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Research Article

Effect of pH Control Strategy on Anaerobic Fermentation of Oxytetracycline Excess Sludge

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ABSTRACT

The high cost of treating Oxytetracycline (OTC) sludge poses a significant risk of secondary pollution, emphasizing the need efficient, eco-friendly, and safe methods. In this study, OTC-containing excess sludge was subjected to continuous pH adjustments to levels of 5, 8, 10, and 12, as well as intermittent regulation to pH 10 and 12 at a temperature of 35 ± 3°C. Notably, intermittent pH regulation was found to be more effective in producing Short-Chain Fatty Acids (SCFAs) compared to continuous regulation at pH 10. The maximum production of SCFAs reached was 1590.5 mg Chemical Oxygen Demand (COD)/g Volatile Suspended Solids (VSS), with acetic acid comprising 82% of the total SCFAs. Additionally, a sludge reduction of 33.1% was achieved. The concentration of the emitted OTC was 22.58 mg/L under intermittent pH regulation was comparable with 18.5 mg/L under continuous pH regulation, while the removal rate of OTC was 62.5% in OTC-containing excess sludge anaerobic fermentation. The qualitative analysis by mass spectrometry revealed that the main metabolites such as 4-Epi-Oxytetracycline (EOTC) and alpha-apo-Oxytetracycline (α-apo-OTC) were formed. The intermittent regulation of pH proved more conducive to the enrichment of hydrolytic acidifying bacteria (*Bacillus* (29.94%), *Hydrotalea* (4.32%) and *Thauera* (13.67%)). Moreover, *Pseudomonas* (1.56%) demonstrated remarkable efficiency in degrading OTC.

Keywords: Anaerobic fermentation; Intermittent pH value; OTC degradation; Sludge reduction; SCFAs

INTRODUCTION

OTC, a broad-spectrum tetracycline antibiotic, is extensively used in aquaculture due to its efficacy, affordability, and widespread availability [1]. However, OTC is readily absorbed through the gastrointestinal tract, resulting in high residues in livestock and poultry products and the potential development of resistance [2]. The concentration of OTC has been found to be at the higher level in Wastewater Treatment Plants (WWTPs) [3]. However, the concentration of OTC is increasing in the excess sludge (≥ mg/L). OTC-containing excess sludge is disposed as hazardous waste, resulting in expensive treatment costs and secondary pollution [4]. As the economy rapidly develops, the harmless disposal of sludge has become a pressing issue, attracting widespread attention [5-6]. Severe challenges are posed by environmental pollution and resource scarcity, the treatment and disposal of sludge have become integral components of the wastewater treatment process [7]. Excess sludge is no longer merely considered a “waste”, but rather as valuable resource rich in potential for regeneration and reuse [8]. This perspective shift enables the utilization of resources and energy, highlighting a recycling-based approach [9]. Moreover, the combination of sludge recycling and reduction is considered the optimal strategy for sludge treatment and disposal. This approach can achieve both economic and environmental benefits.

Anaerobic fermentation has emerged as a highly efficient, safe, and resource-friendly method for treating OTC-containing excess sludge [10]. Anaerobic fermentation mainly includes hydrolytic acidification, hydrogen production for the generation of acetic acid, and methane production. Among these processes, sludge hydrolysis represented the rate-limiting step, and the competition between methanogenic archaea and acid producing bacteria determines whether methane or SCFAs become the primary products [11,12]. SCFAs are valuable biodegradable products with a high market value due to their wide range of applications and ease of transport and storage. Previous studies have demonstrated that the yield and composition of SCFAs produced during fermentation were influenced by factors such as pH value, temperature and sludge residence time [13-15].

Alkaline pH has been shown to enhance the production of

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SCFAs [16]. The alkaline fermentation process promotes protein fragmentation and denaturation, leading to increase the production of SCFAs. Recent studies have found that maintaining an alkaline pH inhibits the activity of methanogenic archaea, thereby reducing the consumption of SCFAs in the fermentation system [17]. While alkaline conditions can enhance SCFAs production and inhibit acid-producing bacteria from converting hydrolyzed organic substrates into SCFAs. This may limit the potential for maximizing SCFAs production [18]. To achieve the dual purpose of increasing the yield of SCFAs and reducing the cost, we need to explore more optimal ways to promote the anaerobic fermentation process.

The major objective of this work was to propose a simple and consumption-saving intermittent pH control strategy based on the natural decrease of pH at moderate temperature (35 ± 2°C) for improving SCFAs production. The study explored changes in SCFAs production, sludge reduction, and OTC content, along with its degradation products in the fermentation of OTC-containing excess sludge. The study confirmed the advantages of intermittent pH phased regulation and revealed alterations in the biodegradability of the fermentation broth. Utilizing high-throughput sequencing analysis, the bacterial community and abundance in the reactor with the highest acid production were examined. Additionally, the study discussed the degradation products and pathways of OTC during the fermentation process, providing practical suggestions for its treatment.

MATERIALS AND METHODS

The source of OTC excess sludge

The OTC-containing excess sludge was sourced from Gansu Qilianshan Pharmaceutical Co., Ltd. The sludge was stored at 4°C for 24 h and the supernatant was subsequently removed. A 40 mesh sieve was used to remove the larger particles and the sludge was rinsed 3 times with water. The primary physical and chemical properties of the sludge are summarized in Table 1.

Table 1: The primary physical and chemical properties of OTC sludge.

pH	SS	VSS	COD	Polysaccharides (PS)	Protein (PN)
8.1	(mg/L) 14956 ± 205	(mg/L) 9743 ± 120	(mg/L) 190.2 ± 5.16	(mg/L) 4.7 ± 1.23	(mg/L) 5.8 ± 1.01

Experimental methods

The experiment consists of two parts. In the first part, OTC sludge was introduced into an anaerobic fermentation flask. pH values were adjusted to 5 (R1), 8 (R2), 10 (R3), and 12 (R4) using 2 mol/L NaOH and HCl solutions. The reaction temperature was set at 35 ± 3°C, and the operation was initiated. The second part of the experiment aimed to investigate the impact of pH on the intensive fermentation of OTC-containing excess sludge. In conjunction with the anaerobic fermentation stage, three sets of anaerobic fermentation bottles were set up with pH control values of 8 (R0), 10 (R5), and 12 (R6). The pH of the control group (R0) and the fermentation bottles R5 and R6 was maintained at the initial sludge pH, without any adjustments throughout the fermentation process and not subjected to control against natural changes in the reaction system. The reaction cycle was established at 24 h with pH adjustments maintained within a narrow range of ± 0.3. The sludge fermentation mixture was sampled every 24 h and centrifuged at 6000 rpm for 5 min to analyze the concentrations of COD, NH^{4+}-N, Protein (PN), Polysaccharides (PS), SCFAs, and other conventional parameters.

Routine indicators and measurement methods

After post-centrifugation at 6000 rpm for 5 min and filtration through a 0.45 μm membrane, the supernatant was analyzed for all indicators and Extracellular Polymers (EPS) extraction method. Diagram of the specific reaction set-up for this experiment was given.

Microbial diversity analysis

In accordance with the experimental design, sludge samples were collected from various fermentation systems at the point of maximum acid production. A volume of 50 mL well-mixed sludge samples was transferred into 50 mL centrifuge tubes and subjected to the centrifugation using a high-speed centrifuge. The freeze-dried sludge samples were stored at -20°C until further analysis. These samples were sent to Guangdong Meagher Biopharmaceutical Technology Co.Ltd. in China, where high-throughput microbial sequencing analysis was performed using the Illumina MiSeq platform.

3D-Excitation-Emission Matrix (EEM) analysis

The three-dimensional fluorescence data were processed using the myremovescater program within the MATLAB R2020b toolbox. During the detection process, excitation wavelengths spanned from 200 to 400 nm with a step size of 5 nm, while emission wavelengths ranged from 250 to 550 nm with a step size of 5 nm. This scanning range covered all complete fluorescence peaks in the sample. The EEM scanning time for each sample was approximately 15 min. To mitigate the effects of Rayleigh scattering and Raman [2].

Quantitative and qualitative analysis of OTC

The quantification of OTC was performed using a liquid chromatography tandem mass spectrometer (model: LC-MS 8040) following these steps. Firstly, the instrument mode was adjusted to be in MS, and the name of the compound to be tested was OTC in the instrument setting panel. The specific setting conditions were as follows, initially set the nebulizing gas flow rate as 3 L/min, DL temperature as 250°C, heating block temperature as 400°C, drying gas flow rate as 15 L/min, data acquisition time as 4.20 min, and set the running time program. Next, set the pump mode as binary high-pressure gradient, the total flow rate as 0.3000 mL/min, and the concentration of pump B as 15.0%. Set the parameters of the column temperature chamber to a temperature of 35°C and a maximum injection temperature of no more than 90°C. The degradation products of OTC were characterized using Shimadzu LC-20ADXR MSD: LCMS-2020 following these steps. With liquid-phase detection conditions: mobile phase A (pure water) and B (acetonitrile), the average temperature was 40°C, the flow rate was 2 mL/min, and the set-up time was 0~10 min; mobile phase B was 10%, 10~20.5 min, and mobile phase B was 100%, 20.5~30.9 min. The metabolites of OTC were analyzed using mass spectrometry to determine the molecular mass and molecular formula of the different degradation products and to introduce the chemical structural formula of the different degradation products. Firstly, the mass spectra of the fermentation system were analyzed at different pH for each stage of fermentation. In the primary mass spectrogram, we can see the peak times of the different structures of OTC, which correspond to different mass spectral amounts. In the secondary mass spectrogram, we define the intensity of the base peak as 100% for the molecular ion peak, at the highest end of the m/z, and all others as fragment ion peaks, based on their corresponding values of m/z. In the secondary mass spectra, we deduce the relative molecular mass based on the different m/z values and then introduce the possible chemical structure based on the possible loss of functional groups and bond breaking.

Statistical analysis

Pearson's correlation analysis is a statistical method used to measure the degree of linear correlation between two variables, enabling the assessment of how a single factor influences the primary variable. Pearson's coefficient index was used to evaluate the degree of correlation between the various pH regulation methods and the concentrations of ammonia, COD, PN, PS and SCFAs production (Table 2). Our analysis revealed significant correlations between the different data sets.

Differentiating factors were searched by Analysis Of Variance (ANOVA) with significance level 0.05. Standard statistical comparisons and graphing were performed in Microsoft Excel, and correlation analyses were performed in IBM SPSS Statistics.

Table 2: Description of correlation coefficients.

	Time	**pH**	**COD**	**NH^{4+}-N**	**SCFAs**	**PN**	**PS**
Time	1	0	-0.08	-0.254	-0.123	0.588**	545**
COD	-0.08	0.464**	1	0.500**	0.705**	0.483**	0.450**
pH	0	1	0.464**	0.635**	0.759**	0.502**	0.534**
SCFAs	-0.123	0.759**	0.705**	0.742**	1	0.522**	0.513**
PN	0.588**	0.502**	0.483**	0.183	0.522**	1	0.876**
NH^{4+}-N	-0.254	0.635**	0.500**	1	0.742**	0.183	0.277**
PS	0.545**	0.534**	0.450**	0.277	0.513**	0.876**	1

Note: The correlations are significant at the 0.01 level.

RESULTS AND DISCUSSION

Stability of the OTC sludge anaerobic fermentation system under continuous and intermittent pH control regulation

pH is widely recognized as important factors influencing anaerobic fermentation. In general, acetogens have an adaptable pH range of 4.5～8.0, while methanogens are most active between 6.6-7.5 [19]. This pH dependence enables continuous monitoring of the fermentation process, providing valuable insights into the ongoing fermentation dynamics and the overall stability of the system.

As shown in Figure 1A, real-time pH measurements indicated that during the initial 2 days of fermentation, the minimum values for the different pH groups, under their respective pH regulations, were 6.470 (R0), 4.560 (R1), 6.958 (R2), 8.726 (R3), and 10.306 (R4). Notably, the pH decreased in the groups with strong acid and alkaline solid addition was more pronounced than in the control group. The pronounced pH decrease was attributed to the disruption of sludge cells by strong acids and bases, which was particularly evident in these groups. This disruption of in the normal microbial activity can lead to the accumulation of SCFAs during the hydrolysis acidification stage, which can persist in the system for an extended period [20,21]. At the end of the fermentation process, the pH values for the blank control group R0 and the other experimental groups were recorded as 7.29 (R0), 5.37 (R1), 7.16 (R2), 9.74 (R3), 11.72 (R4), 9.08 (R5), and 11.35 (R6), respectively. Notably, the fermentation system showed enhanced favorability for the hydrolytic acidification of natural OTC-containing excess sludge under intermittent pH control at pH=10 (R3).

The magnitude of the Oxidation-Reduction Potential (ORP) value served as an indicator of the redox reactions within the anaerobic culture flasks [22]. The effect of OTC on ORP has received limited attention in fermentation studies. Notably, the ORP of each group under both intermittent and continuous pH modes of fermentation exhibited a decreasing trend, as illustrated in Figure 1B. On the second day of fermentation, the ORP values for each group were recorded as -334, -336, -337, -340, and -344 mV, respectively. Notably, the ORP values for each group with different pH regulation were lower compared to the blank control group. This observation could be attributed to the fact that controlled pH regulation enhanced the fermentation process, leading to a higher degree of anaerobiosis within the fermentation system. Following the fourth day, as the hydrolysis acidification stage concluded, the ORP stabilized within a specific range. It indicated that the actual intensive fermentation system of OTC-containing excess sludge was relatively stable under this continuous and intermittent pH regulation, which facilitated the fermentation process towards the hydrolysis-acidification stage and increases the production of short-chain volatile fatty acids while promoting the degradation of OTC [23].

Effect of intermittent and continuous pH control on soluble modified parameters in anaerobic fermentations

Release of NH^{4+}-N, COD, PN, PS, under different pH regulation: In the anaerobic fermentation of sludge, a substantial quantity of NH^{4+}-N is generated. The analysis of NH^{4+}-N release was conducted under various pH regimes. Figures 2A and 2B, illustrated a comparison of NH^{4+}-N release under different pH conditions. On the 4th day of fermentation, the highest concentration was 170.4 mg/L at pH=12. The concentration was 1.14, 1.32, and 2.11 times higher than at pH=10, pH=5, and pH=8, respectively. Consequently, the concentration of NH^{4+}-N in the liquid phase displayed an increasing trend. As depicted in Figure 2B, NH^{4+}-N released from both groups during the four days of fermentation were similar, measuring 150.3 mg/L(R3) and 153.5 mg/L(R5), respectively. However, intermittent pH regulation led to the fermentation persisting in the acid production phase after four days which primarily characterized by the generation of SCFAs. Despite the fermentation's continuation in the acid production phase as time progressed, the analysis of NH^{4+}-N revealed that intermittent pH modulation at pH=10 (R5) released 13.46% more NH^{4+}-N compared to continuous pH regulation (R3). In the fermentation system, the high concentration of NH^{4+}-N would cause to exist Free Ammonia (FA) in the reaction system. However, FA could diffuse from the cell membrane into the cytoplasm, causing the intracellular potassium ions or protons to lose balance and ammonia inhibition to occur, thus inhibiting the process of acid production [24]. This ensured optimal SCFAs production throughout the fermentation process.

The experimental determination of COD accumulation in various pH environments was conducted Figure 2C. Notably, the COD accumulation reached 1654.82 mg/L at pH=12 on the 4th day of the OTC-containing excess sludge fermentation, marking 86.91% increase compared to the blank pH. This suggested that pH=12 had the most significant effect on the decomposition of organic matter in the fermentation of OTC-containing excess sludge in successive pH regulation modes. Moreover, Figure1A, highlighted that the dissolution rate of organic matter significantly varies under different pH conditions. The dissolution rates of organic matter followed the order: pH12＞pH10＞pH5＞pH8 under the four continuous pH regulation conditions. The COD content in the released fermentation broth served as an indicator of the degree of sludge cell lysis [25]. A higher organic matter removal implied a greater degree of sludge lysis, indicated that OTC was fully released during the hydrolytic acidification stage of anaerobic fermentation [19]. The observed phenomenon could be attributed to the fact that both acidic and alkaline conditions to have the capacity to disrupt the sludge floc structure and microbial cell structure. This disruption lead to the release of more substances from the OTC-containing excess sludge, consequently increasing the amount of soluble COD in the sludge that was released into the liquid phase. Therefore, an alkaline environment with pH=12 was more favorable for the leaching and accumulation of organic matter. The anaerobic fermentation of typical activated sludge considered that the leaching of organic matter was better at pH=10 [22]. This difference could be attributed to the presence of challenging-to-biodegrade substances, such as OTC, in the actual sludge. These substances might wrap around the sludge cells, make it more difficult for them to biodegrade and release organic matter. More vital alkalinity was therefore required to break down the floc structure of the sludge cells, releasing intracellular substances into the fermentation broth. This process promoted the anaerobic fermentation of sludge and enhanced the acid production process. Additionally, maximizing the amount of dissolved organic matter in different fermentation systems strengthens the SCFAs production system. On the 4th day of fermentation, it was observed that the concentration of the full amount of COD appeared in the R4/R6 fermentation system. This further illustrated the presence of challenging-to-biodegrade substances in the OTC-containing excess sludge.

Both acidic and alkaline conditions were found to promote the solubilization of PS and PN in the fermentation system. As depicted in Figure 2D, the levels of both PS and PN were notably increased at pH=12. The PS content continued to increase over time after the second day of fermentation. Concurrently, there was a transient decrease in PN content, possibly attributed to the more pronounced denaturation of PN compared to their release during this stage of fermentation. On the fifth day of fermentation, the pH=10 control group exhibited the PS content of 7.74 mg/L and the PN content of 50.92 mg/L. These values demonstrated the most significant regulation by pH=10 compared to several other groups (pH=5/8/12), in contrast to the trend observed in soluble COD. It was speculated that both acid and alkaline could disrupt the structure of sludge cells, leading to the release of more EPS, which were often negatively charged in fermentation systems [26]. In an alkaline fermentation system, the acidic groups within the EPS interact with alkalinity, leading to a faster degradation of these acidic groups. This resulted in EPS exhibiting a more substantial negative charge creating a repulsive effect. Consequently, the floc structure of sludge cells was disrupted, leading to the release of increased amounts of PS and PN. Under acidic or alkaline conditions, the PN and PS content in the system peaked on the 5th and 6th days, followed by a gradual decline as fermentation time extended. This pattern arized from the fact that acid-producing bacteria primarily utilized PS and PN as substrates for SCFAs production. In the later stages of anaerobic fermentation, the consumption rate of PS and PN surpassed in the hydrolysis stage, resulting in a declining trend for both components [27]. In general, intermittent pH=10 conditions favored the solubilization of PS and PS, facilitated their subsequent utilization and degradation by acid producing bacteria. However, the fermentation of actual OTC-containing excess sludge maintained higher pH was not preferable, as it may promote the destruction of the sludge floc structure.

Effect of intermittent and continuous pH control on SCFAs production: SCFAs served as a high-quality carbon source for biological nitrogen and phosphorus removal as well as for the production of other chemicals [28,29]. Consequently, the study aimed to investigate the accumulation performance of SCFAs in the fermentation system. As shown in Figure 3A, the production of SCFAs reached 1590.5 mg COD/g VSS and 1672.8 mg COD/g VSS under intermittent pH=10 and pH=12 conditions, respectively. In contrast, 920.3 mg COD/g VSS and 543.5 mg COD/g VSS were accumulated under continuous pH=5 and pH=8, respectively. As methanogenic bacteria utilized SCFAs as substrates for the process, a decreasing trend was observed.

The delay in reaching the maximum SCFAs production under strong alkaline conditions was attributed to the extensive destruction of the sludge cell floc structure [30]. It was evident that the decline in SCFAs content was more pronounced at pH=5 and pH=8. This was a result of the significant inhibition of methanogenic bacteria activity, leading to a reduced consumption of SCFAs. The study found that intermittent pH=10 regulation had a minimal impact on the dissolution efficiency of organic matter but favored the production of more SCFAs during the hydrolysis acidification stage. As shown in Figure 3B, the proportion of acetic acid was further increased under intermittent pH=10 regulation, reaching up to 82%. However, intermittent pH=12 promoted COD dissolution which was detrimental to acid production. In summary, the forthcoming analyses would solely focus on comparing the differences between intermittent pH=10 and continuous modulation methods.

The preceding discussion indicated that continuous pH control was more favorable for the dissolution of soluble organic matter, further enhancing the hydrolysis process of macromolecular organic matter. Conversely, intermittent pH control ensured that the elevated pH environment promoted the sludge hydrolysis process and mitigated the inhibition of hydrolysis-acidifying and acid-producing bacteria caused by the naturally decreasing pH in the fermentation system. This facilitated the hydrolysis reaction and promotes the production of SCFAs. The specific acid production mechanism required further elucidation through microbiological analysis.

Quantitative and qualitative analysis of OTC under intermittent and continuous regulation of the pH Quantitative analysis of OTC in fermentation systems with different pH regulation: Studies have indicated that hydrolysis was the main degradation pathways of OTC in the environment. Quantitative analysis was important for illustrating the specific degradation process of OTC in the fermentation system, ultimately contributing to the effective degradation of OTC. The OTC have a benzene ring molecular structure. Parts of its attached to the surface of the sludge floc. As shown in Figure 4A, the initial content of OTC sludge was 8.27 mg/L from the sludge dissolution experiments with different oscillation times. Experimental details of different oscillatory desorption times for OTC sludge was found. After two days of fermentation, the OTC content exhibited an initial increase followed by a subsequent decrease (Figure 4B). During the hydrolytic acidification stage, the concentrations of OTC at different pH levels were 2.65 mg/L, 3.69 mg/L, 1.58 mg/L, 17.21 mg/L, and 9.32mg/L. The experiments revealed that the concentration of OTC was higher than that of the blank control group under both acidic and alkaline conditions. As fermentation progressed into the hydrolytic acidification stage, both acidic and alkaline conditions proved effective in disrupting the sludge structure, promoting the release of OTC from the sludge system. Conversely, adjusting the pH externally with alkali at pH=8 may have led to microorganisms adapting to the altered system environment, resulting in a diminished functional impact. As depicted in Figure 4B, the intermittent pH regulation approach alleviated the inhibitory effect of strong bases on microorganisms and promoted more OTC degradation in the process. In essence, the primary pathway for OTC release and degradation in sludge was the stage where OTC was adsorbed to the sludge surface. Various pH adjustment methods were employed to disrupt and break down sludge flocs and cells, releasing OTC into the fermentation liquor. OTC was a broad-spectrum antibiotic with limited biodegradability, its degradation was essential to the breakdown of other metabolites and low molecular weight organic matter in the fermentation system [31].

Analysis of OTC metabolites in different pH-regulated fermentation systems: The presence of OTC residue was a primary factor classifying its sludge as hazardous waste, and it represented a significant limitation to its recyclability. An analysis of the metabolites and potential pathways of the fermentation process was essential in Figure 5. Map of OTC degradation pathways under different pH regulation strategies and Mass spectra of OTC and its intermediates are given. OTC, being an aromatic compound containing a stable benzene ring structure, underwent structural breakdown through continuous and intermittent pH regulated fermentation, resulting in corresponding degradation products [32]. Under the blank control condition at pH=8.05, two compounds with formula weights of 395.4 and 461.1 were produced at the end of fermentation. It is speculated that the metabolic pathway may involve lactonization to form EOTC. Both neutral and alkaline conditions could favor the formation of EOTC, which further be converted to OTC or undergo direct degradation [33]. Another compound with a formula weight of 395.4 was produced through deamidation, decarbonylation, dehydration, and dehydrogenation of OTC. Under pH=5 conditions, OTC retain its benzene ring structure characteristics of aromatic compounds, with a formula weight of 461.1 in the initial stage of fermentation. This was likely to be a α-apo-OTC, and both acidic and alkaline pH conditions were conducive to the formation of α-apo-OTC [34]. Additionally, a compound with a formula weight of 83.3 was identified as a biodegradable low molecular organic matter, certified through 3D-EEM analysis. The original structure of OTC persisted in the initial stage of fermentation under intermittent pH=10 control. The base peaks that did not appear to have 100% intensity were all fragment ion peaks in the hydrolytic acidification. This suggested a hypothesis that OTC underwent degradation, giving rise to multiple metabolites [35]. Mass spectra analysis at the end of the fermentation phase under intermittent pH=10 control revealed no formation of degradation products containing benzene ring. However, under pH=12 conditions, the presence of an OTC degradation product with 100% base peak intensity and a relative molecular weight of 403.2 was indicative of a benzene ring in its structure, suggesting potential resistance to biodegradation. This highlighted the advantage of intermittent pH=10 regulation in OTC-containing excess sludge fermentation. Various pH control approached yield different metabolites through possible pathways Figure 5 Such pH adjustment contributed to reducing the environmental risk of OTC and potential threat to human health [36].

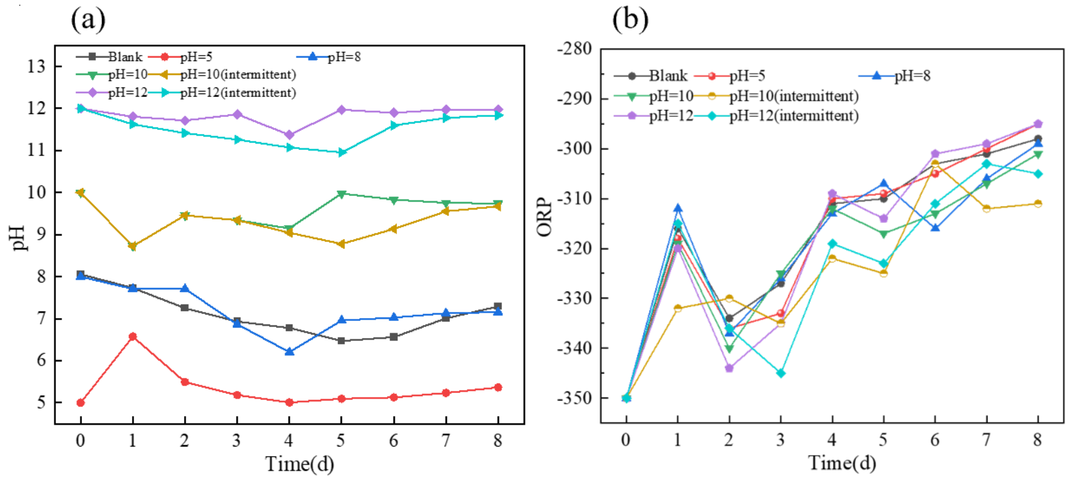

Figure 1: The changes under intermittent and continuous regulation. **Note:** (A): Real time pH; (B): ORP.

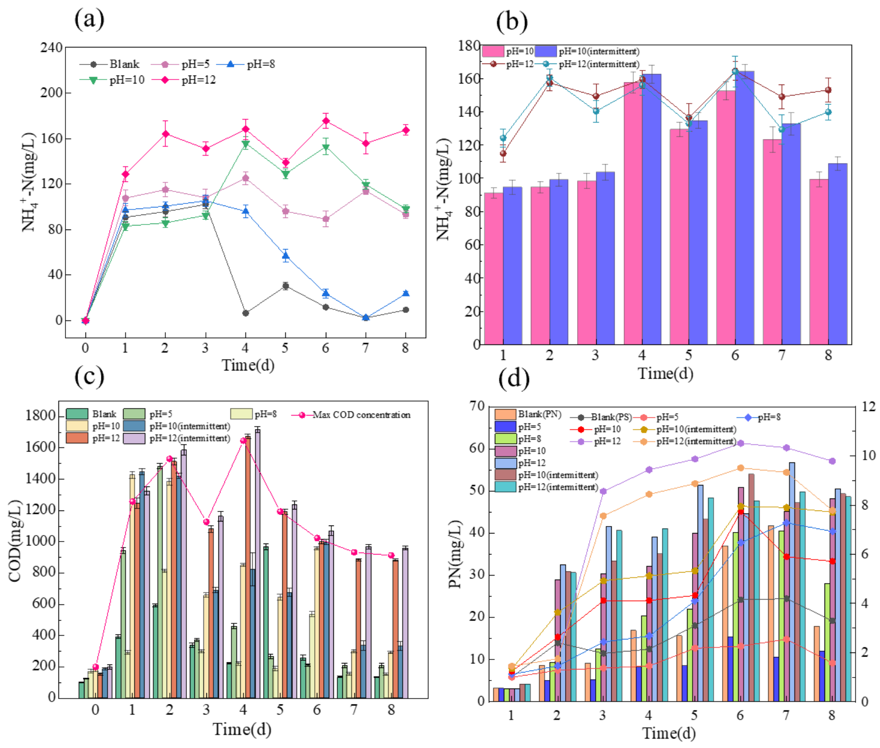

Figure 2: Comparison of NH^{4+}-N release under different pH conditions under. **Note:** (A): Continuous pH regulation; (B): Intermittent pH regulation. Experimental determination of COD accumulation in various pH environments (C): Changes of COD; (D): Plots of PS and PN.

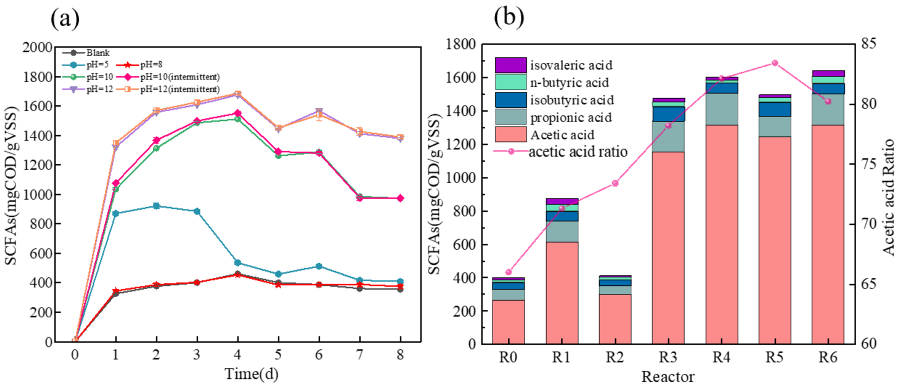

Figure 3: (A) Plot of total SCFAs under intermittent and continuous pH regulation; (B) Percentage of different acids at day 3 of fermentation.

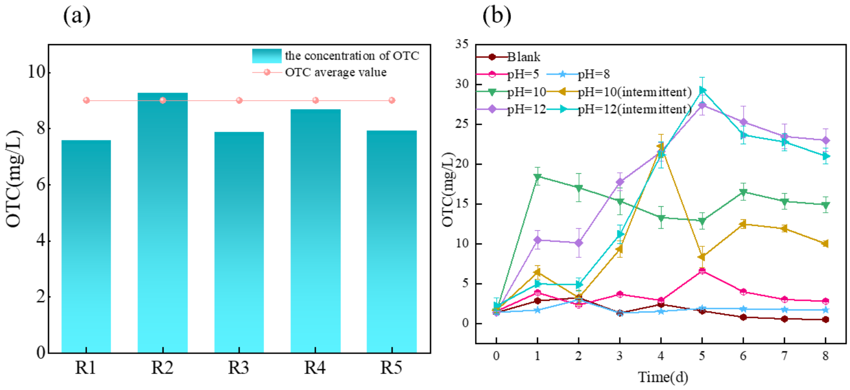

Figure 4: (A) Content of OTC in initial sludge flocs and quantitative analysis of OTC under intermittent; (B) Continuous pH modulation.

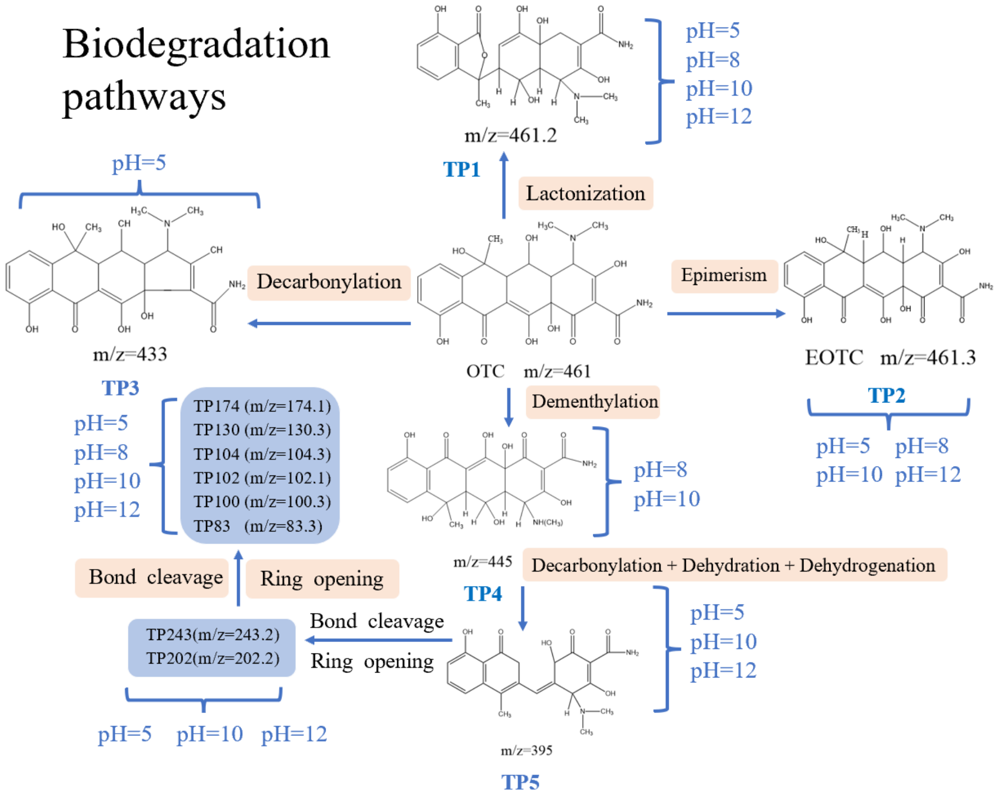

Figure 5: Possible fermentation pathways of oxytetracycline.

Sludge reduction performance under intermittent and continuous pH control

OTC-containing excess sludge was a by-product of OTC production effluent. A significant amount of sludge was generated and disposed of as hazardous waste, incurring high costs. Reducing sludge production was essential for cost and energy savings. Thus, OTC-containing excess sludge reduction was urgent. The most direct manifestation of sludge reduction was the change of VSS concentration after fermentation. The VSS removal rate characterized the tendency of organic matter to be converted to inorganic matter in the fermentation system as well as the reduction of sludge and the hydrolysis effect of particulate organic matter [37]. In Figure 6, both acidic and alkaline conditions contributed to the VSS removal rate with the pH effects ranking as follows pH=8<pH=5<pH=10<pH=12, respectively. At different fermentation stages, the available material in the sludge decreased, leading to endogenous respiration of microbial cells and the autolysis and death of microbial cells, resulting in sludge reduction. Under continuous and intermittent pH=12 conditions, the VSS removal on the 5th day was 30.1% and 30.8%, respectively. There was minimal difference in sludge reduction between intermittent and continuous pH=10 control conditions. The reduction in sludge could be attributed to the presence of hydrolytic acidifying bacteria, which contributed to the lysis of sludge cells [28]. The presence of NO_2^--N in the system during the early stages of anaerobic fermentation could promote *in situ* hydrolysis of sludge to produce organic substrates.

Biodegradability analysis of fermentation both under intermittent and continuous pH regulation

The microbial cells in sludge comprise a diverse range of organic compounds, including antibiotic substances, known for their resistance to biodegradation. Hence, an investigation was conducted using three-dimensional fluorescence spectrum to examine whether the pH regulation method induced alterations in the biodegradability of organic compounds released from the sludge [38]. The 3D-EEM spectrum was broadly categorized into five regions based on excitation and emission wavelengths, as detailed in section 2.5 of the experimental materials and methods. The corresponding abundance of different organic compounds was expressed as a percentage fluorescence response [39]. As demonstrated in section 2.5 of the experimental materials and methods, regions I and IV primarily exhibit the fluorescence intensity of biodegradable substances, while regions II, III, and V display the fluorescence intensity of non-biodegradable substances. At day 4 of fermentation as shown in Figure 7A, the EEM peak fluorescence intensity of the fermentation supernatant was higher than that of the blank control group in both continuous and intermittent pH=10 fermentation systems (R3 and R5). Moreover, the organic matter released by microbial cells in the sludge plays an important role in the production of SCFAs by acid-producing microorganisms [40].

In the blank control, the percentages of fluorescence response in Region-I and Region-IV were 1.17% and 19.25%, respectively. In the continuous pH=10 reactor, these percentages increased to 15.91% and 24.82%, as shown in Figure 7B. Furthermore, the combined sum of $P_{I,n}$ and $P_{IV,n}$ was 40.73%, marking a 1.54-fold increase compared to the blank control. In comparison with the blank reactor (R0), the corresponding percentages in the intermittent pH modulation (R5) were 14.79% and 27.53%, respectively. The sum of $P_{I,n}$ and $P_{IV,n}$ in this case was 42.32%, representing a 1.62 times higher value than that of the blank control. The results indicated that, in comparison with the blank reactor, pH control regulation increased the proportion of biodegradable organic matter in the fermentation supernatant, providing a substantial amount of available organic matter for the production of SCFAs. Furthermore, compared to the blank, PIII,n decreased from 12.57% to 10.29% and 11.94%, and $P_{V,n}$ decreased from 51.58% to 16.99% and 31.88% in R3 and R5, respectively. This suggested that the pH adjustment reduced the content of non-biodegradable substances such as fulvic acid and humic acid in the total organic matter. The potential reason for this reduction was the conversion of these non-biodegradable substances into biodegradable ones through microbial action. However, the precise pathways of reduction and conversion require further investigation.

Analysis of microbial community structure

OTC may alter the microbial community structure of the sludge. To better understand the effects of intermittent and continuous pH regulation strategies on the fermentation system of OTC-containing excess sludge, high-throughput gene sequencing technology was employed to analyze the microflora. As depicted in Figure 8, bacteria belonging to the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, Patescibacteria, and *Chloroflexi* were the most abundant fermenters under both intermittent and continuous pH conditions. Both acidic and alkaline conditions led to an increase in the relative abundance of *Proteobacteria* and *Bacteroidetes* as the first and second dominant phyla. Compared with the blank control, the relative abundances of *Proteobacteria* and *Bacteroidetes* under pH=5 conditions were higher (1.26% *versus* 26.59%, 17.04% *versus* 28.64% respectively). The increased abundance of the dominant fermentation phyla suggested that acidic conditions accelerated fermentation. At pH=8, this sludge sample closely resembled the OTC-containing excess sludge system, and the relative abundance of *Proteobacteria* and *Bacteroidetes* increased from 1.26% and 17.04% to 39.17% and 38.64%, respectively. Additionally, *Acidobacteria* were identified in the pH=8 reactor with a relative abundance of 7.65%, which significantly higher than in sludge samples under other conditions. It was speculated that the potential reason for this observation was the presence of unidentified microorganisms before fermentation with some of them transforming into acid producing microorganisms [41]. Additionally, *Actinobacteria* played an important role in the acidification phase of anaerobic fermentation of sludge. They could utilize carbon sources such as glucose, starch, and cellulose for hydrolysis and acid production [42]. These bacteria have been identified in sludge under various pH conditions except in sludge prior to fermentation. The increased abundance of hydrolytic acidifying bacteria under intermittent pH=10 conditions was considered one of the key factors contributing to the accumulation of SCFAs. In R3, there was nearly no Actinobacteriota, while the abundance of this bacterial type in R5 was 3.05%. This difference was believed to be due to the elimination of poorly adapted bacteria from alkaline environments [43]. There was a slight increase in the relative abundance of *Proteobacteria* and *Chloroflexi* phylum from 23.45% and 1.68% to 34.80% and 3.02% under continuous and intermittent pH=10 conditions, respectively. The increase in *Proteobacteria* in R5 was closely associated with microbial cell lysis and the release of intracellular substances [44]. The *Chloroflexi* phylum primarily degraded carbohydrates and microbial cells with some members contributing to the degradation of substrates into SCFAs [41]. Additionally, it was found that a portion of the *Thermotogae* phylum was capable of degrading complex organic matter, such as xylose and cellulose [38]. The analysis revealed that intermittent pH adjustments led to an increase in the abundance of microorganisms engaged in hydrolytic acidification.

The composition of the bacterial community at the genus level was illustrated in Figure 9. Genera such as *Hydrotalea, Bacillus*, *Acidovorax*, *Enterococcus*, *Enterobacter*, and *Acinetobacter* were identified. The relative abundance of *Bacillus* was 0.409% in the R3 reactor and 29.94% in the R5 reactor, making it the dominant genus under intermittent pH regulation. *Bacillus* have been recognized for its key role in protein degradation and utilization [42-43], where in it hydrolyzed proteins into acetic, propionic, and iso-butyric acids. Furthermore, *Pseudomonas* emerged as a potential tetracycline degrader, exhibiting varying abundances across different pH adjustments: 0.38% (R0), 1.54% (R1), 0.86% (R2), 1.25% (R3), 1.21% (R4), and 1.56% (R5). It was noteworthy that in the continuous pH=10 fermentation system, the abundance of potential OTC degrading bacteria reached 1.25%, which is 3.28 times higher than that of the blank control group (R0). Additionally, in the intermittent pH=10 (R5) fermentation system, the abundance of *Pseudomonas* was found to be 4.12 times higher than the blank control group. This suggested that intermittent pH regulation was conducive to the enrichment of OTC degrading bacteria in the fermentation system. *Thauera*, identified as a parthenogenetic bacterium in both R3 and R5 sludge, which exhibited a relative abundance of 0.00% in R0, which increased to 1.16% and 13.67% in R3 and R5, respectively. In related study, it was determined that *Thauera* played a role in controlling the denitrification process during the nitrite phase, aligning with the detection of nitrite in this experiment. Additionally, Truepera, known for degrading proteins to produce acetic acid and functioning as a specific acid-producing bacterium, showed increased relative abundance under intermittent pH modulation [44]. This indicated that the intermittent pH modulation approach enhanced the overall relative abundance of hydrolytic acidification microorganisms, thereby increasing the diversity of functional microorganisms contributing to OTC degradation.

Continuous pH control was found to be advantageous for the enrichment of various species of hydrolytic acidifying bacteria. In contrast, intermittent pH control not only minimized the harm to acid producing bacteria in the alkaline environment, enhancing the effectiveness of these acid-producing bacteria, but also elevated the gene abundance of specific functional groups of OTC degrading bacteria. Consequently, the microbial community structure under intermittent pH regulation not only supported the accumulation of SCFAs but also contributed to an increased abundance of specific OTC degrading bacteria.

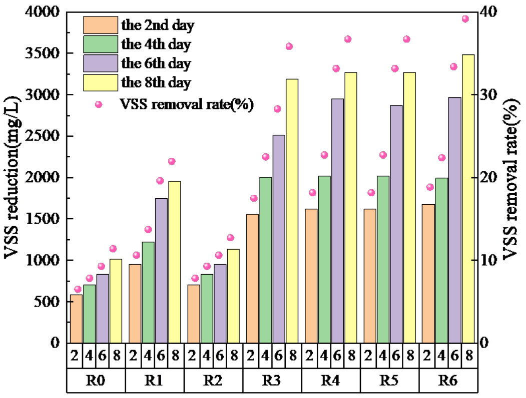

Figure 6: VSS removal under intermittent and continuous pH modulation.

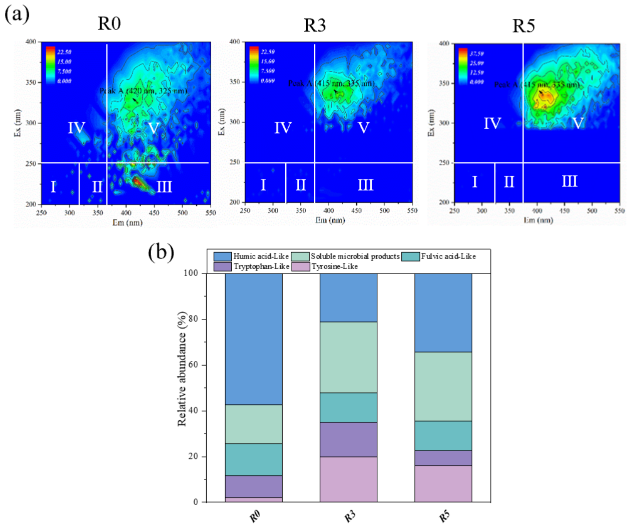

Figure 7: (A) Fluorescence spectra of organic matter release from R0, R3, and R5 after 4 days of fermentation under intermittent and continuous pH regulation; (B) Stacked histograms of the percentage integral in different regions.

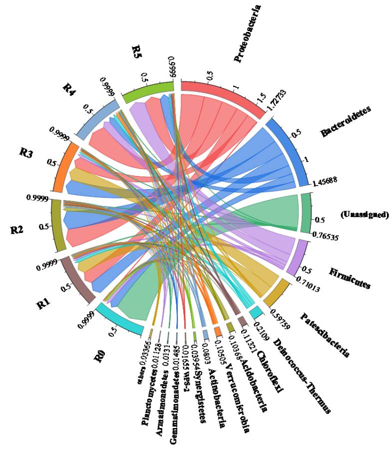

Figure 8: Relative abundance of microbial populations at phylum level under intermittent and continuous pH regulation.

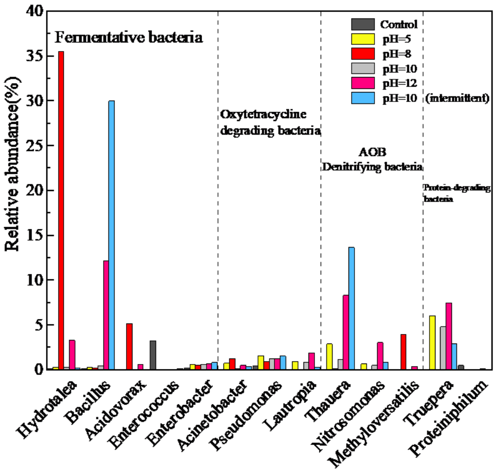

Figure 9: Relative abundance of microbial populations at genus level under intermittent and continuous pH regulation.

CONCLUSION

The study confirmed the advantages of intermittent pH phased regulation. Intermittent pH regulation proved more advantageous to generate SCFAs. And the maximum SCFAs produced was 1590.5 mg COD/g VSS during the fermentation process. Moreover, the sludge reduction achieved to 33.1%. Simultaneously, intermittent pH regulation enhanced the removal of OTC during anaerobic fermentation. Main metabolites such as EOTC and α-apo-OTC were produced through internal esterification and tautomerization. And then the biodegradable organics were further formed. Hydrolytic acidifying bacteria (*Bacillus*, *Hydrotalea*, *Acidovorax*, *Enterococcus*) cooperated with OTC degrading bacteria (*Pseudomonas*) to achieve the release and removal of OTC from the excess sludge. It is beneficial for the OTC-containing excess sludge to address the degradation, succession and reversion of contaminants.

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