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RESEARCH ARTICLE

Anaerobic Fermentation for Ceftriaxone Sodium Degradation in Excess Sludge: pH-Dependent Performance, Metabolite Formation, and Microbial Community Dynamics

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Abstract: The accumulation of ceftriaxone sodium (CTX), a third-generation cephalosporin antibiotic, in wastewater and sludge poses significant environmental and health risks, necessitating effective degradation strategies. This study investigated anaerobic fermentation of excess sludge (12,000-14,000 mg/L) spiked with 15 mg/L CTX under varying pH conditions (4, 7, 10, 12) to evaluate sludge reduction, metabolite formation, and microbial community dynamics. Results demonstrated that pH 10 yielded optimal performance, achieving a 62.09% sludge reduction rate, 129.35 mg/L NH_4^+-N release, and 1,485.84 mg/L volatile fatty acid (VFA) production, alongside efficient CTX degradation via hydrolysis and deamination pathways. Microbial analysis revealed dominant phyla (Proteobacteria, Bacteroidota, Chloroflexi) and genera (Ellin6067, Caldilinea) under pH 4-10, while extreme alkalinity (pH 12) favored Firmicutes but reduced functional diversity. The findings highlight pH 10 as the optimal condition for balancing CTX removal, sludge reduction, and resource recovery, offering a practical pretreatment approach for antibiotic-laden sludge in wastewater treatment.

Keywords: Anaerobic Fermentation, Ceftriaxone Sodium, Degradation Pathway, Microbial Community

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Introduction

Ceftriaxone sodium (CTX; $C_{18}H_{16}N_8Na_2O_7S_3 \cdot 5H_2O$) is a third-generation cephalosporin antibiotic with broad clinical use and strong bactericidal activity [1-3]. Owing to improper disposal and incomplete removal in treatment systems, CTX and other antibiotics can accumulate in wastewater and subsequently enter Wastewater Treatment Plants (WWTPs), where they may disturb microbial metabolism, promote antibiotic resistance, and pose ecological and human-health risks [4-6]. Residues

of β-lactam antibiotics have been detected in diverse settings, including livestock farms, slaughterhouses, hospital effluents, municipal WWTPs, and antibiotic manufacturing facilities, typically at ng/L to μg/L levels in municipal and mixed wastewaters, but reaching mg/L or higher in production wastewater and shock-loading scenarios [4, 7, 8]. Such high-strength antibiotic waste streams remain challenging to treat efficiently and safely.

A variety of physicochemical technologies have been explored for antibiotic removal, including adsorption and Advanced Oxidation Processes (AOPs). Adsorption-based methods rely on van der Waals forces, hydrogen bonding, π-π interactions, and surface electron transfer to capture antibiotics, and removal can be enhanced by material modification [9, 10]. AOPs (e.g., Fenton, ozonation, electrochemical oxidation, and photocatalysis) can degrade recalcitrant antibiotics by generating highly reactive radicals [4, 8]. Nevertheless, these approaches often involve high operational costs, substantial chemical/energy inputs, and potential secondary pollution, which may limit practicality for low-concentration antibiotics in municipal WWTP contexts and motivate the development of more sustainable biological strategies [4, 6-9].

Biological treatment, particularly anaerobic processes, offers the potential to degrade antibiotics while converting organic matter into value-added products. Anaerobic reactors such as anaerobic membrane bioreactors (AnMBRs) and UASB systems have been reported to treat pharmaceutical wastewaters with substantial COD removal, and anaerobic treatment of β-lactam antibiotic wastewater can reduce both organic load and antibiotic residues under suitable conditions [5, 11, 12]. However, antibiotic stress can inhibit key functional guilds in anaerobic systems, and the fate of CTX is not limited to complete mineralization to CO2 and H2O; instead, transformation products may dominate, making quantitative tracking and metabolite characterization essential to elucidate transformation pathways and potential risks [5, 13]. Importantly, antibiotics are not only present in the aqueous phase but also accumulate in sludge, which acts as a major reservoir for antibiotics and their transformation products [13] Excess sludge, therefore, represents a critical compartment where antibiotics can migrate, transform, and potentially be released during sludge handling and reuse. Enhancing antibiotic release from sludge solids and clarifying transformation behavior are necessary for both accurate assessment and effective sludge stabilization [14].

For β-lactam antibiotics such as CTX, pH is a key operational lever because the β-lactam ring is intrinsically unstable and prone to hydrolysis and ring opening, providing a chemical basis for transformation under appropriate conditions and a potential advantage for biologically assisted treatment [6]. Meanwhile, pH profoundly regulates sludge anaerobic fermentation (hydrolysis-acidogenesis) by influencing sludge disintegration/solubilization, enzymatic activity, acidogenic pathways, and microbial community selection. Thus, pH control may simultaneously steer:

(i) The release of sludge-bound CTX into the liquid phase

(ii) CTX transformation behaviors, and

(iii) Fermentation performance, such as VFA production and nitrogen release. Despite prior studies on anaerobic fermentation and antibiotic degradation, an integrated understanding that links pH-dependent fermentation performance with CTX transformation product profiles and microbial community dynamics in excess sludge remains limited

Although alkaline fermentation has been widely used to enhance sludge solubilization and VFA production, most studies primarily emphasize resource recovery and community selection rather than the fate of specific antibiotics within an excess-sludge fermentation matrix. Moreover, existing work on ceftriaxone has largely focused on aqueous/activated-sludge systems (e.g., co-metabolic enhancement) rather than a systematic pH-gradient evaluation coupled with multi-dimensional evidence of transformation mechanisms. pH-dependent fermentation studies on other cephalosporins have been reported, yet an integrated linkage among fermentation performance, metabolite formation, DOM evolution, and microbial community dynamics under a unified pH gradient remains limited. Therefore, this study provides an integrated framework to:

(i) Quantify pH-dependent anaerobic fermentation performance and ceftriaxone transformation in excess sludge

(ii) Identify key transformation products via LC-MS and interpret concurrent DOM changes using 3D-EEM, and

(iii) Associate these outcomes with community restructuring based on 16S rRNA sequencing across pH 4, 7, 10, and 12. A comparison with representative alkaline fermentation and cephalosporin/β-lactam transformation studies is provided in Table 1 to clarify the incremental contribution of this work

In this study, we investigate pH-regulated anaerobic treatment of excess sludge amended with CTX under representative pH conditions, aiming to clarify antibiotic migration and transformation in the sludge matrix. We quantify fermentation performance and sludge reduction, track CTX dynamics, and characterize transformation products to elucidate plausible pathways. In addition, we integrate DOM characterization (3D-EEM) and microbial community analysis (16S rRNA gene sequencing) to connect pH-driven shifts in organic matter features and microbial consortia with CTX fate and fermentation outcomes. By identifying an operationally favorable pH window and providing mechanistic insights, this work is intended to support pretreatment and process optimization strategies for antibiotic-laden waste streams and sludge stabilization in WWTPs.

Materials and Methods

Reaction Apparatus

Batch anaerobic fermentation was conducted in 500 mL serum bottles sealed with butyl rubber stoppers and aluminum crimps. The working volume was 400 mL, providing a headspace of 100 mL. Bottles were incubated at 35 ± 3 °C on an orbital shaker (500 rpm) to ensure adequate mixing. Liquid samples were withdrawn at designated time points using sterile syringes through the stopper, and the removed volume was not replaced to maintain the working volume. All experiments were performed in biological triplicates for each pH condition (pH 4, 7, 10, and 12; n = 3).

All experiments were performed with biological triplicates for each pH condition (n = 3). Results are presented as mean ± SD, and error bars represent SD. Formal hypothesis testing was not performed in the current dataset; future work will incorporate inferential statistics to support comparisons among pH treatments.

Sludge Characteristics

Excess sludge was collected from the sludge storage tank of a wastewater treatment plant in Lanzhou. Prior to the experiment, the sludge was allowed to settle for 24 h, and the supernatant was decanted. The sludge was then washed with deionized water 3-5 times until the supernatant became clear, and subsequently passed through a mesh screen to remove impurities and fine inorganic particles. The pretreated sludge was transferred into fermentation bottles, and its initial concentration was measured to support subsequent condition setting.

Table 1: Common water quality test table

Testing Parameters	Analytical Methods	Testing Parameters	Analytical Methods
SCFAs	Gas Chromatograph	COD	Potassium Dichromate Method
NH_4^+-N	Nessler's Reagent Photometric Method	pH/DO	WTW Multi3420
NO_3^--N	Thymol Spectrophotometric Method	MLSS	Gravimetric Method
SVI	30-Minute Settling Method	MLVSS	Muffle Furnace Combustion-Loss Method
SV	30-Minute Settling Method		

Experimental Conditions

Process definition. The reactors were operated as batch anaerobic fermentation (acidogenic fermentation) systems. Biogas/methane production was not monitored, and the process was not intended to achieve methane recovery; therefore, results are interpreted in the context of pH-regulated anaerobic fermentation rather than methane-producing anaerobic digestion.

pH adjustment strategy. The initial pH of each reactor was adjusted to pH 4, 7, 10, or 12 using NaOH to increase pH and HCl to decrease pH. For the pH 10 treatment, the pH was primarily raised with NaOH, and HCl was used only when necessary for fine correction if slight overshooting occurred during titration. pH was monitored over time to evaluate pH evolution during fermentation.

Anaerobic setup and dosing. To establish anaerobiosis, the headspace was purged with high-purity N_2 for 20 min prior to initiating the reaction. Ceftriaxone sodium was then spiked to an initial concentration of 15 mg/L. Fermentation was conducted under the conditions described in Section 2.1 (35 ± 3 °C, 500 rpm). Sampling was performed at 24-h intervals.

Antibiotic-free sludge controls (sludge without CTX) were not included in the present study. Accordingly, treatment effects on fermentation indicators and DOM characteristics are interpreted as the combined influence of pH regulation and background sludge transformation in the presence of CTX, and rigorous attribution will be strengthened in future work by incorporating matched antibiotic-free controls under each pH condition.

Volatile Fatty Acid (VFA) Analysis Method

Water samples were filtered through 0.22 μm membranes and adjusted to pH 4.0 using diluted 10% H_3PO_4. The processed samples were transferred to 2 mL GC vials and analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID). A DB-WAXer column (30 m × 530 μm × 1 μm) was used, with injector and detector temperatures of 220 °C and 250 °C, respectively. All measurements were completed within 24 h. In this study, VFAs were quantified and reported as total VFA concentration; individual VFA speciation (e.g., acetate, propionate, butyrate) was not determined [15].

Dissolved Organic Matter (DOM) Characterization by 3D-EEM Fluorescence

Fluorescence spectra were acquired using a Techcomp F-1700 spectrophotometer (Japan) with excitation and emission slit widths set at 5 nm. The scanning ranges for both Excitation (Ex) and Emission (Em) were 200-550 nm at a scanning speed of 12,000 nm/min, covering all characteristic fluorescence peaks in the samples. Each EEM scan required approximately 15 minutes, with Rayleigh and Raman scattering effects removed using Milli-qEEM processing. The analyzed water samples were supernatants obtained after solid-liquid separation of the anaerobic fermentation mixture.

Cefalexin and Ceftriaxone Sodium Determination Methods

Water samples were filtered through 0.22 μm membranes and analyzed using a Waters 1525-2996-TCM HPLC system equipped with a C18 column (4.6 × 250 mm, 5 μm). Standard curves were established using reference standards. Processed samples were stored in sealed vials at 4°C and analyzed within 24 hours following instrument-specific preparation protocols [16].

Metabolite Analysis Method

Qualitative analysis of cephalosporin transformation products was performed using a Shimadzu LC-20ADXR high-performance liquid chromatography system coupled with an LCMS-2020 mass spectrometer (Shimadzu, Japan). Ultrapure water (mobile phase A) and acetonitrile (mobile phase B) were used as the mobile phases. The column temperature was maintained at 40 °C, and the flow rate was 0.4 mL/min. The gradient program for mobile phase B was: 10-100% from 0-10 min, 100% from 10-20.5 min, and 100-10% from 20.5-30.9 min for column re-equilibration.

Mass spectrometric detection was carried out using an Electrospray Ionization (ESI) source operated in both positive and negative ion modes. Full-scan mass spectra were acquired over m/z 50-600. Putative transformation products were identified based on their mass-to-charge ratios (m/z) and, where applicable, characteristic fragmentation features and comparison with literature-reported transformation products of cephalosporins. Identified features were reported as putative products and used to propose transformation pathways under different pH conditions ADDIN ZOTERO_ITEM CSL_CITATION [15, 16].

Microbial Community Analysis

To characterize microbial community shifts under different pH conditions, sludge samples were collected from each batch reactor at [the end of fermentation / the designated sampling time point]. Fermentation mixtures were subjected to solid-liquid separation by centrifugation, and the sludge pellets were collected. The pellets were frozen at −80 °C and subsequently freeze-dried prior to DNA extraction. A total of 4 sludge samples were submitted for sequencing, corresponding to four pH treatments (pH 4, 7, 10, and 12).

The V3-V4 region of the 16S rRNA gene was amplified using primers 338F (5′-ACTCCTACGGGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). High-throughput sequencing was performed on an Illumina MiSeq platform by Guangdong Magigene Technology Co., Ltd. Sequencing data were processed and analyzed using the MagCloud platform. Alpha-diversity indices (Richness, Chao1, Shannon, and Simpson) and taxonomic composition at multiple levels were obtained following the MagCloud workflow.

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Results and Discussion

Characteristics of Ceftriaxone Removal Via Anaerobic Fermentation

Batch anaerobic fermentation of excess sludge was conducted at four initial pH conditions (pH 4, 7, 10, and 12) with CTX dosed at 15 mg/L (35 ± 3 °C, 500 rpm; N_2 purging for 20 min). All measurements are presented as mean ± SD (n = 3). Temporal profiles of pH and NH_4^+-N are shown in Fig. 1, and soluble COD and total VFA dynamics are summarized in Fig. 2. Across all treatments, total VFAs accumulated, indicating an acidogenic fermentation regime. At pH 4, pH gradually increased and stabilized around 6.1-6.2 by the end of fermentation (Fig. 1), while NH_4^+-N increased from 15.97 to 51.06 mg/L. At pH 7, pH decreased slightly and remained relatively stable (Fig. 1), with NH_4^+-N increasing and stabilizing around ~80 mg/L. At pH 10, NH_4^+-N increased steadily and reached 129.35 mg/L (Fig. 1); soluble COD peaked at 2,357.25 mg/L on day 3, and total VFA accumulated to 1,485.84 mg/L (Fig. 2). At pH 12, soluble COD release remained pronounced (Fig. 2), while NH_4^+-N stabilized at 98.85 mg/L and total VFA accumulation was lower than that at pH 10 (Fig. 2).

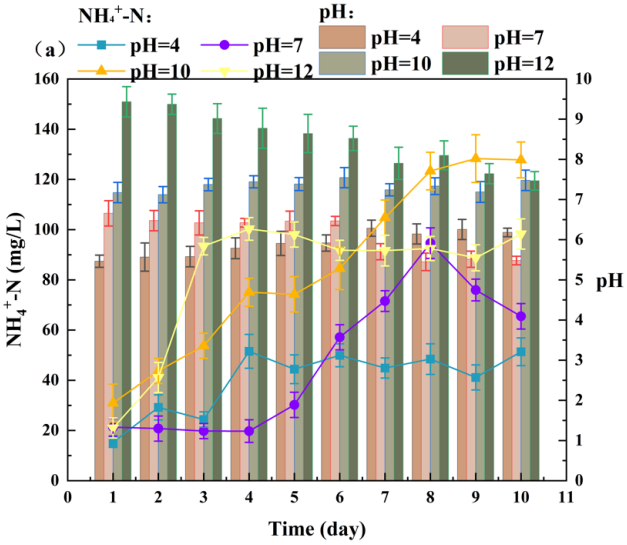

Fig. 1: Temporal profiles of pH and NH_4^+-N during batch anaerobic fermentation of excess sludge at pH 4, 7, 10, and 12 with an initial ceftriaxone sodium concentration of 15 mg/L (35 ± 3 °C, 500 rpm; N_2 purging for 20 min). Data are presented as mean ± SD (n = 3). Error bars indicate SD

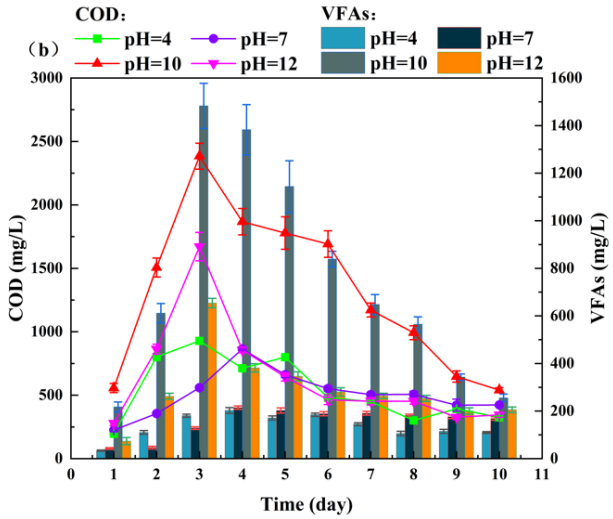

Fig. 2: Changes in COD and total VFA concentration during batch anaerobic fermentation of excess sludge at pH 4, 7, 10, and 12 with an initial ceftriaxone sodium concentration of 15 mg/L (35 ± 3 °C, 500 rpm; N_2 purging for 20 min). Data are presented as mean ± SD (n = 3). Error bars indicate SD

Because VFAs accumulated and biogas/methane was not monitored, the system is interpreted as pH-regulated anaerobic fermentation (hydrolysis-acidogenesis) rather than methane-producing anaerobic digestion. The data indicate a clear pH dependence: pH 10 provided the most favorable balance between sludge solubilization and downstream acidogenesis (highest total VFA and NH_4^+-N release), whereas pH 12 promoted solubilization but did not translate into maximal VFA production. This pattern is consistent with the notion that extreme alkalinity can impose alkaline/ionic stress on fermentative activity and reduce conversion efficiency from solubilized organics to VFAs. The pH-conditioned fermentation milieu also provides the process context for CTX attenuation and transformation products discussed in Sections 3.4-3.6.

Design limitations relevant to interpretation. Total VFAs were measured without individual VFA speciation; therefore, pathway-level interpretation (e.g., acetate-/propionate-/butyrate-type shifts) cannot be resolved. In addition, antibiotic-free sludge controls (without CTX) were not included; thus, some changes in fermentation indicators and DOM characteristics likely reflect combined effects of pH regulation and background sludge fermentation in addition to CTX-related processes. Accordingly, CTX attenuation is primarily supported by direct concentration tracking (HPLC) and transformation-product evidence (Section 3.4), while fermentation/DOM indicators are interpreted as the evolving process environment.

Analysis of Sludge Reduction

The initial MLSS was ~12,000 mg/L. After fermentation, MLSS decreased to 6,496 mg/L (pH 4), 5,246 mg/L (pH 7), 4,690 mg/L (pH 10), and 4,252 mg/L (pH 12), corresponding to sludge reduction rates of 47.49%, 56.54%, 62.09%, and 66.45%, respectively (Fig. 3). CTX decreased to below the detection limit under all tested conditions (HPLC), indicating effective attenuation during fermentation.

The monotonic increase in sludge reduction rate with pH is consistent with stronger sludge disintegration/solubilization under alkaline conditions, while the VFA results (Section 3.1) suggest that maximal acidogenesis occurred at pH 10 rather than pH 12. From a practical perspective, these results support the potential of alkaline-regulated fermentation as a pretreatment option to couple sludge reduction/resource recovery with antibiotic attenuation, while acknowledging that attribution of some process indicators to CTX-specific effects is constrained by the lack of antibiotic-free controls.

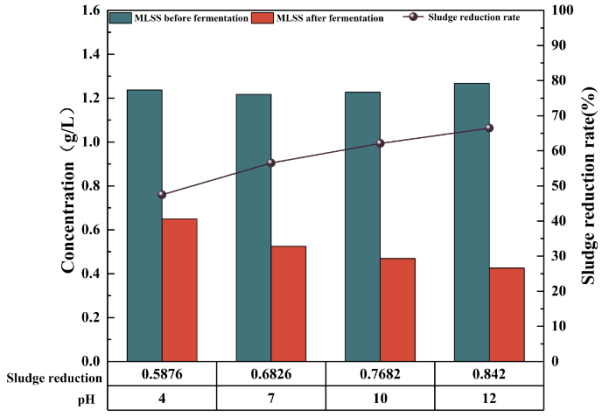

Fig. 3: Variation of sludge concentration in the fermentation system during batch anaerobic fermentation of excess sludge under different pH conditions (pH 4, 7, 10, and 12) with an initial ceftriaxone sodium concentration of 15 mg/L (35 ± 3 °C, 500 rpm; N_2 purging for 20 min). Data are presented as mean ± SD (n = 3). Error bars indicate SD

Three-Dimensional Fluorescence Spectroscopy Reveals Characteristics of Organic Matter Transformation

Three-dimensional excitation-emission matrix (3D-EEM) fluorescence was used to characterize DOM evolution during fermentation. Across the pH gradient, overall fluorescence intensity/peak areas increased with alkalinity (Fig. 4a-d), consistent with enhanced sludge solubilization and accumulation/release of fluorescent DOM components. Using a commonly applied regional classification, fluorescence was described as Region I (tyrosine-like), Region II (tryptophan-like), Region III (fulvic-like), Region IV (soluble microbial product-like), and Region V (humic-like). Two major peak domains were observed: a humic-like/microbial byproduct domain (Ex/Em ≈ 250-400/380-500) and a protein/SMP-related domain (Ex/Em ≈ 250-300/280-380). Region IV showed relatively weaker signals at pH 7 and stronger signals at pH 10, while Region V increased prominently at pH 12 (Fig. 4).

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In complex sludge matrices, EEM signatures primarily reflect sludge-derived DOM fractions rather than serving as a direct fingerprint of CTX. Therefore, EEM is interpreted here as complementary evidence describing the DOM environment accompanying fermentation, and discussed together with fermentation performance (Section 3.1) and LC-MS-derived transformation products (Section 3.4). The stronger Region IV signal at pH 10 aligns with intensified hydrolysis-acidogenesis and higher VFA accumulation, suggesting active production/turnover of soluble microbial byproducts and labile intermediates. In contrast, the pronounced Region V increase at pH 12 indicates accumulation/release of more humic-like, potentially less readily fermentable DOM fractions, consistent with lower VFA accumulation at pH 12 than at pH 10. Peak shifts (e.g., blue shifts) may reflect changes in functional groups and humification degree [17, 18], and these DOM changes may indirectly influence CTX fate by altering sorption/complexation behavior and microbial metabolic context. Because EEM signals are dominated by background DOM, CTX transformation is primarily supported by direct concentration tracking and LC-MS-based product identification (Section 3.4).

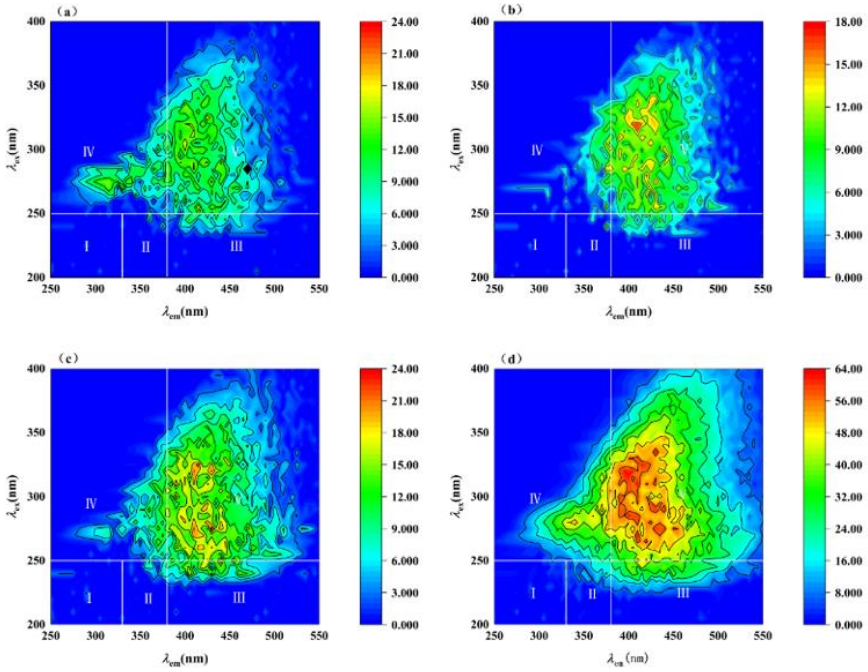

Fig. 4: Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectra of dissolved organic matter (DOM) during batch anaerobic fermentation of excess sludge under different pH conditions: (a) pH 4, (b) pH 7, (c) pH 10, and (d) pH 12 (initial CTX = 15 mg/L; 35 ± 3 °C, 500 rpm; N_2 purging for 20 min)

Analysis of Ceftriaxone Metabolites in Anaerobic Fermentation

As a third-generation cephalosporin antibiotic, previous studies have demonstrated that ceftriaxone sodium can be effectively removed through anaerobic fermentation under various environmental conditions. However, further analysis is required to determine whether any metabolites remain after removal. Post-reaction analysis of degradation pathways was conducted at different pH levels (4, 7, 10, 12). During the anaerobic fermentation process, sodium ions in ceftriaxone sodium are rapidly replaced by hydrogen ions, forming hydroxyl and carboxyl derivatives, while the β-lactam ring undergoes hydrolysis, followed by deamination and decarboxylation to yield a product with a molecular weight of 468.57 ($C_{16}H_{20N8}O_{3S3}$).

Under acidic conditions (pH 4), the 468.57 Da product ($C_{16}H_{20N8}O_{3S3}$) degrades through deamination and C-N bond cleavage into products with molecular weights of 453.55 ($C_{16}H_{19N7}O_{3S3}$) and 129.18 (C_5H_7NOS). The 453.55 Da product ($C_{16}H_{19N7}O_{3S3}$) further degrades through C-C bond cleavage, ring opening, and deamination into products with molecular weights of 313.39 ($C_{11}H_{15N5}O_{2S2}$) and 259.34 ($C_9H_{13N3}O_{2S2}$). The 259.34 Da product is subsequently degraded through C-C bond cleavage into a 128.15 Da product ($C_4H_{4N2}OS$). These three degradation pathways collectively contribute to ceftriaxone sodium degradation. Although fermentation performance is relatively poor under acidic conditions, the combined effects of acidity and anaerobic fermentation promote hydrolysis and decomposition of ceftriaxone sodium, with multiple factors synergistically enhancing cephalosporin antibiotic removal and degradation. Similar findings were observed in studies of first-generation cefalexin, where acidic conditions favored simultaneous multiple metabolic pathways for synergistic treatment.

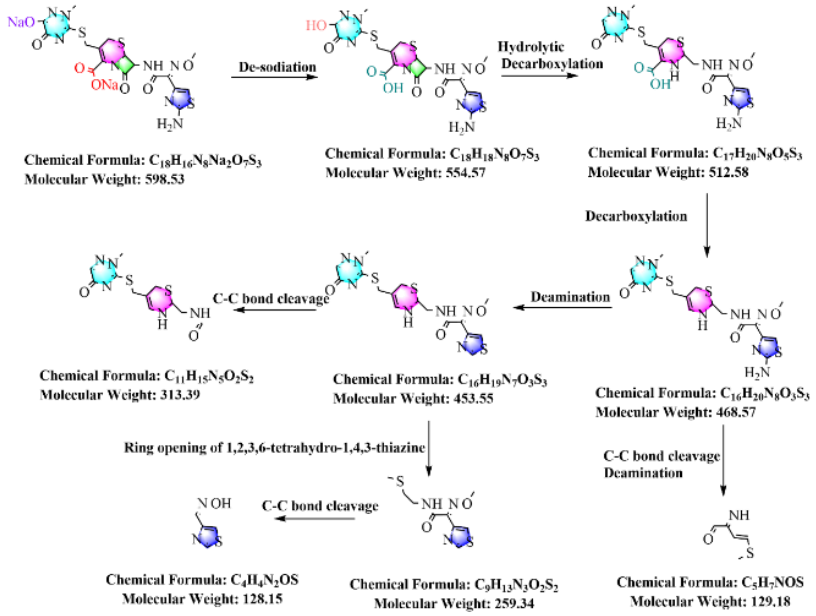

Fig. 5: Proposed transformation pathways of ceftriaxone sodium during batch anaerobic fermentation of excess sludge at pH 4, inferred from LC-MS/MS-based metabolite identification (initial CTX = 15 mg/L; 35 ± 3 °C, 500 rpm; N_2 purging for 20 min)

At neutral pH (7), the 468.57 Da product ($C_{16}H_{20N8}O_{3S3}$) undergoes deamination and C-N bond cleavage to yield products with molecular weights of 453.55 ($C_{16}H_{19N7}O_{3S3}$) and 129.18 (C_5H_7NOS). The 453.55 Da product ($C_{16}H_{19N7}O_{3S3}$) then degrades through C-C bond cleavage, ring opening, and deamination into products with molecular weights of 313.39 ($C_{11}H_{15}N_5O_2S_2$) and 259.34 ($C_9H_{13N3}O_{2S2}$). The latter 259.34 Da product ($C_9H_{13N3}O_{2S2}$) is further degraded through C-C bond cleavage into a 128.15 Da product ($C_4H_{4N2}OS$), which is ultimately broken down into an 85.12 Da product (C_3H_3NS) through additional C-C bond cleavage. Compared to acidic conditions (pH 4), the degradation process under neutral fermentation (pH 7) is more complete, with $C_4H_{4N2}OS$ being further degraded to C_3H_3NS. While the other two degradation pathways yield essentially the same metabolites, potential differences in metabolite concentrations require further analysis. The similar metabolic pathways observed under these two different anaerobic fermentation conditions demonstrate the feasibility of using anaerobic fermentation for cephalosporin antibiotic removal. Optimization of the anaerobic fermentation process can effectively transform large cephalosporin molecules into small molecules that can be directly utilized by microorganisms.

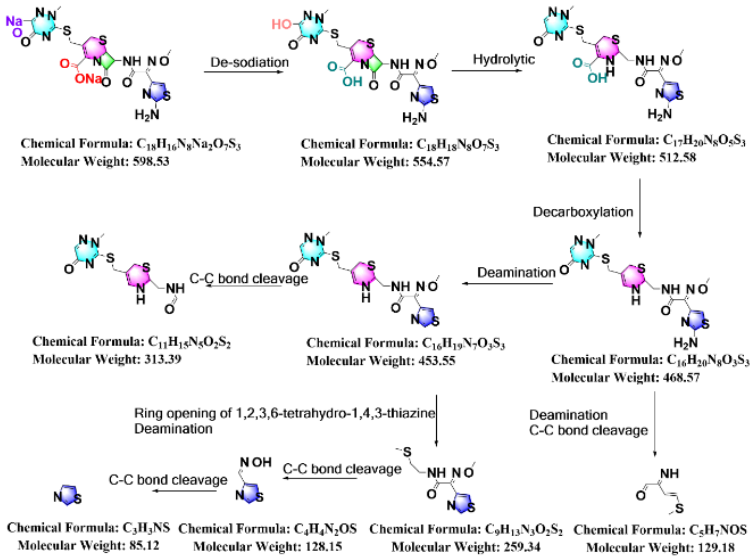

Fig. 6: Proposed transformation pathways of ceftriaxone sodium during batch anaerobic fermentation of excess sludge at pH 7, inferred from LC-MS/MS-based metabolite identification (initial CTX = 15 mg/L; 35 ± 3 °C, 500 rpm; N_2 purging for 20 min)

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Under alkaline conditions (pH 10), the 468.57 Da product ($C_{16}H_{20N8}O_{3S3}$) undergoes deamination to yield a 453.55 Da product ($C_{16}H_{19N7}O_{3S3}$), which is further degraded through demethylation, dehydroxylation, and C=C bond cleavage into a 396.50 Da product ($C_{14}H_{16N6}O_{2S3}$). Subsequent 1,2,4-triazin-5(2H)-one ring opening and deamination yields a 356.48 Da product (C13H16N4O2S3), which is then degraded through N-((methylthio) methyl) vinylamine ring opening into a 358.49 Da product ($C_{13}H_{18N4}O_{2S3}$). This 358.49 Da product is broken down through C-C bond cleavage and thiazole ring opening into products with molecular weights of 129.18 (C_5H_7NOS) and 159.25 ($C_7H_{13}NOS$), with the latter being further degraded through C-N bond cleavage into a 99.13 Da product (C_5H_9NO).

When the pH is increased to 12, the 468.57 Da product ($C_{16}H_{20N8}O_{3S3}$) undergoes deamination to yield a 453.55 Da product ($C_{16}H_{19N7}O_{3S3}$), which is then degraded through 1,2,3,6-tetrahydro-1,4,3-thiazine ring opening and deamination into a 259.34 Da product ($C_9H_{13N3}O_{2S2}$). This product is further degraded through thiazole ring opening and deamination into a 159.25 Da product ($C_7H_{13}NOS$).

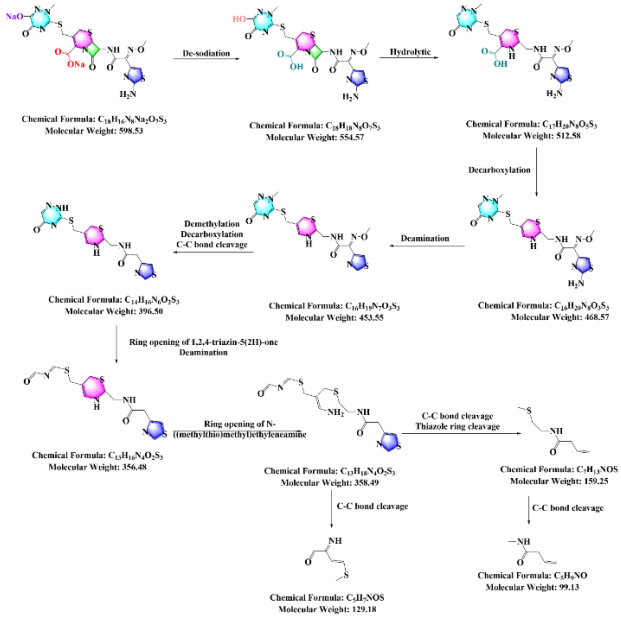

Fig. 7: Proposed transformation pathways of ceftriaxone sodium during batch anaerobic fermentation of excess sludge at pH 10, inferred from LC-MS/MS-based metabolite identification (initial CTX = 15 mg/L; 35 ± 3 °C, 500 rpm; N_2 purging for 20 min)

Analysis at the end of alkaline condition reactions revealed more detectable metabolites. While the degradation pathways were essentially similar, more large molecules were present. When the initial pH was increased from 10 to 12, the removal process of ceftriaxone sodium significantly deteriorated, with fewer small molecules produced and metabolic pathways markedly inhibited, resulting in only a single metabolic process that yielded fewer types of small molecules. This clearly demonstrates that excessive alkalinity is not conducive to the degradation of ceftriaxone sodium into small molecules during anaerobic fermentation. However, for standalone anaerobic fermentation processes, alkaline conditions are more favorable for fermentation performance and yield better results in terms of short-chain fatty acid production and methanogenesis, though they show poorer removal efficiency for substances like ceftriaxone sodium.

In summary, ceftriaxone sodium can be effectively removed through anaerobic fermentation, with gradual degradation occurring as fermentation progresses. Under acidic and neutral conditions, ceftriaxone sodium exhibits more diverse metabolic pathways and yields more small molecules. In contrast, alkaline conditions result in simpler metabolic processes that produce more metabolites but fewer small molecules.

To move beyond descriptive comparisons, we interpret the pH-dependent metabolite patterns together with the concurrent community restructuring and fermentation intensity. Across the pH gradient, the emergence/attenuation of major LC-MS features was evaluated alongside changes in alpha diversity and dominant taxa, providing a coherent process-product-microbiome linkage under pH regulation. Nevertheless, we acknowledge that a fully quantitative causal attribution would benefit from additional targeted measurements (e.g., VFA speciation, functional gene quantification, and time-resolved metabolite kinetics), which will be pursued in future work.

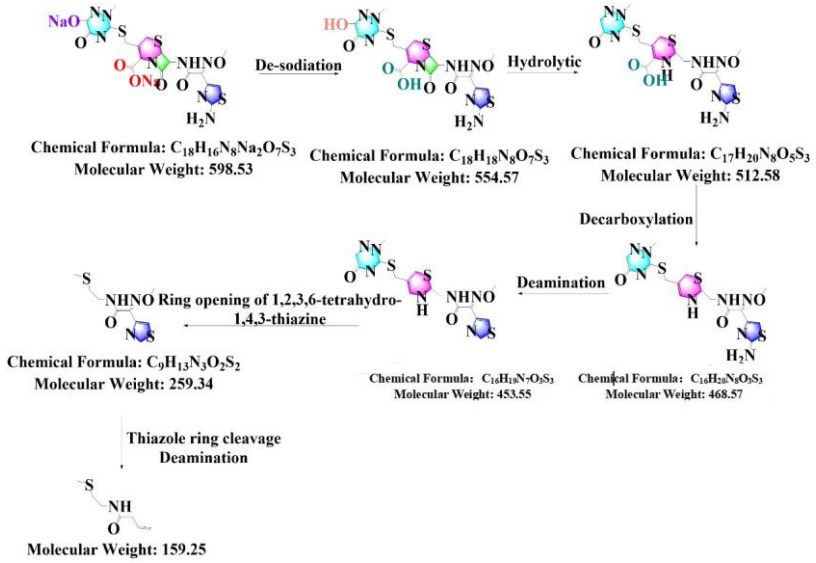

Fig. 8: Proposed transformation pathways of ceftriaxone sodium during batch anaerobic fermentation of excess sludge at pH 12, inferred from LC-MS/MS-based metabolite identification (initial CTX = 15 mg/L; 35 ± 3 °C, 500 rpm; N_2 purging for 20 min)

Microbial Community Composition and Diversity

Sequencing reads exceeded 80,000 across samples (80,998-88,457 reads; Table 2). Alpha diversity declined under extreme alkalinity: at pH 12, Richness/Chao1 decreased to 2134, and Shannon decreased to 7.94, while Simpson increased to 0.0217 (Table 2). In contrast, pH 4-10 maintained higher richness (2626-2842) and Shannon indices (8.57-8.84), with lower Simpson values (0.0104-0.0122).

Table 2: Sequencing reads and alpha diversity indices (Richness, Chao, Shannon, and Simpson) of microbial communities under different pH conditions (T1-T4 correspond to pH 4, 7, 10, and 12, respectively)

Sample \ Assessment Value	Richness	Shannons	Reads	Simpson	Chao
T1	2842	8.84	83262	0.0115	2842
T2	2626	8.65	80998	0.0104	2626
T3	2629	8.57	88457	0.0122	2629
T4	2134	7.94	82419	0.0217	2134

At the phylum level, Proteobacteria remained dominant across treatments, with Bacteroidota and Chloroflexi also abundant at pH 4-10, whereas Firmicutes increased at pH 12 (Fig. 9).

The diversity pattern indicates that extreme alkalinity constrained community richness/evenness, consistent with alkaline stress selecting for alkali-tolerant taxa while reducing overall functional diversity. The persistence of phyla commonly linked to hydrolysis/acidification and organic matter transformation (including Proteobacteria, Bacteroidota, and Chloroflexi) at pH 4-10 is consistent with robust fermentation performance in this pH range and aligns with their reported roles in degrading complex organics and supporting SCFA production [19, 20-22]. The enrichment of Firmicutes under pH 12 is consistent with their strong environmental tolerance and involvement in organic matter degradation under harsh conditions [23-25]. Importantly, because antibiotic-free controls were not included, community shifts are interpreted primarily as responses to the combined pH-regulated fermentation environment and CTX-amended conditions rather than as CTX-specific causal effects.

At the genus level, pH 4-10 exhibited broadly similar dominant genera (Ellin6067, Caldilinea, and Stenotrophobacter), whereas pH 12 showed pronounced community restructuring with enrichment of Thauera, Symbiobacterium, and Bacillus (Fig. 10). These shifts are interpreted in the context of established functional roles in sludge hydrolysis-acidogenesis and stress adaptation, which collectively shape the biochemical milieu in which β-lactam transformation may occur.

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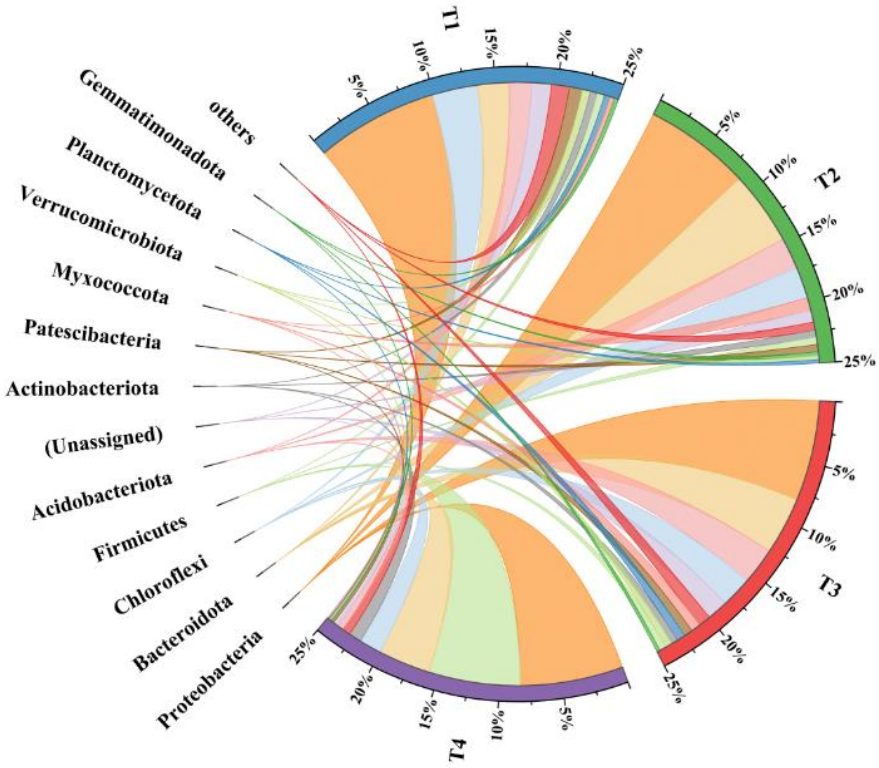

Fig. 9: Changes in relative abundance at the phylum level (T1, T2, T3, and T4 pH values of 4, 7, 10, and 12, respectively)

Microbial Functional Interpretation

The occurrence of Caldilinea (Chloroflexi) among the dominant taxa at pH 4-10 is consistent with reports from anaerobic/fermentative sludge systems, where Chloroflexi-related populations contribute to the turnover of complex organics and participate in syntrophic networks that support acidogenesis and SCFA accumulation [19, 20-22]. Such functionality can indirectly facilitate CTX-related transformation by increasing the availability of soluble substrates and by sustaining an enzyme-rich, metabolically active community under moderate pH conditions.

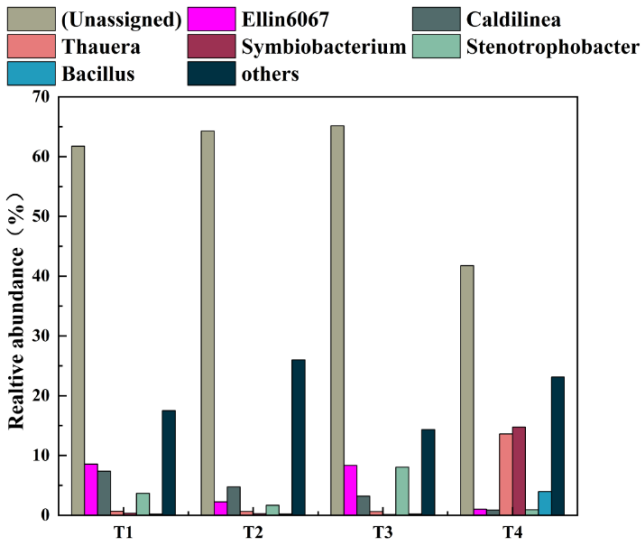

Fig. 10: Changes in relative abundance at the genus level (T1, T2, T3, and T4 pH 4, 7, 10, and 12, respectively)

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In contrast, the enrichment of Bacillus under pH 12 is consistent with the strong stress tolerance of Firmicutes and their reported capacity for extracellular enzymatic activity and broad organic matter conversion under harsh operational conditions [23-25]. The concurrent increase of Thauera (Proteobacteria), a genus frequently detected in engineered wastewater systems and associated with the transformation of diverse organic compounds, further suggests a shift toward metabolically versatile taxa under extreme alkalinity [19, 20-25]. While these observations do not demonstrate CTX-specific degradation, they provide literature-consistent context for potential community-level capacity related to β-lactam inactivation and transformation in pH-regulated fermentation matrices.

β-Lactam antibiotics are commonly deactivated via β-lactam ring hydrolysis mediated by β-lactamase/cephalosporinase enzymes; therefore, enrichment of taxa frequently observed in antibiotic-impacted microbiomes may indicate an increased potential for β-lactam inactivation at the community level. Nevertheless, because this study is based on 16S rRNA amplicon profiling and lacks antibiotic-free controls, the current evidence supports association rather than causation. Future work will incorporate matched CTX-free controls and functional validation, including quantification of β-lactamase/ARG markers (e.g., bla genes) by qPCR/metagenomics and time-resolved coupling of transformation products with targeted activity assays.

Environmental Implications, Cost Considerations, and Limitations

While pH regulation—particularly under alkaline conditions—can enhance sludge solubilization and accelerate ceftriaxone transformation during anaerobic fermentation, the practical implementation of strongly alkaline operation (e.g., pH ≥ 10-12) requires critical evaluation. First, maintaining high pH would necessitate continuous or staged dosing of alkaline chemicals (typically NaOH), which implies non-negligible chemical consumption and associated operational costs, and may increase the salinity/ionic strength of the treated sludge liquor. Second, the neutralization demand after alkaline treatment (e.g., prior to downstream biological processes or discharge) would further contribute to chemical use and cost, and could increase the overall environmental footprint of the process. Third, from a sustainability perspective, the present system was operated as acidogenic anaerobic fermentation with VFA accumulation and without methane/biogas monitoring; therefore, direct energy recovery (as in methanogenic anaerobic digestion) was not demonstrated. This indicates a trade-off between antibiotic attenuation/sludge disintegration and energy recovery potential, and highlights the need to evaluate the process within an integrated sludge-management train (e.g., coupling alkaline fermentation as a pretreatment step followed by downstream digestion or VFA valorization).

In addition, two methodological limitations should be noted:

(i) VFAs were quantified only as total VFA, and individual VFA speciation (acetate/propionate/butyrate) was not determined, limiting pathway-level interpretation

(ii) Antibiotic-free sludge controls (without CTX) were not included, which may constrain rigorous attribution of some fermentation and DOM changes solely to CTX-related processes. Future work should

a. Quantify VFA speciation

b. Include matched antibiotic-free controls under each pH condition

c. Track pH evolution and chemical dosing demand more explicitly, and

d. Evaluate coupled configurations that improve sustainability (e.g., subsequent methane recovery or targeted VFA/product valorization) under environmentally relevant antibiotic levels

Conclusion

Batch anaerobic fermentation of excess sludge attenuated ceftriaxone sodium to below the detection limit under all tested pH conditions (4, 7, 10, and 12). pH strongly regulated fermentation behavior, with pH 10 providing the most favorable balance for hydrolysis-acidogenesis and yielding the highest total VFA production and NH_4^+-N release. LC-MS profiling indicated pH-dependent transformation patterns, while EEM and 16S results collectively suggested that extreme alkalinity (pH 12) altered the DOM environment and reduced microbial diversity, which may constrain acidogenesis. Overall, pH-regulated anaerobic fermentation at pH 10 is a promising pretreatment option for antibiotic-laden sludge, but practical implementation should consider chemical demand, neutralization needs, and the need for matched antibiotic-free controls and VFA speciation in future work.

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Author's Contributions

Weiwei Li: Writing original draft, methodology, investigation.
Hong Niu: Investigation, formal analysis.
Yonghui Zhang: Methodology, investigation.
Hong Liu: Writing review and editing.
Yong Zhi Chen: Writing review and editing, writing original draft, funding acquisition, Conceptualization.

Ethics

This study did not involve human participants, human data, or animals. The excess sludge samples used in this work were collected from a municipal wastewater treatment plant as environmental materials, and no personal or sensitive information was collected. Therefore, formal ethical approval and informed consent were not required for this study.

Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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