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Bioconversion of food waste into short-chain organic fatty acids: Influence of biochar on acidogenesis

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Utilization of food waste (FW) via anaerobic digestion (AD) has emerged as an efficient and sustainable method for producing value-added bioproducts, such as volatile fatty acids (VFA). The present study focuses on combining the effects of substrate pretreatment, microbial culture, and biochar additive in acidogenic FW fermentation to optimize VFA production. Various pretreatment methods, including acidic, alkaline, and heat pretreatment, were studied to assess FW solubilization and its subsequent relationship to acidogenic digestion efficiency. Acidic pretreatment yielded a total VFA concentration of 987 ± 32.6 mg/L and improved substrate solubilization. Subsequently, the effect of microbial inocula was evaluated by using *B. licheniformis, K. pneumoniae,* and a co-culture of both bacteria. The co-culture presented an effective fermentation process and well-developed synergistic effects, showing a maximum VFA concentration of 1600.2 ± 63 mg/L. The addition of biochar to the tested experimental setup at a loading of 3 g/L had a greater effect on fermentation, with a maximum VFA accumulation of 2523.5 ± 130 mg/L. The overall increased performance of the biochar-amended experimental setup was correlated with increased substrate conversion, microbial attachment, and continued fermentative activity. Further, FE-SEM examination revealed the porous, fibrous structure of the biochar and its capacity to support microbial growth and biofilm development. Overall, the study has shown that implementing a combination of acidic pretreatment, selective co-culture of bacteria, and biochar addition can enhance acidogenic fermentation, resulting in higher VFA production from FW.

Keywords: Anaerobic fermentation, Biomass, Carbon support, Organic waste, Valorization

Introduction

Rapid growth and urbanization led to a dramatic increase in food waste (FW) generation[1]. These large amounts of waste need to be managed efficiently and properly. Additionally, the problems associated with FW management, from collection to disposal, demand a sustainable solution considering the efficacy and environmental friendliness. Generally, once these wastes are collected from various sources, they are disposed of in landfills[2]. Large stacks of these landfill waste products produce methane, which could contribute to increased greenhouse gas (GHG) emissions. The most commonly used traditional waste treatment methods, such as landfills and incineration, are not sustainable. Hence, it is necessary to develop efficient technologies to convert such waste materials into energy forms[3]. The urban waste hierarchy strongly emphasizes recovering energy and other value-added products from waste to better use waste materials and ultimately create a cleaner environment[4].

There are many ways to use biodegradable waste materials to generate renewable energy, and anaerobic digestion (AD) is one of the most effective biological approaches[5]. It is a biological process that involves four stages of hydrolysis, acidogenesis, acetogenesis, and methanogenesis[6]. Volatile fatty acids (VFA) are short-chain fatty acids (SCFA), i.e., C_2-C_6 compounds, which are produced mainly during acidogenesis and then during acetogenesis. Both steps involve complex consortia of obligate and facultative microorganisms, including *Bacteriocides, Clostridia, Streptococci,* and *Enterobacteriaceae*[7]. These SCFAs have a wide range of industrial applications, including the synthesis of bioplastics, biosurfactants, lipids, biofuels, and various other industrially relevant chemicals. The current industrial VFA production relies on petrochemical synthesis from coal or crude oil-based products. Interestingly, the overall cost of VFA produced through organic waste bioprocesses is lower than that of petrochemical-based production methods[8]. Thus, FW is an appropriate feedstock for an acidogenic

fermentation process due to its availability, ease of biodegradation, and higher organic matter and moisture content[9]. However, the overall VFA concentration and the type of VFA produced will depend on the composition of the feedstock used, its pretreatment method, the available microbial consortia within the reactor, and the operating parameters (such as pH, temperature, hydraulic retention time, organic loading rate, etc.)[10–12].

In addition to the aforementioned bioprocess parameters, carbonaceous conductive materials can serve as stabilizing agents to improve reactor performance and enhance the efficiency of the acidogenic fermentation process. Due to their large surface area and porous nature, they promote bacterial adsorption and provide a growth matrix for microorganisms. They also improve Direct Interspecies Electron Transfer (DIET) mechanisms between microorganisms[13]. Using carbon-based additives helps shorten the lag phase in microorganisms, thereby accelerating microbial metabolism and richness. Biochar is a promising conductive material that is much more economically sustainable and environmentally friendly[14]. It is a porous carbonaceous material with a high surface area and a rich array of functional groups, derived from waste biomass (such as rice husk, sawdust, lignocellulosic biomass, corn straw, and waste sludge) via high temperature pyrolysis in the absence of oxygen[15]. It demonstrated sufficient conductivity to support interspecies electron exchange via a conduction-based mechanism. In this mechanism, electrons migrate from the electron donor to the acceptor cell via biochar[16].

In this study, the influence of substrate pretreatment using physicochemical methods (acid, alkali, and heat) and the type of microbial culture on acidogenic fermentation is investigated. Further, the effect of biochar on VFA metabolism and production is studied, which is the novelty of this study.

Experimental Section

Substrate characterization

Food waste (FW) used as a substrate in this study was collected from the institute hostel and consisted mainly of rice, roti, mixed vegetables, potato curry, various pulses, and fresh salad residues. After collection, the FW was distributed evenly on the drying tray and heated in a hot-air oven for 24-48 h at 60-65 °C, depending on the initial moisture content. Afterwards, the dried FW was ground in a laboratory mixer-grinder into a uniform powder and stored in airtight containers until further use. The physicochemical characteristics, such as moisture content, total solids (TS), volatile solids (VS), total lipids, total carbohydrates, total proteins, CHNS elemental composition, total organic carbon (TOC), and total Kjeldahl nitrogen (TKN), were determined according to the standard analytical procedures[17]. Estimation of substrate biodegradability and potential for acidogenic fermentation is usually based on compositional parameters[18]. FW is identified as an ideal feedstock for VFA production owing to its high organic matter and easily degradable composition.

Experimental setup

FW leachate was prepared by adding dried FW powder to distilled water at a concentration of 5 g/L. For acid pre-treatment (PT_acid), the substrate was treated with 1 N HCl, and the pH was maintained at 2.5-3.0 for 24 h. In alkali pretreatment (PT_alkali), 1 N NaOH was added to raise the pH to 11, followed by a 24 h incubation. Wet heat pre-treatment (PT_heat) was performed by autoclaving in water at 121 °C and 15 psi for 15 min. The untreated control (UT_control) was run under the same conditions with no pre-treatment. Pre-treatments with acid and base can aid in the deconstruction of lignocellulosic material and the subsequent release of the soluble organic fraction, which is readily available for microbial attack. Acid or base pretreatments are also reported to enhance soluble COD (sCOD) and VFA formation during FW fermentation[19]. After the chemical treatment, the substrates were adjusted to neutral pH (7.0) and then sterilized. The sterilization process was carried out using a MAC autoclave at 121 °C and 15 psi for 15 min before inoculation. All experimental setups were performed in triplicate.

Preparation of inoculum and biochar

Bacillus licheniformis (MTCC-429) was purchased from the Microbial Type Culture Collection (MTCC), Chandigarh, India. Additionally, a bacterial isolate capable of producing VFA, identified by Sanger sequencing as *Klebsiella pneumoniae*, was obtained from an in-house anaerobic digester culture. Both bacteria were grown in nutrient broth at 37 °C until they reached the mid-exponential phase. These were subsequently used as inocula for the experiments. These microbes were selected for their capacity to degrade complex polysaccharides and for their involvement in metabolic pathways that biosynthesize

short-chain fatty acids such as acetate, propionate, and butyrate[18]. Garden waste biomass collected from the campus premises was used as feedstock for biochar production. The biomass was initially dried at 65 ℃ for 24 h and then ground to reduce particle size. The grounded ground material was then pyrolyzed in a muffle furnace at 350 ℃ for 4 h under oxygen-limited conditions. After cooling to room temperature, the biochar was ground to a fine powder and sealed in airtight containers. The surface morphology and elemental composition of the biochar were characterized using field-emission scanning electron microscopy (FE-SEM) and energy-dispersive spectroscopy (SEM-EDS). Previous studies have demonstrated that biochar can enhance anaerobic fermentation by promoting microbial attachment, facilitating electron transfer, and stabilizing microbial communities[20].

Experimental methodology

A systemic workflow for the experimental methodology used in this research is shown in Fig. 1.

Screening of inoculum combination

Batch fermentation experiments were performed using pretreated FW in separate 300 mL working-volume setups to evaluate the most effective inoculum combination for VFA production. Individual experimental setups were inoculated with 4 % (v/v) *Bacillus licheniformis* and 4% (v/v) *Klebsiella pneumoniae,* respectively, while another setup was inoculated with a 4% (v/v) bacterial co-culture (*Bacillus licheniformis* and *Klebsiella pneumoniae*). All preparations for the experimental setups were carried out under aseptic conditions in a laminar airflow cabinet to minimize contamination. The fermentation systems were sealed withrubber septa and maintained at 37 ℃ using a magnetic hot plate stirrer with agitation at 120 RPM for 5 days. Samples were collected every 24 h for further biochemical analysis. The inoculum configuration yielding the highest VFA concentration was identified and selected for all subsequent experimental studies. Each experiment was performed in triplicate, and the obtained results are reported as mean ± standard deviation (SD).

Influence of biochar addition

Based on the screening results, acid pretreatment was chosen as the most effective method for hydrolysis and was therefore selected for further experimentation. Subsequent studies were carried out under two experimental conditions: acid-pre-treated substrate inoculated with a 4% co-culture consortium, and acid-pre-treated substrate inoculated with a 4% co-culture consortium supplemented with biochar at 3 g/L. The fermentation systems were operated with a working volume of 300 mL and maintained at 37 ℃ using a hot plate magnetic stirrer at 120 rpm for 5 days. Samples were collected every 24 h and stored at 4 ℃ in the refrigerator until analysis. Biochar was added to explore its effect on acidogenic fermentation, as conductive carbon materials have been shown to promote microbial activity and

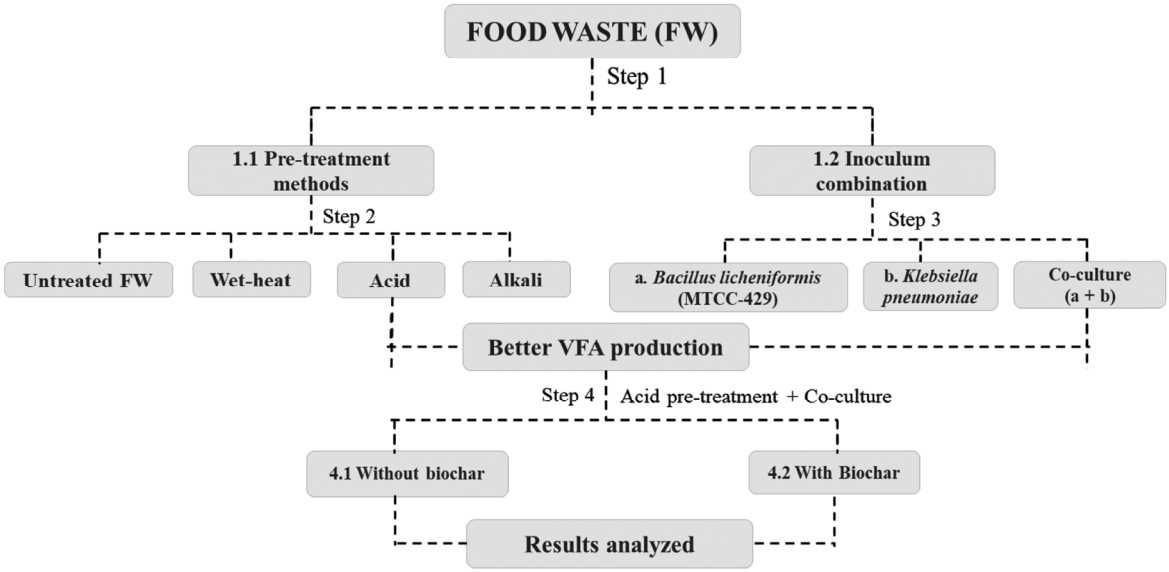

Fig. 1 — A systematic representation of the experimental workflow carried out in the current study

improve VFA production. The addition of biochar was also shown to improve the efficiency and stability of anaerobic digestion systems in batch tests[21,22].

Biochemical analysis

Volatile fatty acids analysis

Samples were collected at different time intervals during fermentation, centrifuged (10,000 rpm, 15 min), and the supernatants were filtered through 0.22 µm nylon membranes (Hi-Media). Subsequently, the VFA concentrations were estimated using an HPLC system (Agilent 1200 Infinity Series) equipped with a diode-array detector (DAD) and a Hi-Plex-H column maintained at 50 °C. The eluent was 0.005 M sulfuric acid, with a flow rate of 0.6 mL/min. The injection volume was 20 µL, and the analysis duration was 30 min. Standard external calibration curves were prepared from analytical-grade acetic, butyric, lactic, and propionic acids. Chromatographic data were processed using the OpenLAB CDS program[20,23].

Soluble chemical oxygen demand estimation

Soluble chemical oxygen demand (sCOD) was measured using the standard dichromate reflux method[17,24]. This method measures the amount of oxygen required to chemically oxidize the organic matter in the samples. The sample organics are first reacted with potassium dichromate ($K_2Cr_2O_7$) in a strongly acidic medium containing concentrated sulphuric acid, and then refluxed for 2 h. During the reaction, most of the oxidizable organic matter is converted into carbon dioxide and water. To measure the oxygen equivalent, the excess dichromate remaining after oxidation is titrated with ferrous ammonium sulfate (FAS), and the amount of dichromate reduced by reaction with organics is determined. The sCOD values were calculated from the volume of FAS used during titration and the results were expressed as mg/L of oxygen demand.

Calculations

The influence of biochar on acidogenic fermentation was evaluated by calculating the percentage increase in total VFA concentration in biochar-supplemented systems relative to the control without biochar. The percentage rise in VFA concentration was determined by the formula below:

$$VFA\ increment\ (\%) = \frac{VFA\ with\ biochar - VFA\ without\ biochar}{VFA\ without\ biochar} \times 100$$

where, VFA with biochar indicates the total VFA concentration (mg/L) measured in the biochar-supplemented experimental setup; VFA without biochar corresponds to the total VFA obtained from the experimental setup without biochar addition (mg/L). Through this calculation, the increase in metabolite production resulting from the addition of conductive materials such as biochar, which has also been reported to enhance microbial activity during anaerobic fermentation[25].

All experiments were repeated three times, so all results in this study are expressed as mean standard deviation (SD). The objective of the statistical analysis was to examine whether there were significant differences between treatments with and without biochar addition. A one-way ANOVA was used to test for differences in the parameters among treatments and biochar addition. The pairwise comparisons of the treatment means were carried out using Tukey's Honest Significant Difference (HSD) post hoc test to make pairwise comparisons between treatment means. This also identified the specific groups that differed significantly. The following equation calculated the value of HSD:

$$Tukey's\ HSD = q\sqrt{\frac{MS_{within}}{n}}$$

In this equation, "q"equals the studentized range at a specified level of significance and number of groups, "MS_{within}" equals the mean square within groups of the ANOVA, and "n" is the number of observations per treatment. A difference between two treatment means was considered statistically significant if the absolute value of the difference was greater than the calculated HSD. A p-value < 0.05 was used as the level of statistical significance[26]. These statistical methods are widely used in fermentation and anaerobic digestion studies to find treatment effects and optimize processes.

Results and Discussion

In the current study, the acidogenic fermentation of FW was investigated as a promising alternative for producing VFA under different operational conditions. The influence of different substrate pretreatment methods, inoculum conditions, and biochar addition on substrate solubilization and efficient VFA production was investigated. Various ultimate and proximate analyses were performed to analyze the composition of food waste (Table 1).

Influence of pre-treatment methods

Production of volatile fatty acids and changes in pH profile

Different pretreatment techniques had different effects on VFA production and pH (Fig. 2(a) and (b)). There was a clear divergence in fermentation performance among the various pretreatment types. This demonstrated that pre-treatment strongly affected the hydrolysis of the substrate and the formation of low-molecular-weight compounds. As a result, subsequent VFA production was affected during fermentation. Overall, acid and alkali pre-treatment showed higher levels of VFA throughout the fermentation stages, indicating that the substances were more effectively hydrolyzed (Fig. 2a). The initial (Day 0) VFA concentrations in samples pre-treated by acid, alkali, and heat were 548.1±30.8 mg/L, 472.5±31.1 mg/L, and 321.3±27.2 mg/L, respectively.

Table 1 — Physicochemical characteristics of food waste used in this study.

Sl. No.	Parameter	Value (% w/w)
1.	Moisture content	76
2.	Total solids (TS)	30 ± 2
3.	Total lipids	4 ± 2
4.	Total carbohydrates	20 ± 2.33
5.	Total proteins	12 ± 0.9
6.	Total organic carbon	41.60
7.	Total Kjeldahl nitrogen	0.0657
8.	C	46.320
9.	H	7.196
10.	N	1.660
11.	S	0
12.	O	38.004
13.	Volatile solids	93 ± 0.18

The untreated sample showed a much lower level of 434.7±28.9 mg/L. The relatively high VFA concentration in chemically pretreated systems implies that under acid and alkali conditions, partial hydrolysis of the complex organic portion of the feedstock occurred prior to active fermentation. While the wet-heat pretreated system produces relatively lower breakdown of the substrate matrix, resulting in the release of less soluble organics. In the early stage of fermentation, both pretreated and non-pretreated experimental setups exhibited high VFA accumulation, indicating active hydrolysis and acidogenesis. Clearly, at Day 1, the acid-pretreated system reached 748.2±27.4 mg/L, compared with the untreated control (591.6±24.8 mg/L).The alkali-pretreated system also showed a significant accumulation of VFA (678.6±27.34 mg/L), whereas the heat-pretreated system showed a lower value (574.2±32.2 mg/L). The increase in VFA concentration over the first 24 h indicates active metabolism and acidogenesis of the released biodegradable fractions during acid pretreatment in anaerobic fermentation systems. The increased VFA in the acid pre-treatment process may be due to the disruption of structural recalcitrance through breaking down lignocellulosic linkages in biomass. Volatile fatty acid production reached a maximum on Day 2 across all treatment regimes, suggesting that the first few days are the most metabolically active in acidogenic conversion. The acid-pretreated substrate showed the highest VFA (987±32.6 mg/L), followed by the alkali-pretreated substrate (764.4±28.4 mg/L), the untreated substrate

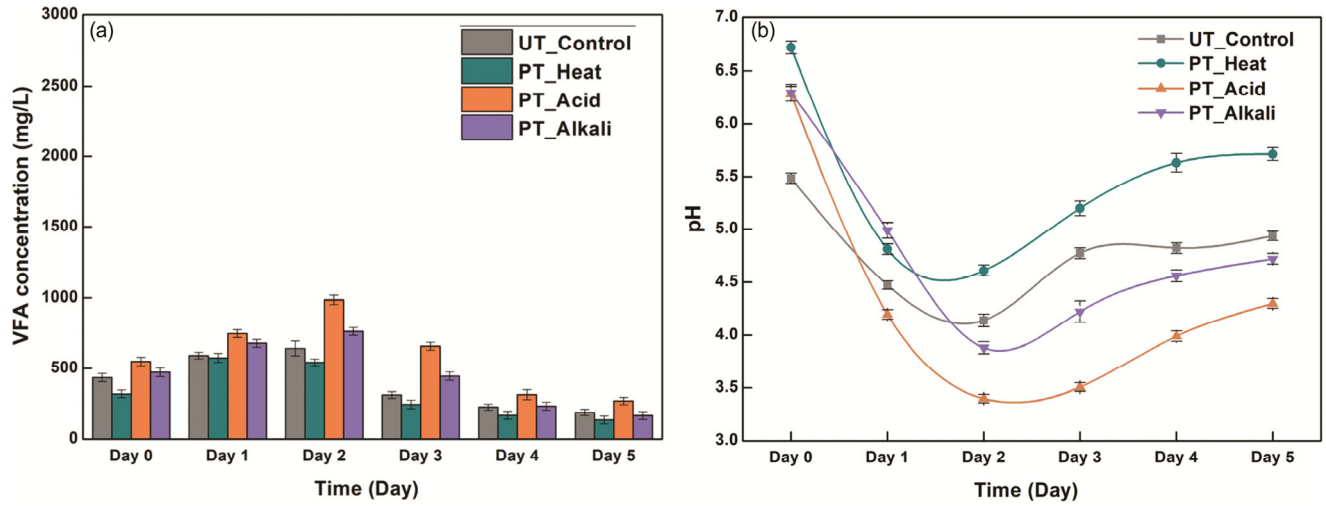

Fig. 2 — (a) Comparison between volatile fatty acids produced from different pre-treatment methods and (b) pH profile of the experimental conditions at different pre-treatment methods

(642.6±53 mg/L), and the heat-pretreated substrate (541.7±24.7 mg/L).

Pretreatment with acids promoted the enzymatic hydrolysis of cellulosic and amorphous carbohydrate fractions, diminished the crystallinity of cellulose, and displayed an increased release of soluble organics. This resulted in enhanced microbial acidogenesis. Alkali pretreatment increased VFA yield compared with the raw feedstock, likely due to lignin solubilization and improved access to structural carbohydrates. Yet, the relatively lower VFA concentration than in the acid-pre-treated system indicates that alkaline pre-treatment was ineffective under the test conditions used. Thermal pre-treatment resulted in the lowest VFA concentration among all pre-treated systems; however, this indicated that thermal disruption alone was not an effective means of solubilizing the substrate.

Following the peak observed on Day 2, VFA concentrations declined gradually across all treatments. On Day 3, the acid-pretreated substrate decreased to 658.35±28.15 mg/L, while the untreated control, alkali-pretreated, and heat-pretreated systems recorded 313.5±23.3 mg/L, 445.5±29.9 mg/L, and 247.5±29.9 mg/L, respectively. This declining trend continued through Day 4 and Day 5. By the end of fermentation (Day 5), VFA concentrations decreased to 271.32±25.96 mg/L in the acid-pretreated system, 190.19±21.33 mg/L in the untreated substrate, 167.58±27.98 mg/L in the alkali-treated system, and 135.66±27.98 mg/L in the heat-treated system. The progressive reduction in VFA concentration after the peak phase may be attributed to secondary utilization of VFA by microbial populations, depletion of easily biodegradable substrates, and possible conversion of intermediate acids into other metabolic products during prolonged fermentation. Similar observations of VFA accumulation followed by gradual consumption were reported during batch acidogenic fermentation of organic wastes[27].

The pH profiles observed during fermentation further supported the VFA production trends (Fig. 2b). All treatments showed a gradual decrease in pH from the first to the last day of fermentation, which is characteristic of acidogenic fermentation due to the accumulation of organic acids. The acid-pretreated system showed the most pronounced pH reduction, decreasing from 6.285±0.07 on Day 0 to 3.4±0.04 on Day 2, which coincided with period of maximum VFA accumulation. On the other hand, the untreated control decreased from 5.485±0.05 to 4.143±0.06, and both the alkali- and heat-pretreated systems achieved pH values of 3.88±0.06 and 4.61±0.05, respectively, on Day 2. The significant acidification of the acid-pretreated substrate shows a much-enhanced fermentative process and higher acid production. A small pH increase was observed in all treatments after Day 2, likely due to decreased acidogenesis and partial utilization of accumulated VFA in the latter stages of fermentation.

The experimental results were also subjected to statistical analysis using one-way ANOVA and Tukey's HSD post hoc test. The one-way ANOVA showed that pretreatment has a statistically highly significant impact on VFA production ($F = 100.98$, $p = 1.07 \times 10^{-6}$). The calculated F-value substantially exceeded the critical F-value of 4.067 (Table 2 and 3). These findings confirm that the pretreatment strategy significantly affects the overall acidogenic performance. Tukey's HSD post hoc test at the 95% confidence level revealed statistically significant differences among all pairwise treatment comparisons, with a calculated HSD critical value of 86.4 mg/L (Table 4). Slightly smaller differences between pairs, such as the one between the untreated control and the wet-heat pretreated one (100.77 mg/L), were larger than the HSD and were statistically significant. Pretreatment is particularly important for acidogenic fermentation, because FW consists of structurally complex organic constituents, such as cellulose, hemicellulose, and lignin, that are

Table 2 — Summary statistics of experimental treatments under different pretreatment conditions

Group	Count	Sum	Average	Variance
UT_Control	3	1927.80	642.60	3969
PT_Heat	3	1625.50	541.8333	166.4233
PT_Acid	3	2961.20	987.0667	161.2933
PT_Alkali	3	2293.62	764.54	70.6188

Table 3 — One-way ANOVA results of experimental treatments under different pretreatment conditions

Source of variation	SS	df	MS	F	P-value	$F_{critical}$
Between groups	330771.3	3	110257.4	100.9837	1.07E-06	4.067
Within groups	8734.671	8	1091.834			
Total	339506.9	11				

Table 4 — Tukey's Honest Significant Difference (HSD) post hoc test showing pairwise comparisons of VFA production among different pretreatment conditions

Comparison	Mean 1 (mg/L)	Mean 2 (mg/L)	Mean difference (mg/L)	HSD critical value	Significance ($p<0.05$)
UT_Control vs PT_Heat	642.60	541.83	100.77	86.40	Significant
UT_Control vs PT_Acid	642.60	987.07	344.47	86.40	Significant
UT_Control vs PT_Alkali	642.60	764.54	121.94	86.40	Significant
PT_Heat vs PT_Acid	541.83	987.07	445.23	86.40	Significant
PT_Heat vs PT_Alkali	541.83	764.54	222.71	86.40	Significant
PT_Acid vs PT_Alkali	987.07	764.54	222.53	86.40	Significant

not directly consumed by microorganisms. Acidic pretreatment enhances hydrolysis by disrupting lignin-carbohydrate complexes, decreasing cellulose crystallinity, and converting particulate organic matter into soluble, easily biodegradable forms[28].

The improved substrate accessibility, in turn, accelerates acidogenesis and increases VFA production. Comparable improvements in VFA production through chemical pretreatment have been reported for waste-activated sludge and various organic substrates in the literature, substrate solubilization has been shown to greatly enhance fermentation efficiency[29]. Overall, the current results show that acidic pretreatment is an effective strategy for enhancing FW hydrolysis and acidogenic fermentation, yielding the highest VFA accumulation across all tested conditions. The amount produced was 15.3-fold higher after acid pretreatment and 12.5-fold higher after alkali pretreatment than in the control (no pretreatment). Acidic pretreatment may be a promising approach for producing VFA via acidogenic fermentation.

Substrate utilization

A clear difference in solubilization was observed across the various experimental systems. The acid pretreatment yielded the highest initial sCOD concentration and a large overall reduction during fermentation, demonstrating its ability to significantly enhance substrate solubilization and microbial conversion. The acid-pretreated fermented broth had the highest initial sCOD concentration (15472.5±10.5 mg O_2/L) on Day 0, followed by the alkali-pretreated setup (14783± 47 mg O_2/L). The untreated and heat-pretreated FW had initial concentrations of 12878±150 mg O_2/L and 12898.5±85.5 mg O_2/L, respectively. The high initial total sCOD values for the chemically pretreated systems indicate that alkali and acid pretreatments promoted extensive solubilization of particulate organic matter in the FW prior to microbial fermentation. Acid hydrolysis cleaves lignocellulosic linkages, hydrolyzes structural carbohydrates, and releases intracellular soluble metabolites into the aqueous phase.

Significant sCOD removal was observed over the first 24 h of fermentation under all treatments, during which a rapid VFA build-up was noted. On Day 1, the acid-pretreated system showed a sharp decrease from 15472.5±10.5 mg O_2/L to 6346±22 mg O_2/L, demonstrating the microbial turnover of the soluble organics released during pretreatment. Similarly, alkali-pretreatment decreased to 6908.5±14.5 mg O_2/L, while untreated FW and heat-pretreated FW recorded 9579±21 mg O_2/L and 7551±659 mg O_2/L. The rapid sCOD reduction on Day 1 indicated high hydrolysis and acidogenic activity, with soluble sugars, proteins, and amino acids rapidly converted to VFA. The maximum sCOD reduction happened from Day 1 to Day 2, concurrent with maximum VFA production. On Day 2, the acid-pretreated system still had the lowest sCOD of 2897.5 ± 4.5 mg O_2/L, while the untreated FW, alkali pretreatments, and heat pretreatment showed sCOD values of 3847 ± 815 mg O_2/L, 3828.5 ± 13.5 mg O_2/L, and 6267 ± 1 mg O_2/L, respectively.

The more pronounced decrease under acidic conditions further confirmed the increased conversion efficiency of dissolved organic matter into fermentation intermediates and VFA. The rate of sCOD reduction gradually stabilized after Day 2 in all systems, reflecting the depletion of readily biodegradable substrates and the onset of slower degradation of recalcitrant compounds. On Day 3, the acid-pretreated system recorded 2627.5 ± 13.5 mg O_2/L, whereas untreated FW, heat pretreatment, and alkali pretreatment recorded 3098 ± 102 mg O_2/L, 3125 ± 3 mg O_2/L, and 3296 ± 20 mg O_2/L, respectively. By Day 5, the final sCOD concentrations decreased to 2361.25 ± 19.75 mg O_2/L in the acid-pretreated substrate. At the same time, the values for untreated FW, alkali pretreatment, and heat pretreatment remained steady at

2870 ± 6 mg O_2/L, 2870.5 ± 25.5 mg O_2/L, and 3008 ± 10 mg O_2/L, respectively. This could be linked to a slower rate of reduction in the later stages of fermentation due to depletion of readily available substrates and reduced metabolic activity after the peak acidogenesis phase. Some of the dissolved organics might also have remained as stable intermediate metabolites that were not further degraded during the experiment[4]. The calculated sCOD removal efficiencies also emphasized the superiority of acidic conditions for substrate conversion. Acid-pretreated FW was observed with the highest sCOD removal efficiency of 84.74%, followed by alkali pretreatment (80.58%), unpretreated FW (77.71%), and heat pretreatment (76.68%). An acidic environment would favour the solubilization of more organic matter, which microbes could then utilize for acidogenic fermentation. It is known that increased sCOD removal efficiencies are often associated with efficient hydrolysis of solid samples into soluble intermediates, which acidogenic microbes may then utilize for high-efficiency VFA production. A similar association of increased solubilization, higher sCOD reduction, and VFA accumulation was often observed during acidogenic fermentation of FW and sludge substrates[30,31]. These systems with enhanced sCOD depletion also exhibited elevated VFA accumulation and a more pronounced pH drop, indicating increased acidogenic activity. For example, the acid-pretreated system with the highest extent of VFA production also showed the most remarkable sCOD removal. This signifies enhanced conversion of dissolved organic matter into short-chain fatty acids by acidogenic bacteria. This relationship confirmed that the solubilization of complex organic matter is a rate-limiting step in acidogenic fermentation. Recent pretreatment-assisted food waste fermentation research also found that, due to improved substrate solubilization, increased organic bioavailability strongly stimulated acidogenic conversion efficiency[32]. Similarly, in waste-activated sludge fermentation research, pretreatment increased the extent of solubilization, thereby directly enhancing VFA accumulation by catalyzing hydrolysis and releasing intracellular organics[33]. However, lower levels of thermal pretreatment may explain the lesser improvement in stimulated substrate solubilization, except for subsequent chemical and catalytic additions, which are consistent with the relatively lower sCOD removal efficiency in this study[32,34].

The present findings reveal that substrate pretreatment governs the efficiencies of hydrolysis and fermentation during acidogenic FW fermentation. Acidic pretreatment emerged as the most beneficial method for enhancing substrate solubilization, generating soluble organics, and promoting microbial acidogenesis, resulting in the highest sCOD removal efficiency, the highest VFA accumulation, and the most significant decrease in pH. Based on the results of the initial screening experiment, acid pretreatment was applied in subsequent experiments to recover short-chain fatty acids from food waste.

Influence of inoculum combination

Production of volatile fatty acids and variation in pH profile

The impact of different inoculum combinations on VFA during acidogenic fermentation and pH variations is presented in Fig. 3(a) and Fig. 3(b),

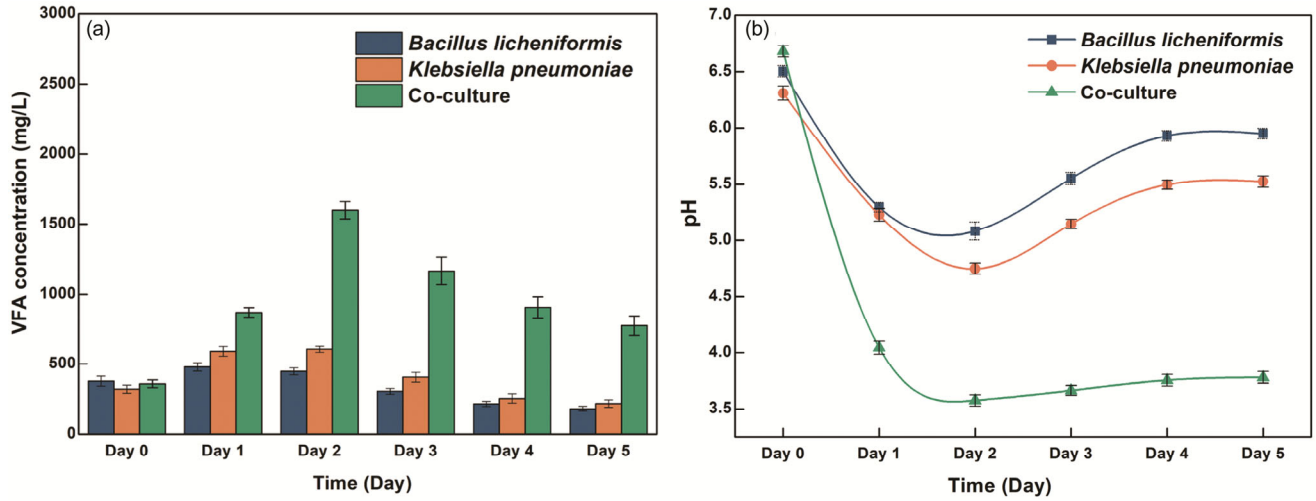

Fig. 3 — (a) Comparison ofvolatile fatty acids produced from individual vs. co-culture experimental setups and (b) pH profile of the experimental conditions at different individual vs. co-culture systems

respectively. All experiments were performed using the FW substrate as the inoculums after acid pretreatment, and the substrate was sterilized before inoculation. VFA production with various inocula demonstrated fermentation performance varied not only between bacterial strains but also among mixed co-culture systems, suggesting that inocula configuration played a significant role in regulating substrate hydrolysis, microbial metabolism, and VFA production. Compared to other systems, the mixed co-culture system yielded the highest VFA concentration throughout the fermentation period. On Day 1, it yielded a maximum of 870.5 ± 34.3 mg/L VFA, which were nearly 80% and 47% higher than those in individual cultures (*B. licheniformis* (478.5 ± 30.3 mg/L) and *K. pneumoniae* (591.6 ± 34.8 mg/L)). The rapid increase in VFA concentration during the initial half of the fermentation period indicated that simultaneous hydrolysis and acidogenesis by the mixed co-culture microbes created a more efficient metabolic environment for other bacteria to access. As a result, the optimal synergistic effects played an important role in improving system performance.

Maximum VFA accumulation was attained on Day 2 across all systems. It clearly indicates that the initial 48 h were the most active phase of acidogenic fermentation. The co-culture system attained a maximum VFA concentration of 1600.2 ± 63 mg/L, an order of magnitude higher than those of *K. pneumoniae* (606.9 ± 22.1 mg/L) and *B. licheniformis* (447.3 ± 24.7 mg/L) alone. The close to 3-fold increase in the co-culture compared to the monocultures demonstrates microbial synergy during fermentation. *Bacillus licheniformis* is known for its robust hydrolytic activity and secretion of extracellular enzymes, such as proteases, amylases, cellulases, and lipases, which aid in the degradation of complex organic polymers into soluble intermediates[35]. These hydrolysis products are then immediately made available to *Klebsiella pneumoniae*, which efficiently directs soluble substrates to VFA via fermentative metabolic pathways. During microbial fermentation, carbohydrates are first processed by glycolysis (the Embden-Meyerhof-Parnas pathway), in which glucose is converted to pyruvate and reducing equivalents, such as NADH. Pyruvate is the key metabolic intermediate and can be converted to acetyl-CoA through oxidation by pyruvate ferredoxin oxidoreductase (PFOR) in the presence of ferredoxin (Fd) during butyric acid fermentation. The NADH: ferredoxin oxidoreductase (NFOR) catalyzes the formation of reduced ferredoxin in the reaction with NADH. The acetyl-CoA is transformed into short-chain fatty acids (acetate, butyrate, lactate, propionate) by the activity of a diverse set of enzymes. In addition to glycolysis, other routes, such as the Entner-Doudoroff pathway, can produce pyruvate. Glyceraldehyde-3-phosphate and fructose-6-phosphate, two compounds produced during glycolysis, are also produced in the pentose phosphate pathway, resulting in pyruvate synthesis[36]. The coexistence of hydrolytic and acidogenic metabolisms in the co-culture system, therefore enabled continuous substrate degradation and efficient conversion into VFA.

Following the peak production phase on Day 2, VFA concentrations gradually declined in all systems. On Day 3, maintained a comparatively high VFA concentration of 1168.2 ± 99 mg/L, whereas K. pneumoniae and B. licheniformis individually showed concentrations of 405.9 ± 34.3 mg/L and 306.9 ± 19.9 mg/L, respectively. Although VFA concentration decreased after Day 2, the co-culture maintained significantly higher VFA levels than the individual cultures for the remainder of the fermentation period. By Day 4, the co-culture system reached a total VFA concentration of 907.2 ± 75.6 mg/L, whereas the individual bacterial systems reached 256.5 ± 32.7 mg/L (*K. pneumoniae*) and 218.7 ± 18.1 mg/L (*B. licheniformis*). By the end of fermentation (Day 5), VFA concentrations in the co-culture system stabilized at 775.39 ± 70.49 mg/L. In contrast, the *K. pneumoniae* and *B. licheniformis* systems remained at levels of 220.78 ± 26.72 mg/L and 183.54 ± 17.98 mg/L, respectively. This observed rapid increase in VFA concentration during batch acidogenic fermentation of food waste and organic residues has frequently been followed by mild decreases. In addition, recent experiments with mixed microbial consortia have demonstrated that co-cultures can be maintained for extended periods at higher metabolite concentrations, driven by metabolic cooperation and enhanced substrate conversion[37].

The statistical analysis confirmed that the inoculum configuration significantly affected VFA production. One-way ANOVA yielded a highly significant difference between the four experimental treatments. The analysis gave an F value ($F = 768.42$, $p = 5.88 \times 10^{-8}$), much greater than the critical value of 5.1432 (Table 5 and 6). Tukey's HSD post hoc analysis further revealed statistically significant differences

among all pairwise comparisons at the 95% confidence level (Table 7). The co-culture system showed significantly higher VFA production than either *B. licheniformis* or *K. pneumoniae* alone, with mean differences of 1125.77 mg/L and 993.93 mg/L, respectively. Even the comparison between the two individual cultures cultures showed significant variation, indicating that *K. pneumoniae* exhibited a comparatively stronger acidogenic capability than *B. licheniformis* under the tested conditions.

The pH variations observed during fermentation further supported the VFA production trends (Fig. 3b). All setups showed a gradual decline in pH during the initial fermentation period, driven by the accumulation of organic acids produced via acidogenesis. However, the co-culture system showed the sharpest decrease in pH, declining from 6.68±0.05 on Day 0 to 3.575±0.05 on Day 2. In comparison, *K. pneumoniae* and *B. licheniformis* individually reached pH values of 4.75±0.05 and 5.08±0.08, respectively, during the same period. The stronger acidification observed in the co-culture setup corresponded directly with the higher VFA accumulation, confirming intensified fermentative metabolism and enhanced organic acid synthesis. After Day 2, a slight increase in pH was observed in all systems. At Day 5, the pH was 5.9475±0.048 for *B. licheniformis*, 5.525±0.05 for *K. pneumoniae*, and 3.78±0.052 for the co-culture system. This gradual pH recovery can be explained by reduced acidogenic activity, limited availability of free hydrolyzable substrates, and production of alkaline metabolites from protein hydrolysis[38]. Similar phenomena have been reported in recent anaerobic fermentation studies involving food waste substrates[39,40].

Overall, the present work revealed that the inoculum configuration plays an important role in acidogenic fermentation. Compared with individual bacterial cultures, the co-culture of *Bacillus licheniformis* and *Klebsiella pneumoniae* showed a significant increase in VFA production, which may be attributed to synergism between hydrolytic and acidogenic metabolism. The improved substrate degradation, the highest accumulated VFA, and the evident decrease in pH indicated that the co-culture system was promising for the production of short-chain fatty acids from food waste via acidogenic fermentation.

Substrate utilization

Substrate solubilization and organic matter consumption varied markedly between individual bacterial cultures and the co-culture system. This demonstrates the strong influence of inoculum composition on fermentation efficiency. Overall, the co-culture system exhibited the highest substrate solubilization and organic matter consumption rate throughout the experiment due to the synergistic effects of multiple bacteria in degrading various organic compounds. For all setups, the co-culture system demonstrated the highest initial sCOD concentration of 12924±152 mg O_2/L at Day 0, followed by the *B. licheniformis* (12740±145 mg O_2/L) and *K. pneumoniae* (9549±118 mg O_2/L) setups. The elevated initial sCOD observed in the co-culture system indicates improved substrate

Table 5 — Summary statistics of experimental treatments under different inoculum combinations

Group	Count	Sum	Average	Variance
Bacillus licheniformis	3	1423.3	474.43	461.70
Klebsiella pneumoniae	3	1818.8	606.27	5.613
Co-culture (*B. licheniformis* + *K. pneumoniae*)	3	4800.6	1600.2	3969

Table 6 — One-way ANOVA results of experimental treatments under different inoculum combinations

Source of variation	SS	df	MS	F	P-value	$F_{critical}$
Between groups	2272634	2	1136317	768.42	5.88E-08	5.1432
Within groups	8872.63	6	1478.772			
Total	2281507	8				

Table 7 — Tukey's Honest Significant Difference (HSD) post hoc test showing pairwise comparisons of VFA production among different inoculum combinations

Comparison	Mean 1 (mg/L)	Mean 2 (mg/L)	Mean difference (mg/L)	HSD critical value	Significance ($p<0.05$)
B. licheniformis vs *K. pneumoniae*	474.43	606.27	131.83	96.40	Significant
B. licheniformis vs Co-culture	474.43	1600.20	1125.77	96.40	Significant
K. pneumoniae vs Co-culture	606.27	1600.20	993.93	96.40	Significant

hydrolysis immediately after inoculation. This may be attributed to the rapid secretion of extracellular hydrolytic enzymes by *B. licheniformis*, which facilitated the breakdown of particulate organic matter into soluble intermediates that became readily available for fermentative metabolism. A substantial decline in sCOD was observed during the initial phase of fermentation in all setups, coinciding with an increase in VFA concentration. By Day 1, the co-culture system exhibited a marked reduction in sCOD from 12924 ± 152 mg O_2/L to 9578.5 ± 125 mg O_2/L. Similarly, setups inoculated with *B. licheniformis* and *K. pneumoniae* individually showed reductions to 9742.5 ± 132 mg O_2/L and 6298 ± 96 mg O_2/L, respectively. The rapid reduction during the first 24 h suggests active microbial utilization of soluble organic compounds generated during substrate hydrolysis. At this stage, fermentative microorganisms likely metabolized soluble sugars, amino acids, and other low-molecular-weight compounds to generate VFA and other intermediate metabolites. The most significant changes in sCOD were observed between Day 1 and Day 3, which also corresponded to the period of maximum VFA accumulation. On Day 2, the co-culture system showed a further decline to 6299.5 ± 104 mg O_2/L, while *B. licheniformis* and *K. pneumoniae*setups recorded 9538.5 ± 126 mg O_2/L and 6038 ± 88 mg O_2/L, respectively. The sharper decrease in the co-culture system indicates more efficient conversion of dissolved organics into fermentation products. This trend became even more evident on Day 3, when the co-culture setup showed a dramatic reduction to 3275 ± 54 mg O_2/L. In contrast, the *B. licheniformis* and *K. pneumoniae* systems maintained comparatively higher concentrations of 6306 ± 92 mg O_2/L and 3949.75 ± 61 mg O_2/L, respectively. The rapid depletion of soluble organics in the co-culture system strongly supports the existence of synergistic microbial interactions that enhanced substrate utilization efficiency. After Day 3, the rate of sCOD reduction slowed in all experimental systems, indicating depletion of readily biodegradable substrates and gradual stabilization of microbial metabolism. On Day 4, the co-culture setup recorded 3193.5 ± 49 mg O_2/L, while *B. licheniformis* and *K. pneumonia* setups showed 6217 ± 85 mg O_2/L and 3658.5 ± 58 mg O_2/L, respectively. By Day 5, the final sCOD concentrations further decreased to 3089 ± 45 mg O_2/L in the co-culture system, 6142 ± 79 mg O_2/L in the *B. licheniformis* system, and 3221 ± 52 mg O_2/L in the *K. pneumonia* system. It also showed the highest sCOD removal efficiency of 76.11% using the co-culture system, indicating that the co-culture system had better substrate-conversion ability than single-species bacteria. The higher the percentage of sCOD removal during acidogenic fermentation, the higher is the degree of hydrolysis and active microbial metabolism of the organic fraction of the substrate. sCOD is an index of soluble organics in the fermentation systemthat bacteria can utilize, and it is directly related to the conversion of soluble organics to VFA and other fermentation products. The positive correlation between sCOD depletion and increased VFA production here confirms that increased substrate solubilization enhanced the fermentation process. Another study also indicated that inoculum type and effective hydrolysis affected both sCOD removal and VFA accumulation, such that optimized microbial conditions led to improved fermentation[41]. Similarly, another study reported that effective hydrolysis and the maintenance offavourable fermentation conditions enhanced soluble organic conversion and increased VFA production from food waste substrates[42].

Overall, this study concluded that inoculum composition is an essential influence on fermentation performance in food waste acidogenic conversion. The bacterial co-culture system experiments resulted in a dramatically greater reduction in sCOD and VFA production than either single bacterial inoculum, suggesting that cooperative synergism within the microbial community during anaerobic fermentation greatly enhanced the capacity to produce short-chain fatty acids. That means, by utilizing the bacterial consortium's brilliant hydrolytic and acidogenic characteristics, the bacterial co-culture system, combined with acid-pretreated food wastes, was used in a subsequent experimental setup to shorten the fermentation retention time and achieve the maximum recovery of short-chain fatty acids.

Influence of biochar

This section assessed the effects of biochar additions on acidogenic fermentation by examining the performance of experimental setups using acid-pretreated food waste and a bacterial co-culture, in the absence of biochar (PT_acid + co-culture) and in its presence(PT_acid + co-culture + biochar). Changes in total VFA accumulation, individual VFA profiles, and

pH profiles throughout the fermentation process were monitored. HPLC was used to analyse daily samples collected from both setups to determine total concentrations and the distribution of individual VFA profiles.

Production of volatile fatty acids and changes in pH profile

The total VFA production profiles for both systems are presented in Fig. 4. The distribution of each VFA is represented in Fig. 5 (a) and (b). Both experimental setups showed distinct differences in fermentation behaviour, suggesting that biochar significantly altered microbial activity and metabolite production. In the setup operated without biochar supplementation (PT_acid + co-culture), the accumulated VFA reached its maximum concentration on Day 2 at 1445.16 ± 80 mg/L. In the beginning (Day 0), the VFA concentration was low (329.3 ± 35 mg/L), which increased sharply to 939.36 ± 55 mg/L on Day 1. The high rate in the first 48 h reflects active hydrolysis of the substrate and efficient transformation of soluble organics into fermentation intermediates. However, after peaking on Day 2, VFA concentration gradually declined to 1059.69 ± 65 mg/L on Day 3, then to 816.32 ± 50 mg/L and 649.3 ± 45 mg/L on Day 4 and Day 5, respectively. The decreasing VFA concentrations observed in the later stages of fermentation could also be due to the exhaustion of easily degradable substrates and secondary microbial utilization of excess acid. The individual VFAs were plotted to investigate the metabolic pathway during the fermentation. Small amounts of lactic acid (678 ± 20 mg/L) and acetic acid (290 ± 25 mg/L) were detected at Day 0, indicating that lactic acid fermentation was very rapid after inoculation, as the easily biodegradable substrates were rapidly utilized. All VFAs were detected together on Day 2, with the dominant metabolites being lactic acid (750 ± 35 mg/L) and propionic acid (665 ± 30 mg/L). Propionic acid increased even after Day 2, reached a maximum value of 985 ± 40 mg/L on Day 4, then declined slightly on Day 5. This implied that the acidogenic bacteria could continually utilize the lactic acid to produce propionates through the second fermentation process. Propionic acid can be produced via anaerobic fermentation through two pathways: the acetyl-CoA pathway and the lactate utilization pathway[43]. In the former pathway, acetyl-CoA is sequentially converted to propionyl-CoA by propionyl-CoA synthetase. Further, it is converted to propionate via propionyl-CoA kinase. Alternatively, lactate can be used as a direct precursor via lactate-utilizing bacteria that act on lactate dehydrogenase and propionate-CoA transferase to produce propionate[44]. The drop in lactic acid and the increase in propionic acid from Day 2 onwards are evidence that lactate acts as an intermediate substrate for secondary propionate production.

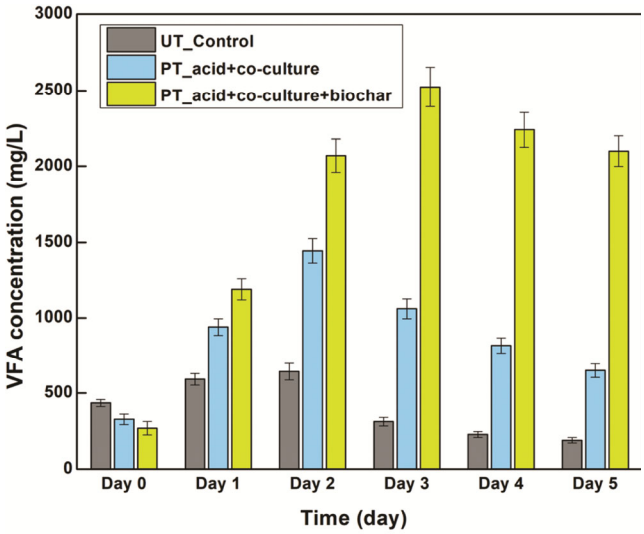

Fig. 4 — Total amount of volatile fatty acids produced in the respective experimental setups: untreated control without any treatments (UT_control), acid-pretreated food waste with bacterial co-culture (PT_acid+co-culture), and acid-pretreated food waste with bacterial co-culture and biochar (PT_acid+co-culture+biochar)

The fermentation profile of the experimental setup with biochar (PT_acid+co-culture+biochar) showed a markedly different trend. Initial VFA concentration at Day 0 was relatively lower (270 ± 45 mg/L).It exhibited substantially higher VFA accumulation during the subsequent fermentation period. The total VFA concentration increased sharply to 1186.5 ± 70 mg/L at Day 1. The highest VFA concentration was observed at Day 3 (2523.5 ± 130 mg/L), which was significantly higher than the highest value without biochar supplementation. Even during the later stages of fermentation, elevated VFA concentrations of 2240.5 ± 115 mg/L and 2099.5 ± 100 mg/L were observed on Day 4 and Day 5, respectively. The increase in total VFA concentration with biochar addition was reflected in the percentage increase relative to the experimental setup without biochar. The VFA concentration was increased by 20.83% on Day 1 and by 30.19% on Day 2 in the presence of biochar. The increase in VFA concentration with biochar addition during the later stage of fermentation

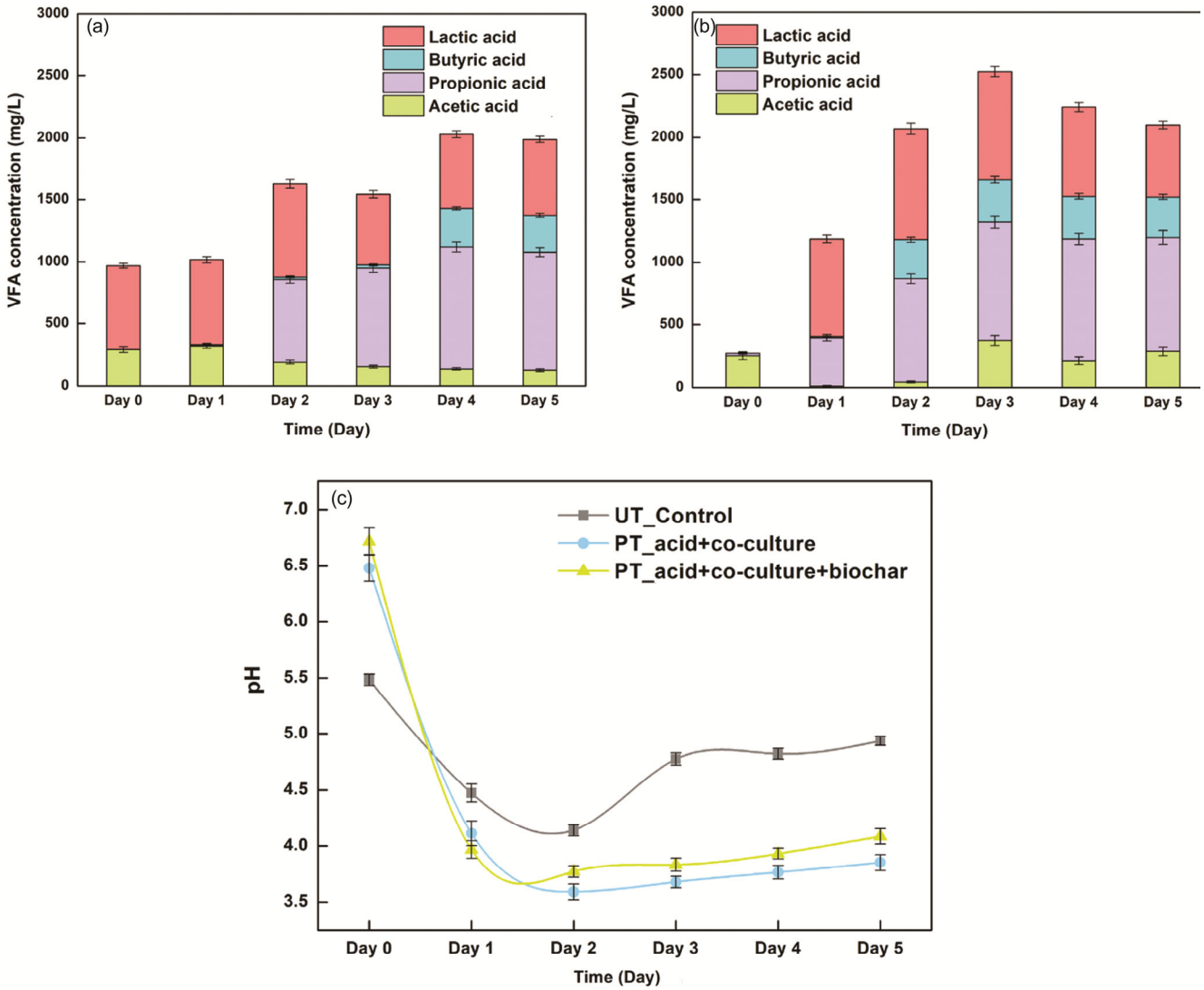

Fig. 5 — Individual amountsof volatile fatty acids produced in (a) acid-pretreated food waste with bacterial co-culture (PT_acid+co-culture), (b) acid-pretreated food waste with bacterial co-culture and biochar (PT_acid+co-culture+biochar), and (c) pH variation with time in different experimental conditions

was 58.01%, 63.57%, and 69.07% on Days 3, 4, and 5, respectively.

The prolonged increase in all VFA in the experimental setup with biochar indicated that biochar facilitated not only the initial fermentation process but also prolonged it by stabilizing the system and enhancing substrate utilization efficiency. Individual VFA analysis revealed that net changes in the metabolite profile were highly significant in the biochar-supplemented setup. At the commencement of fermentation, small quantities of acetic acid (250 ± 30 mg/L) and propionic acid (20 ± 8 mg/L) formed. On Day 1, all four VFA were recorded, with lactic acid at 781 ± 30 mg/L and propionic acid at 383 ± 25 mg/L. Lactic acid increased to a maximum of 890 ± 45 mg/L on Day 2 and then declined. In contrast, the other three VFA increased later to a maximum level of 975 ± 45 mg/L (propionic acid) and 340 ± 22 mg/L (butyric acid), respectively, on Day 4. Acetic acid also increased sharply after Day 2, and reached 374 ± 40 mg/L on Day 3. The combined production of acids indicated a change in the material's overall metabolism, with increased conversion of lactate to acids such as propionate and butyrate. The increase in VFA production after biochar addition can be explained by biochar's physicochemical and microbiological properties. Due to its porous structure and large surface area, biochar can serve as a good carrier for microbes involved in biofilm formation, thereby increasing microbial retention. Also, biochar

adsorb soluble organic matter, nutrients, and fermentation intermediates, thereby creating microenvironments. The conductive properties of biochar have also been reported to promote direct interspecies electron transfer (DIET), thereby enhancing syntrophic interactions among fermentative microorganisms and improving substrate conversion efficiency[45]. A similar study investigated the effect of biochar on anaerobic digestion performance using two inoculum sources: cellulose-peptone-swine inoculum and swine manure inoculum[20]. The results showed a 20.3-38.7% increase in the accumulation of VFA with the addition of biochar. Moreover, microbial community analysis showed that its addition could facilitate the growth and enrichment of microorganisms, thereby improving fermentation. Even supplementation with sludge biochar from the anaerobic digestion of sludge supernatant resulted in a 40.4% increase in VFA yield[46]. The statistical analysis further confirmed the significant effect of experimental configuration on VFA production. One-way ANOVA demonstrated a highly significant difference among the experimental systems ($F = 1805.68$, $p = 4.56 \times 10^{-9}$), with the calculated F-value substantially exceeding the critical value of 5.143 (Tables 8 and 9). These statistical analyses show that the variations in VFA production were not random. Tukey's HSD post hoc analysis indicated significant differences at an HSD critical value of 96.1 mg/L (Table 10) among all pairs of treatments for the experiment. The largest gap in the means occurred between the untreated control and the biochar-supplemented setup (1880.9 mg/L). This illustrates the level of extent of enhancement achieved by the combined effects of acidic pretreatment, co-culture inoculation, and biochar supplementation.

The pH changes during fermentation largely mirrored that of the VFA production profiles (Fig. 5(c). In all experimental systems, the initial pH was adjusted to 7 to provide optimal conditions for acidogenic fermentation. The setup without biochar showed an initial pH drop to 3.59±0.07 on Day 1, after which the maximum total VFA production was observed on Day 2, coinciding with peak VFA accumulation. The pH increased slightly from 3.59±0.07 to 3.85±0.068 by Day 5, then declined again as the total VFA concentration fell. This trend may have reflected a decreased acidogenic rate and the secondary re-consumption of acids accumulated during the longer fermentation period. In the biochar-supplemented experimental setup, the pH decreased more rapidly and remained comparatively lower throughout the fermentation period, reaching 3.77±0.05 on Day 2 and stabilizing near 4.09±0.07 by Day 5. The greateracidification during fermentation in the biochar-supplemented experimental setup was consistent with the observed VFA accumulation[47]. In addition, it was demonstrated that an optimal neutral pH during acidogenic fermentation, conducive to hydrolysis and acidification, was consistent with current results[48]. Overall, compositional and statistical analyses, along with pH measurements, collectively showed that the acid pretreatment step, a selective bacterial co-culture, and biochar supplementation efficiently enhance VFA production. The biochar elicited superior VFA yield by improving substrate

Table 8 — Summary statistics of experimental treatments under different experimental conditions

Group	Count	Sum	Average	Variance
UT_Control	3	1927.80	642.60	3969
PT_Acid + co-culture	3	4875	1625.00	100
PT_Acid + co-culture + biochar	3	7570.50	2523.50	342.25

Table 9 — One-way ANOVA results of experimental treatments under different experimental conditions

Source of variation	SS	df	MS	F	P-value	$F_{critical}$
Between groups	5310197	2	2655098	1805.678	4.56E-09	5.143
Within groups	8822.5	6	1470.42			
Total	5319019	8				

Table 10 — Tukey's Honest Significant Difference (HSD) post hoc test showing pairwise comparisons of VFA production among different tested experimental conditions (control (without any pretreatment), acid-pretreated food waste with bacterial co-culture, and acid-pretreated food waste with bacterial co-culture supplemented with biochar)

Comparison	Mean 1 (mg/L)	Mean 2 (mg/L)	Mean difference (mg/L)	HSD critical value	Significance ($p<0.05$)
UT_Control vs PT_acid+co-culture	642.60	1625.00	982.40	96.10	Significant
UT_Control vs PT_Acid+co-culture+biochar	642.60	2523.50	1880.90	96.10	Significant
PT_acid+co-culture vs PT_acid+co-culture+biochar	1625.00	2523.50	898.50	96.10	Significant

utilization efficiency, boosting microbial activity over prolonged periods, and altering metabolite distribution. This resulted in markedly higher VFA accumulation compared with the non-supplemented systems. This research clearly proves that the biochar-assisted acidogenic fermentation method has outstanding potential for efficient short-chain fatty acids-powered food waste valorization.

Soluble chemical oxygen demand variation

Changes in sCOD during acidogenic fermentation were used to monitor the degree of substrate solubilization and microbial growth on the dissolved organic matter with and without the addition of biochar during fermentation in the different experimental systems. The profiles of sCOD indicated that the different experimental systems had distinct efficiencies for substrate conversion, suggesting that the addition of biochar significantly affected fermentation, particularly in hydrolysis and acidification. At the beginning of fermentation (Day 0), the biochar-amended system had a higher sCOD concentration of 11267.7 ± 192 mg O_2/L, whereas the system without biochar recorded 10502 ± 185 mg O_2/L. The higher initial sCOD concentration may result from increased dissolution and accumulation of soluble organic matter. This could be explained by the physical and chemical attributes of biochar, which has a large surface area and a high capacity to adsorb chemical species, resulting in increased accumulation of soluble organic species and greater availability of organics for fermentation. These results align with other studies on biological fermentation using conductively enhanced carbonaceous materials, which have reported increased organic dissolution and availability during anaerobic fermentation[49].

A significant drop in sCOD was observed during the first few days of fermentation in both experimental setups, highlighting the direct, early microbial utilization of the soluble substrate for VFA formation. In the experimental setup without biochar (PT_acid+co-culture), sCOD decreased from 10502 ± 185 mg O_2/L on Day 0 to 8333 ± 142 mg O_2/L on Day 1, followed by a further decline to 6248 ± 104 mg O_2/L on Day 2. This period saw the maximum VFA accumulation, resulting in the highest VFA value (1445.16 mg/L). pH during this period fell to 3.59±0.07, again confirming increased acidogenic activity and active organic acid production during this time. The extent of the drop in sCOD during this phase indicates rapid conversion of soluble organic material into by-products, predominantly VFA and other fermentation metabolites. After Day 2, the reduction in sCOD plateaued for the condition without biochar. The sCOD values decreased marginally from 6172 ± 96 mg O_2/L on Day 3 to 6084 ± 101 mg O_2/L on Day 4, and then to 6036 ± 88 mg O_2/L on Day 5. This slowly plateauing behaviour suggests that readily biodegradable substrates were being exhausted, and that microbial conversion efficiency was slow from Day 4 onwards. The simultaneous decrease in VFA concentration, coupled with a slight increase in pH after Day 3, may also suggest that the system is shifting to a less active acidogenic stage. This is typical of batch fermentation, where acid production slows down once the more readily fermentable organic matter has been exhausted. The biochar-augmented system had a dramatically different sCOD profile. The sCOD decreased from 11267.7 ± 192 mg O_2/L to 7369 ± 131 mg O_2/L on Day 1. This indicates a faster rate of organic matter conversion than in the other experimental groups. The value was halved the next day again to 6142 ± 98 mg O_2/L, and a dramatic halving was observed on Day 3 to 3070 ± 72 mg O_2/L. This sharp decline in sCOD occurred during the peak VFA accumulation stage, when total VFA concentration reached 2523.5 ± 130 mg/L. The strong relationship between sCOD depletion and VFA accumulation clearly indicates increased system efficiency due to biochar addition.

Although sCOD also remained low in the later stage of fermentation, it decreased to 2944.67 ± 61 mg O_2/L on Day 4 and then stabilized at 2787.33 ± 56 mg O_2/L on Day 5. The overall sCOD removal efficiency of the biochar-supplemented setup was 75.26%, compared to the non-supplemented setup. These results proved that biochar enhanced hydrolysis and microbial metabolism in acidogenic fermentation. The biochar-amended bio-digestion demonstrates superior fermentation performance for several physicochemical reasons. Biochar is a highly conductive medium that can connect various microbes these conductive biochar-coupled electron pathways are established, electrons transfer during metabolism to enhance the energetics of anaerobic fermentation[50]. Additionally, biochar has high electrical conductivity, biocompatibility, and a large reactive surface area, which can enhance microbial attachment and long-term retention [3]. These characteristics create a suitable

microenvironment for microbial metabolism and stabilize syntrophic fermentation, enabling efficient substrate degradation and VFA enrichment. Although biochar has been shown in the literature to positively influence VFA production from FW digestion, studies examining its effects on VFA accumulation in acidogenic fermentation remain limited. Previous studies have reported that adding biochar to FW fermentation can increase VFA production by 1.93-fold[51]. Another study showed that the addition of biochar synergistically improved solubilization, hydrolysis, and acidification, resulting in accelerated VFA accumulation during organic-substrate anaerobic fermentation[52]. Recent research also confirmed that conductive biochar materials could not only enrich functional fermentative microorganisms but also improve carbon conversion efficiency, essentially through promoted direct interspecies electron transfer (DIET)[53]. The individual VFA distributions in this study further indicated that biochar promoted acidogenic activity. Lactate, acetate, propionate, and butyrate were the most significant products inboth experimental setups, revealing that fermentation processes involving hydrolysis and acidification occurred. The production of these metabolites was relatively elevated in the biochar-supplemented system, demonstrating biochar's capacity to promote both microbial hydrolysis of organic substrates and the sequential conversion to VFA. To gain insight into the mechanisms underlying biochar-mediated microbial attachment and interfacial interactions, the material's physical and structural parameters were investigated using field-emission scanning electron microscopy (FE-SEM). These microstructures provide direct evidence of how biochar's specific morphology enhances microbial immobilization and fermentation performance.

Biochar analysis

To explore how biochar improves fermentation performance, the surface topography and elemental composition of biochar were investigated using FE-SEM combined with energy-dispersive X-ray spectroscopy (EDS).The biochar-added experimental setupproduced more VFA than the control system, suggesting that biochar's structural features may enhance microbial activity. Therefore, the biochar sample recovered from the setup was analyzed by FE-SEM. Before analysis, the sample was dried at 60 °C overnight to remove moisture. The FE-SEM image (Fig. 6a) depicts the surface texture of biochar prior to its interaction with microbes. It shows a clear, fibrous, layered structure composed of long, plate-like pieces, with rough ridges spread across the surface. Several areas contain parallel channel-likegrooves and micro-pores embedded in the carbon matrix. All these characteristics combine to form a highly irregular, sponge-like mesh of interconnected cavities. This structure provides a sufficiently high surface area, which can be advantageous for capturing dissolved organic compounds in the fermentation broth. The channels and pores may also serve as protective microenvironments for microbes and nutrients within the system. Compared to an unaltered biochar surface,

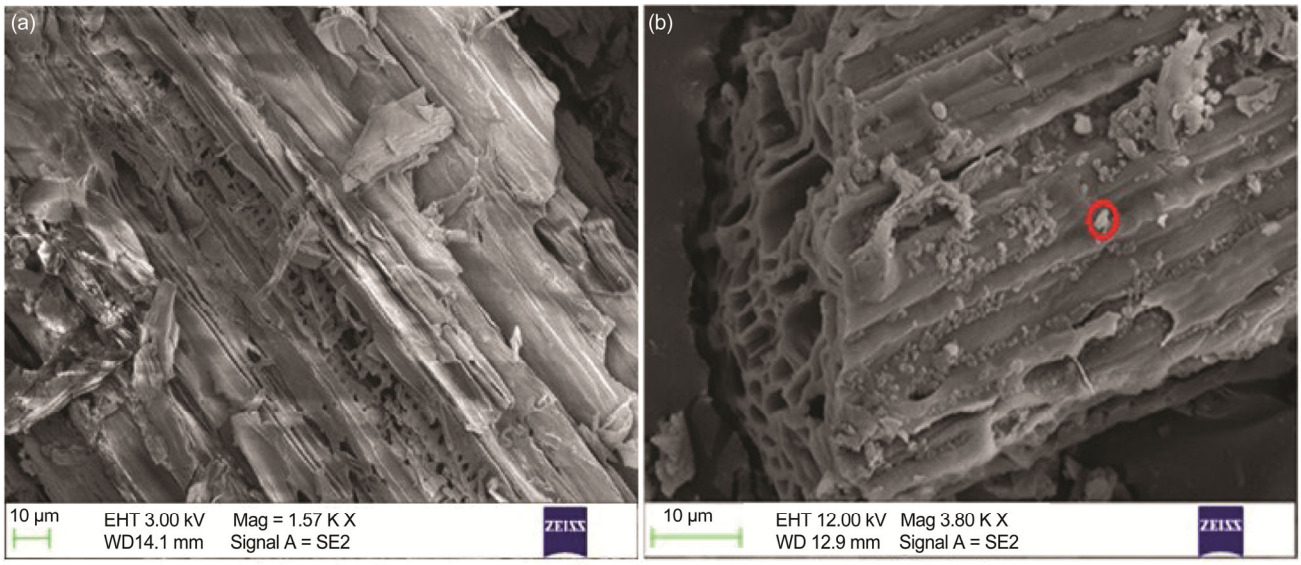

Fig. 6 — Surface images of biochar (a) before and (b) after bacterial interaction

Table 11 — Elemental composition of biochar obtained from Energy Dispersive X-Ray Spectra corresponding to Region A of the biochar sample

Element	Weight %	Method detection limit (MDL)	Net intensity
C K	63.98	0.63	369.52
N K	5.78	1.74	13.52
O K	28.26	0.81	180.26
Si K	1.40	0.15	57.77
K K	0.57	0.34	12.04

Table 12 — Elemental composition of biochar obtained from Energy Dispersive X-Ray Spectra corresponding to Region B of the biochar sample

Element	Weight %	Method detection limit (MDL)	Net intensity
C K	59.11	0.72	292.89
N K	6.11	1.68	15.03
O K	31.19	0.73	206.11
Si K	3.01	0.15	113.38
K K	0.58	0.29	11.06

the micrograph (Fig. 6b) shows the colonizing microbial groups as closely confined to the biochar matrix, mostly adhering to pits or slot openings. In a few areas, the cells appear to be surrounded by the pores of the material, suggesting that the internal voids serve as sites for microbial immobilization. The binding of microbes to these surfaces likely results in close association between cells and adsorbed substrates, thereby enabling efficient metabolic activity.

The comparison between the SEM images (Fig. 6a and b) clearly demonstrates the transformation of the biochar surface from a clean, fibrous, porous matrix to a microbially colonized surface containing cells and deposits. In addition to the morphological study, the elemental composition of the biochar surface was determined by EDS. The subsequent EDS spectra based on the elemental composition of biocharare tabulated (Tables 11 and 12). Carbon (C) was the predominant element, accounting for 63.98% and 59.11% by weight in the two sample areas, respectively. These results further confirm the carbon-rich nature of biochar. Notably, Oxygen (O) was 28.26% and 31.19%, indicating the presence of oxygen functional groups on the surface, which might increase adsorption capacity and provide more sites for microbial interaction. The nitrogen content was 5.78-6.11%, which may be due to residual organic material or to nitrogen functional groups formed during biochar production, whereas silicon (1.40-3.01%) and potassium (0.57-0.58%) were very low. The elemental composition of the two sample areas was very similar, suggesting that elements were evenly distributed on the biochar surface. SEM images of the biochar surface, combined with chemical analysis, revealed the abundance of carbon and its porous, fibrous morphology. These structural and compositional characteristics of biochar could enhance the performance of the biochar-supplemented experimental setup during fermentation and improve VFA yield.

Conclusion

The present study confirmed that the use of acidic pretreatment, bacterial co-culture, and biochar addition improved the acidogenic fermentation of food waste towards VFA production. Acidic pretreatment was the most efficient among the tested pretreatment strategies, achieving a maximum VFA concentration of 987 ± 32.6 mg/L and 84.74% sCOD removal. This was achieved through more effective solubilization and hydrolysis of complex food waste fractions, following alterations in their chemical composition that increased the availability of soluble organic molecules for microbial metabolism. Among the inoculum combinations tested, the co-culture of *Bacillus licheniformis* and *Klebsiella pneumoniae* produced significantly more VFA than the individual cultures. The maximum VFA concentration of 1600.2 ± 63 mg/L was observed in the co-culture system, suggesting a synergistic effect between hydrolytic and acidogenic bacteria on the enzymatic decomposition of complex organic components in food waste. Biochar supplementation further enhanced fermentation, with a maximum VFA accumulation of 2523.5 ± 130 mg/L at a biochar loading of 5 g/L. Increased VFA production in the biochar-amended experimental setup was associated with greater substrate consumption, enhanced sCOD removal, and extended fermentative activity. The porous and conductive properties of biochar's surface might enhance microbial attachment, promote biofilm growth, provide adsorbed nutrients, and accelerate interspecies electron transfer, thereby improving microbial metabolism and acidogenic conversion. Lactate, acetate, propionate, and butyrate were identified as the predominant VFA throughout the fermentation process, which indicated active hydrolysis and acidification pathways. FE-SEM images further confirmed that biochar supported sufficient microbial immobilization and biofilm

formation. One-way ANOVA and Tukey's HSD test were used for comparative statistical analysis among treatment experimental systems, and a significant difference was observed ($p < 0.05$). To wrap up, this study presents the promising application of an integrated process of acidogenic fermentation, including acidic pretreatment, microbial co-culture, and biochar addition, for maximizing SCFA recovery from food waste. Continued investigation and fine-tuning of these factors are required to fully harness the potential of these approaches and further advance the field of acidogenic fermentation.

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Conflict of Interest

The authors declare no conflict of interest.

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