to the published version of the manuscript.

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