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Exploring 2,4,6-Trichlorophenol Degradation Characteristics and Functional Metabolic Gene Abundance Using Sludge Fermentation Broth as the Carbon Source

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Abstract: The use of sludge fermentation broth (FB) as a co-metabolic carbon source for treating 2,4,6-trichlorophenol (2,4,6-TCP) wastewater is a novel strategy. The key to the feasibility of this strategy is whether the FB can promote the growth of functional microorganisms that are capable of degrading 2,4,6-TCP. This study focused on long-term acclimatized sludge and investigated the impact of key operating parameters such as the sludge FB concentration and the influent concentration of 2,4,6-TCP on the removal efficiency of chlorophenol. The research findings revealed that when the influent concentration of sludge FB exceeded 300 mg COD/L, it significantly inhibited the degradation of 2,4,6-TCP. Simulation experiments using individual VFA components as influent carbon sources showed that excessive propionic acid addition can inhibit the degradation of 2,4,6-TCP, indicating the need to control the concentration of propionic acid in the fermentation conditions. Metagenomic analysis further showed that sludge FB can promote the enrichment of microbial chlorophenol degradation genes, including *PcpA*, *pcaF*, *pcaI*, *Mal-r*, *chqB*, and *fadA*. The abundances of these six chlorophenol degradation genes were as follows: 1152 hits (*PcpA*), 112 hits (*pcaF*), 10,144 hits (*pcaI*), 12,552 hits (*Mal-r*), 8022 hits (*chqB*), and 20,122 hits (*fadA*). Compared with other types of carbon sources, sludge FB demonstrates distinct advantages in terms of leading to the highest chlorophenol degradation concentration and the abundance of functional microbial communities. This study has successfully demonstrated the feasibility of using sludge FB as a co-metabolic carbon source for the degradation of 2,4,6-TCP.

Keywords: 2,4,6-trichlorophenol; functional gene; microbial community; co-metabolism

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1. Introduction

2,4,6-Trichlorophenol (2,4,6-TCP) is a persistent organic compound. Its molecular formula features three chlorine substituents, which significantly increase its biotoxicity compared with mono- and dichlorophenols [1,2]. Its remarkable chemical stability has led to a wider range of applications [3] than other chlorophenols, resulting in higher environmental concentrations of 2,4,6-TCP [4]. Consequently, many researchers have focused on developing techniques for removing 2,4,6-TCP, including biological co-metabolism [5], and bio-electrochemical methods [1,6–8]. The bio-co-metabolism technique involved enhancing the biodegradability of 2,4,6-TCP wastewater by adding readily degradable carbon sources [2]. Over time, the technique has evolved from using commercial carbon sources like sucrose and glucose to employing other organic wastewater streams as co-metabolic carbon sources [9]. This shift has significantly reduced the application cost of the co-metabolic technique, thereby increasing its practicality. A crucial aspect of this progress has been the enrichment and cultivation of 2,4,6-TCP functional microbial communities.

Research on the co-metabolism of biological processes can employ mixed microbial cultures. Wang, J et al. [5] investigated the degradation of 2,4,6-TCP using sucrose as a co-metabolic carbon source. They utilized mixed cultures from a wastewater treatment plant as the inoculum sludge and identified Norank_p_Saccharibacteria as the predominant taxa after domestication. Additionally, they observed varying abundances of these taxa with different sucrose concentrations. Similarly, Zhao, J et al. [10] used sludge from a wastewater treatment plant as inoculum and studied the degradation mechanism of 4-chlorophenol with starch and sodium acetate as carbon sources. The microbial communities utilizing starch and sodium acetate both had Proteobacteria as the dominant phylum, with abundances of 32.15% and 43.43%, respectively.

In contrast, other researchers employed single strains to investigate the degradation mechanisms of 2,4,6-TCP. *Cupriavidus necator JMP134* was frequently employed as an aerobic co-metabolism bacterium with strong 2,4,6-TCP degradation capabilities. It was often utilized to investigate the functional genes that are involved in the aerobic metabolism pathway of 2,4,6-TCP. *Cupriavidus necator JMP134* contained all the tcp degradation genes, enabling the efficient synthesis of metabolic enzymes not only for 2,4,6-TCP but also for its different metabolic by-products, resulting in the complete degradation of 2,4,6-TCP [11,12]. The degradation process by *Cupriavidus necator JMP134* did not lead to the accumulation of metabolic intermediates, thus greatly enhancing the mineralization efficiency of 2,4,6-TCP. *Ralstonia pickettii DTP0602* is another extensively tested strain, sharing the same phenol degradation pathway as *Cupriavidus necator JMP134*. However, the functional genes that were responsible for metabolism were entirely different [13]. Wang, C C et al. [14] isolated pure strains that are capable of utilizing 2,4,6-TCP from a mixed microbial community grown on substrates containing chlorophenolic compounds: *Pseudomonas spp. strain 01* and *Pseudomonas spp. strain 02*. These two aerobic bacteria individually exhibited low removal rates for 2,4,6-TCP. When 200~400 mg/L of phenol was added as a co-metabolic substrate, the removal rates for 2,4,6-TCP could be increased to 65% and 48%, respectively. However, further evidence is needed to confirm whether the 2,4,6-TCP degradation active sludge, adapted by adding a mixed carbon source, contains the aforementioned microbial communities.

Research on 2,4,6-TCP-degrading bacteria has primarily concentrated on changes in the microbial community [15,16]. However, there has been limited investigation into the expression of functional genes. The continuous introduction of mixed wastewater into the co-metabolic system could potentially alter the functional bacterial community due to the presence of numerous suspended microorganisms. This could, in turn, impact the degradation efficiency of 2,4,6-TCP. Metagenomic technology, a technique that is capable of analyzing microbial abundance at the gene level [17], presents a viable solution to this issue. It facilitates the examination of microorganisms that are involved in carbon source metabolism and 2,4,6-TCP degradation within the reactor. Importantly, this method allows for the exploration of the potential relationship between the bacteria introduced through mixed wastewater and the original bacterial community. This approach not only enhances our understanding of the degradation process but also provides valuable insights into the impact of introduced bacteria on the functional bacterial community.

In our previous research, the feasibility of utilizing sludge fermentation liquid as a carbon source to domesticate bacteria that are capable of degrading 2,4,6-TCP has been meticulously demonstrated [18]. Based on the above research background, this study focuses on 2,4,6-TCP-degrading bacteria cultivated using sludge fermentation broth as a carbon source. Batch experiments are conducted to validate the influence of different reactor operating conditions on the degradation properties of the microbial community. Simultaneously, both high-throughput and metagenomic analysis techniques were employed to examine the original sludge, fermentation sludge, and chlorophenol-degrading bacteria. This approach allowed us to understand the impact of sludge fermentation broth on the abundance of 2,4,6-TCP degradation genes.

2. Materials and Methods

2.1. Seeding Sludge

Three types of activated sludge were employed in this research. The excess sludge (CS) was collected from the secondary clarifier of a wastewater treatment plant. The degradation sludge (DS), which was acclimated from the CS and had the ability to degrade 2,4,6-TCP, was used with sludge fermentation liquid as the carbon source. The fermentation sludge (FS) was used to provide the sludge fermentation liquid.

CS does not possess the capability to degrade 2,4,6-TCP or ferment acid. The DS demonstrated a maximum degradation concentration of 240 mg/L for 2,4,6-TCP. The FS exhibited an acid production rate of 80.40%, with a peak acid concentration of 974.5 mgCOD/L.

2.2. The Batch Experiment

Batch experiments were conducted within a small-scale sequential batch reactor (SBR), as illustrated in Figure 1. The effective volume of this reactor was 750 mL. The operating conditions for the batch experiments were as follows: the temperature was set at 28 °C, dissolved oxygen was maintained at 4–5 mgO_2/L, and the sludge operational stirring speed was set at 200 rpm. During the operation of the batch experiments, sampling was conducted using a syringe, with each sampling event collecting 10 mL. The pretreatment conditions for batch experiment samples involved centrifugation at 4000 rpm and membrane filtration (0.22 μm). Subsequently, various water quality parameters were further analyzed.

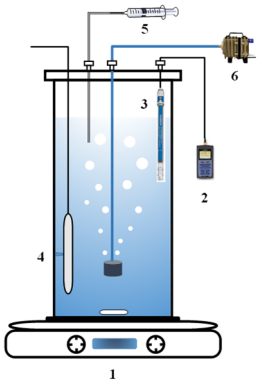

Figure 1. The schematic diagram of batch test SBR (1. magnetic stirrer; 2. WTW water quality monitor; 3. dissolved oxygen and temperature probes; 4. constant temperature heater; 5. sampling syringe; 6. air pump).

2.2.1. Test 1: Studying the Impact of the Dosage of Sludge Fermentation Liquid on the Degradation of 2,4,6-TCP

The influent concentration of the fermentation liquid was calculated in terms of COD equivalents, with concentrations set at 0, 150, 300, and 600 mg COD/L. Sampling intervals ranged from 10 to 30 min. The influent concentration of 2,4,6-TCP was 70 mg/L.

2.2.2. Test 2: Exploring the Influence of the Fermentation Liquid Composition on the Degradation of 2,4,6-TCP

The primary components of the sludge fermentation liquid are VFAs, with the major constituents being acetic acid, propionic acid, butyric acid, valeric acid, and other volatile fatty acids, along with a small portion of lipids and proteins [19]. Given the difficulty in purifying volatile components, sodium acetate, sodium propionate, sodium butyrate, and sodium valerate were used to simulate the VFAs' composition. The alteration of the solution pH induced by sodium acetate, sodium propionate, sodium butyrate, and sodium valerate was rectified by adjusting the pH to neutral using hydrochloric acid. The influent concentration was calculated in terms of COD and was set at 150 mg COD/L for each of these components. The influent includes a simulated carbon source at 150 mg COD/L and 2,4,6-TCP at 100 mg/L. Sampling intervals were set at 60 min.

2.2.3. Test 3: The Impact of Varying Concentrations of 2,4,6-TCP on the Degradation Characteristics of Sludge

The dosage of sludge fermentation liquid was set at 150 mg COD/L. The influent concentrations of 2,4,6-TCP were established at 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 230 mg/L, and 250 mg/L, respectively. Sampling intervals were set at 60 min.

2.2.4. Test 4: Investigating the Influence of Chloride Ion Concentration on the Degradation Characteristics of Sludge

2,4,6-TCP contains three chlorine atoms within its molecular structure. As the reaction progresses, 2,4,6-TCP is continuously oxidized, releasing chlorine ions into the reaction solution. Therefore, it is essential to investigate the influence of the accumulating chlorine ion concentration on the metabolism of 2,4,6-TCP. Sodium chloride served as the primary compound providing chlorine ions, and the impact of chloride ion concentration on the degradation of 2,4,6-TCP in sludge was examined. Batch experiments were conducted under the same conditions as the long-term operation of the SBR, with chloride ion concentrations set in the range of 1000 mg/L, 2000 mg/L, 3000 mg/L, and 5400 mg/L. Sampling intervals were set at 60 min. The influent concentration of 2,4,6-TCP was 100 mg/L.

2.2.5. Test 5: The Effect of Temperature on 2,4,6-TCP Degradation

Prior to the commencement of the reaction, the influent concentration of 2,4,6-TCP was 100 mg/L, while the influent concentration of sludge fermentation liquid was 150 mg COD/L. The investigation was carried out at temperatures of 10 °C, 20 °C, 28 °C, and 40 °C. The experiment lasted for 10 h, with sampling intervals of 60 min.

2.3. Analytical Methods

2.3.1. Standard Parameters

2,4,6-TCP was detected using liquid chromatography, chloride ions were detected using ion chromatography, and volatile fatty acids were detected using gas chromatography. Specific testing conditions for these three parameters are detailed in the attached document.

2.3.2. Sludge Microbial Community and Genetic Analysis

In this study, a total of three types of sludge samples were tested. The activated sludge from the secondary sedimentation tank of the wastewater treatment plant, when used as the original biological sample, was named excess sludge (ES). The active sludge sample, stabilized through the utilization of sludge fermentation liquid as a carbon source for the degradation of 2,4,6-TCP, was named degradation sludge (DS). The fermentation active sludge remaining within the sludge fermentation reactor was named fermentation sludge (FS).

High-throughput methods and metagenomics were employed for the analysis of microbial community and gene abundance. Detailed analytical procedures are available in the Supplementary Materials. The raw data from the metagenomic analysis have been deposited in the NCBI database under accession number PRJNA707137.

3. Results and Discussion

3.1. The Impact of Sludge Fermentation Liquid Dosage

Degradable organic compounds were found to enhance the degradation of 2,4,6-TCP, and this enhancing effect was intensified with an increase in the concentration of the carbon source [5]. It was also demonstrated that 2,4,6-TCP was not adversely affected in the short term when mixed carbon sources containing recalcitrant components were present [9]. In this study, FB served as a typical mixed carbon source with a VFA content reaching 88.4%. Therefore, it was imperative to investigate the impact of adding FB on the degradation of 2,4,6-TCP.

As shown in Figure 2a, there was no significant difference in the degradation of 2,4,6-TCP when the influent FB was either 0 or 150 mgCOD/L. In both cases, complete

degradation was achieved within 300 min. However, when the influent FB concentrations were increased to 300 mgCOD/L and 600 mgCOD/L, the degradation times for 2,4,6-TCP extended to 420 min and 540 min, respectively.

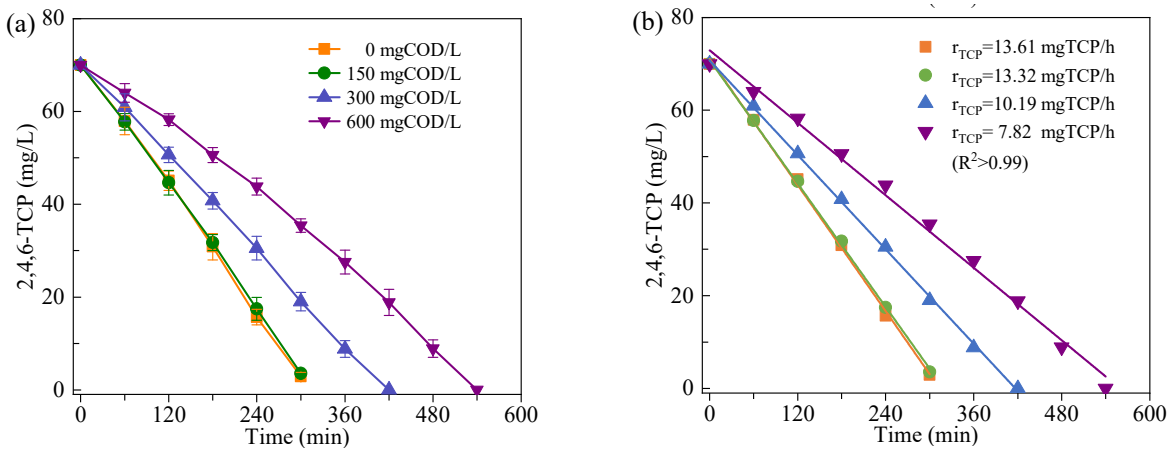

Figure 2. (**a**) The effects of adding different fermentation broth levels on 2,4,6-TCP degradation, (**b**) average degradation rate.

As depicted in Figure 2b, calculations through fitting provided the average degradation rates of 2,4,6-TCP at different FB levels, which were 13.61 mgTCP/h, 13.32 mgTCP/h, 10.19 mgTCP/h, and 7.82 mgTCP/h. It can be observed that there is a competitive metabolic phenomenon between FB and 2,4,6-TCP. This differs from the case when sodium acetate and sodium propionate are used. The reason for this phenomenon may be the interference caused by other organic compounds that are present in the FB on the degradation of 2,4,6-TCP. In the short term, not adding FB can achieve the best removal efficiency of 2,4,6-TCP. However, this is not suitable for the long-term stable operation of the reactor [5]. An FB concentration of 150 mgCOD/L can maintain a stable sludge concentration and has a relatively minor inhibitory effect on 2,4,6-TCP degradation.

3.2. Effect of VFA Compositions on 2,4,6-TCP Degradation

It remains to be further demonstrated which specific component among VFAs, including acetic acid, propionic acid, butyric acid, and valeric acid, may be inhibited in the degradation of 2,4,6-TCP when added in excessive amounts. To explore the impact of the addition of these components, the simulation of the above four VFA components was achieved using different fatty acid salts, and the effect of their concentration on the degradation of 2,4,6-TCP was investigated. The influent concentration of 2,4,6-TCP was set at 100 mg/L, while the influent concentration of fatty acid salts was uniformly set at 150 mgCOD/L.

As shown in Figure 3, when sodium acetate, sodium butyrate, and sodium valerate were used as carbon sources, 2,4,6-TCP was completely degraded within 240 min, with average degradation rates of 24.93 mgTCP/h, 24.98 mgTCP/h, and 24.94 mgTCP/h, respectively, and there were no significant differences. However, when sodium propionate was used as a carbon source, 2,4,6-TCP could only be completely degraded by the 300th minute, with an average degradation rate of 20.19 mgTCP/h, which is significantly lower than the other three carbon sources. These results indicate that among the VFAs, acetic acid, butyric acid, and valeric acid can enhance the removal of 2,4,6-TCP, while propionic acid has a certain degree of inhibition on the degradation of 2,4,6-TCP. Based on these observations, by adjusting the sludge fermentation conditions, it is possible to increase the content of acetic acid, butyric acid, and valeric acid in the total VFAs, thereby enhancing the removal efficiency of 2,4,6-TCP.

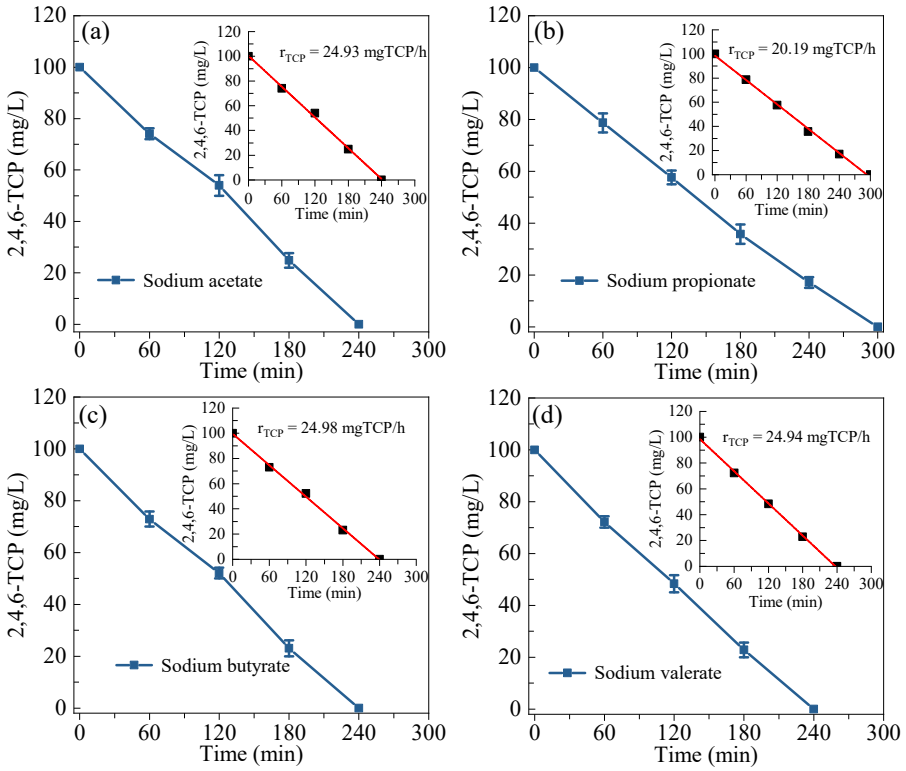

Figure 3. The effects of different VFA components on 2,4,6-TCP degradation, (**a**) sodium acetate, (**b**) sodium propionate, (**c**) sodium butyrate, and (**d**) sodium valerate.

3.3. The Impact of Influent 2,4,6-TCP on Its Degradation

As shown in Figure 4, the specific degradation rates at different 2,4,6-TCP concentrations were investigated. It can be observed that at influent concentrations of 50 mg/L, 100 mg/L, and 150 mg/L of 2,4,6-TCP, the corresponding specific degradation rates were 8.28 mgTCP/g·VSS·h, 8.34 mgTCP/g·VSS·h, and 8.37 mgTCP/g·VSS·h, respectively. When the influent concentration of 2,4,6-TCP was raised to 200 mg/L and 230 mg/L, the specific degradation rates increased to 9.48 mgTCP/g·VSS·h and 9.63 mgTCP/g·VSS·h, respectively. However, when the concentration of 2,4,6-TCP was further increased to 250 mg/L, the specific degradation rate rapidly decreased to 8.32 mgTCP/g·VSS·h.

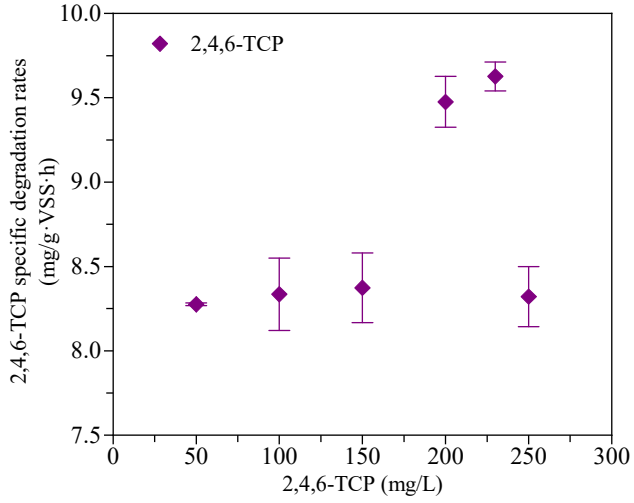

Figure 4. Variation in specific degradation rates of 2,4,6-TCP with its initial concentration (all values are means (n = 3)).

These results indicate that, at a certain influent concentration of 2,4,6-TCP, the specific degradation rate increases with the increase in influent concentration. However, due to the toxicity of 2,4,6-TCP, even with long-term acclimation of the activated sludge, excessively high influent concentrations can still inhibit the microbial degradation activity. The floccular sludge used in this study exhibited significantly higher degradation efficiency for 2,4,6-TCP compared with the use of granular sludge [20].

3.4. Effect of Chloride Ion on 2,4,6-TCP Degradation

Dechlorination is an important indicator for the environmentally friendly treatment of 2,4,6-TCP, and whether the removal of chloride ions will affect the further degradation of 2,4,6-TCP needs further investigation. Using sodium chloride to simulate the concentration of chloride ions released by 2,4,6-TCP, the influence of its influent concentration on the degradation of 2,4,6-TCP was studied. As shown in Figure 5a, when the influent chloride ion concentration ranged from 1 to 3 g/L, 2,4,6-TCP was completely removed within 240 min. However, when the chloride ion concentration was increased to 5.4 g/L, the degradation of 2,4,6-TCP was significantly inhibited. The tolerance threshold of activated sludge to chloride ions was below 5.4 g/L. Furthermore, the chloride ion concentration varied with the influent 2,4,6-TCP and the reactor's operational mode.

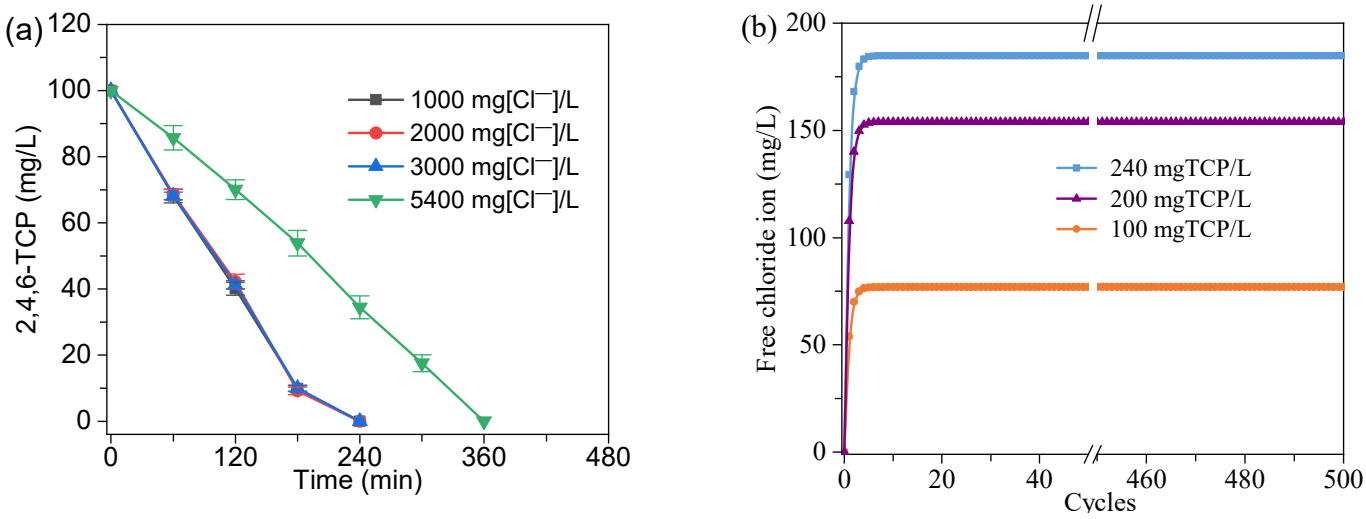

Figure 5. (**a**) The effect of different chloride ion concentrations on 2,4,6-TCP degradation; (**b**) the relationship between the release of chloride ions and the operating cycle with the addition of different 2,4,6-TCP (SBR drainage ratio of 0.7).

In Figure 5b, under the conditions of different influent 2,4,6-TCP (100 mg/L, 200 mg/L, and 240 mg/L) and an SBR effluent ratio of 0.7, the relationship between Cl^{-1} in the reaction liquid and the SBR operational cycles was calculated. It can be observed that within the first four operational cycles, the Cl^{-1} increased rapidly. However, starting from the fifth cycle, the production and discharge of Cl^{-1} reached equilibrium. The final equilibriums of Cl^{-1} were 184.77 mg/L, 153.89 mg/L, and 76.95 mg/L, respectively. Therefore, under a constant SBR effluent ratio, the Cl^{-1} generated by the dechlorination of 2,4,6-TCP reached an equilibrium. In this study, the maximum degradation concentration of 2,4,6-TCP was 240 mg/L, with an SBR effluent ratio of 0.7, resulting in Cl^{-1} below 185 mg/L, while the highest Cl^{-1} tolerance of the activated sludge was below 5.4 g/L. Consequently, the Cl^{-1} generated by the dechlorination of 2,4,6-TCP will not have a long-term impact on the reactor's operation.

3.5. Effect of Temperature on 2,4,6-TCP Metabolism

At different temperatures, the degradation characteristics of 2,4,6-TCP are illustrated in Figure 6. At 28 °C, which was the long-term acclimation temperature for the activated sludge, it was observed that the sludge exhibited the highest degradation activity for 2,4,6-TCP. It could remove 100 mg/L of 2,4,6-TCP within 300 min. However, when the temperature was raised to 40 °C, the initial degradation rate of 2,4,6-TCP was faster, but the degradation significantly slowed down in the later stages. This may have been due to the higher external temperature accelerating the metabolic rate of the carbon source, leading to a lack of necessary carbon source supply in the later stages of operation. When the temperature was lowered to 10 °C and 20 °C, at the end of the cycle, 2,4,6-TCP still remained incompletely degraded.

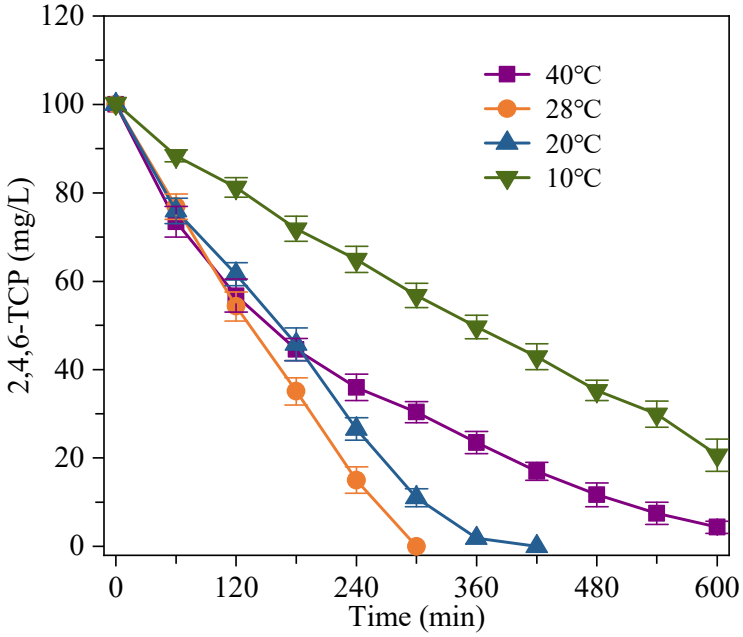

Figure 6. Degradation characteristics of 2,4,6-TCP at different operating temperatures.

3.6. Analysis of FSBR-DSBR Process Bacterial Community

The active sludge tested in this experiment includes three types: residual sludge, which serves as the feedstock for the FSBR; fermentative sludge; and sludge capable of degrading 2,4,6-TCP. Residual sludge acts as the substrate for fermentation, and the sludge FB serves as the carbon source for 2,4,6-TCP degradation. Therefore, it is possible that both residual sludge and the FB may have a certain impact on the microbial community within the target unit.

3.6.1. Analysis of Functional Microbial Community

As shown in Figure 7, at the phylum level of anaerobic digestion, there are a total of 12 dominant bacterial phyla among the three sludge samples. Among these, *Proteobacteria* is the predominant phylum in the residual sludge, accounting for 31.61% of the total abundance. In contrast, *Firmicutes* dominates in the fermentative sludge, representing a substantial proportion at 55.56%. *Firmicutes* is a commonly observed phylum in sludge fermentation [21–25]. The higher abundance of *Firmicutes* observed in this study aligns with the effective acid production during FSBR. Other phyla with relatively high abundances include *Proteobacteria* (29.31%) and *Actinobacteria* (2.73%).

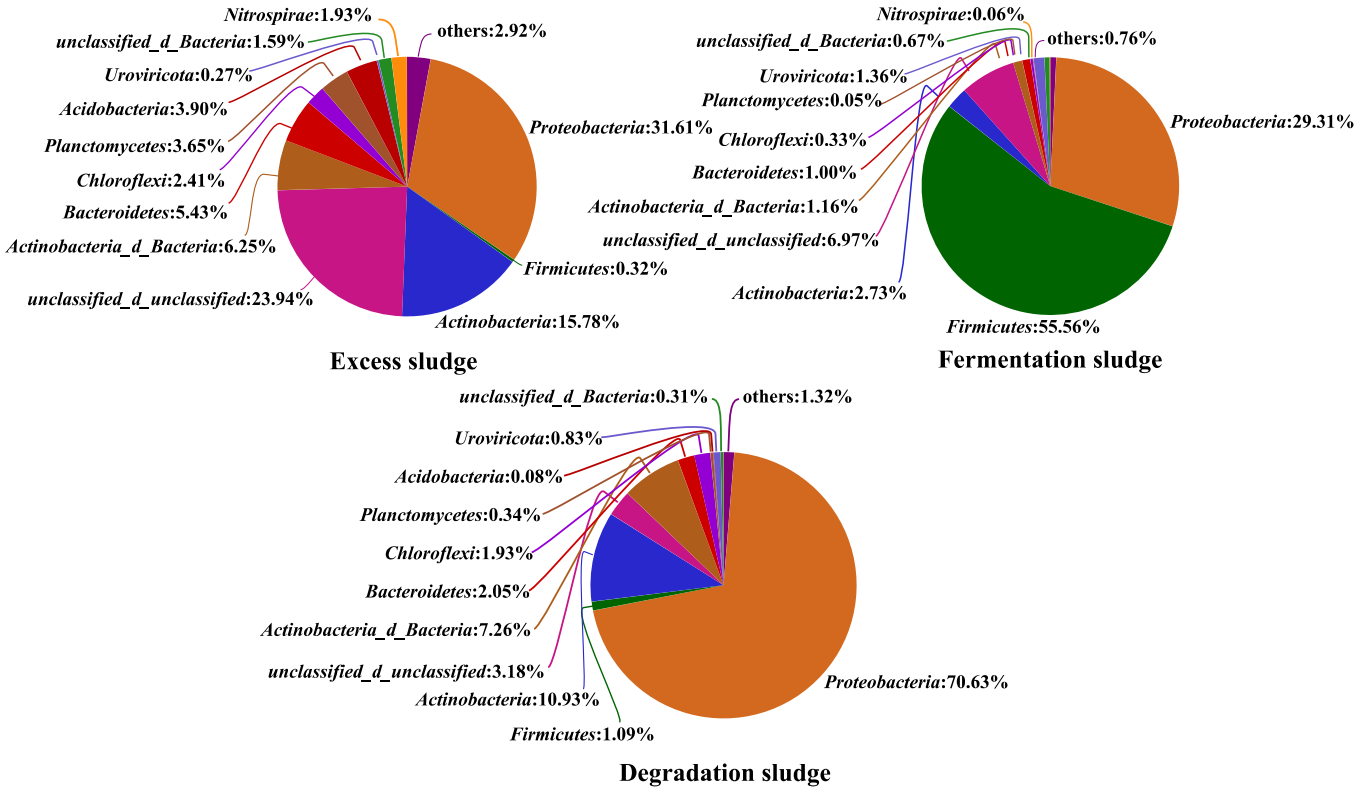

Figure 7. Bacterial community evolution of residual sludge, fermented sludge, and 2,4,6-TCP-degraded sludge at phylum level.

In the sludge that is capable of degrading 2,4,6-TCP, the dominant bacterial phylum is *Proteobacteria*, representing a high proportion of 70.63%. *Proteobacteria* is a key phylum and is associated with the metabolism of 2,4,6-TCP [26], and this finding aligns with the efficient 2,4,6-TCP degradation observed in DSBR. Furthermore, in residual sludge, *Proteobacteria* (31.61%) is the dominant phylum, while fermentative sludge is dominated by *Firmicutes* (55.56%). This suggests that the use of residual sludge as a feedstock substrate did not significantly affect the microbial community structure in the fermentative sludge. This is likely because the heat treatment of residual sludge, its use as the fermentation substrate in FSBR, and the anaerobic fermentation environment favored the dominance of *Firmicutes*. Simultaneously, the suspended sludge in the sludge FB did not significantly impact the microbial community in DSBR.

As shown in Figure 8, at the genus level, *Lactobacillus* was detected in the fermentation sludge, representing 54.06% of the total, and many studies have reported it as a dominant genus in anaerobic activated sludge and acid-producing fermentation processes [27–29]. Therefore, the presence of the *Lactobacillus* genus is favorable for the acid production in sludge fermentation, resulting in an acid production rate of over 88.4%. Additionally, 3.49% of the *Dechloromonas* genus was detected, which plays a significant role in the hydrolysis and acidification of organic matter under anaerobic conditions [22,30,31]. In this experiment, heat-treated residual sludge was used as the feedstock sludge, which contained a substantial amount of polysaccharides and protein components. *Dechloromonas* genus can effectively hydrolyze these organic substances and promote hydrolytic acidification, accelerating acid production.

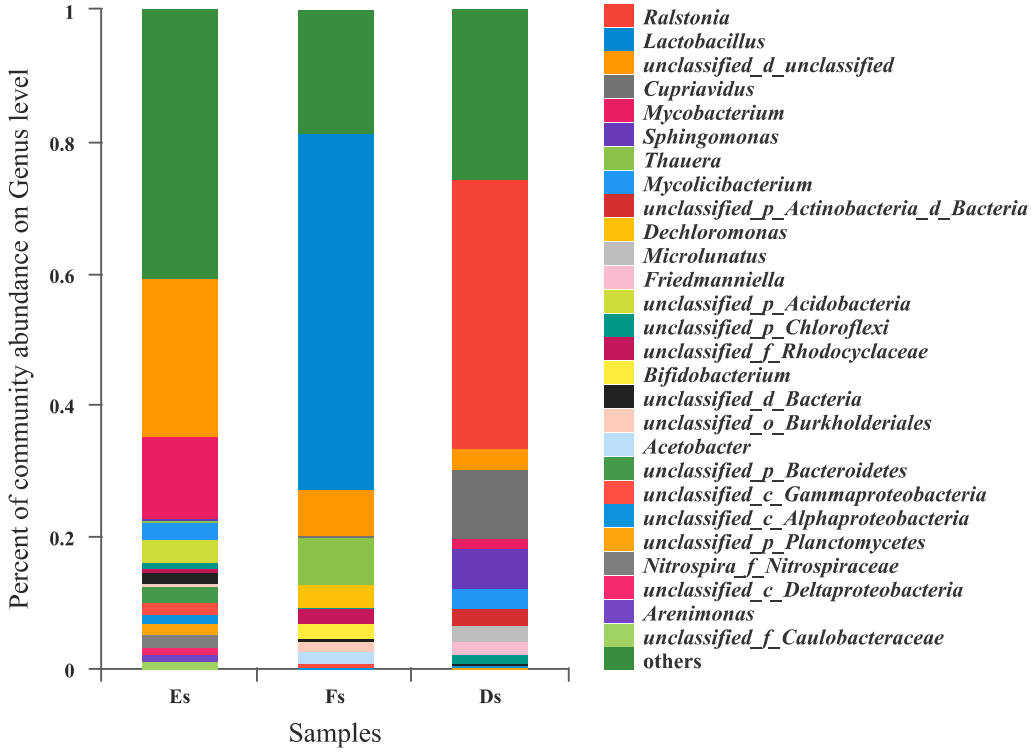

Figure 8. Bacterial community evolution of residual sludge, fermented sludge, and 2,4,6-TCP-degraded sludge at genus level (Es: excess sludge; Fs: fermentation sludge; Ds: degradation sludge).

Through high-throughput analysis of the sludge involved in the degradation of 2,4,6-trichlorophenol, it was discovered that the *Ralstonia* genus was the most abundant genus in the reactor, accounting for as much as 40.89%. Many studies have found a close metabolic relationship between the *Ralstonia* genus and phenol, pentachlorophenol, 4-chlorophenol, and 2,4,6-trichlorophenol [13,32–35]. Other genera with abundances exceeding 1% include *Cupriavidus* (10.52%), *Sphingomonas* (6.18%), *Mycolicibacterium* (2.97%), *Microlunatus* (2.39%), *Friedmanniella* (1.94%), and *Mycobacterium* (1.35%). Among these, the *Cupriavidus genus* [36–39], the *Sphingomonas* genus [40–42], and the *Mycobacterium* genus [43–45] are known to possess the ability to degrade phenol, 4-chlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol, and pentachlorophenol.

Compared with other organic carbon sources, the use of sludge fermentation liquid as a carbon source significantly promoted the enrichment of these chlorophenol-degrading bacteria, particularly the *Ralstonia genus*, which reached a high proportion of 40.89%. The enrichment of these chlorophenol-degrading bacteria is consistent with the removal efficiency of 2,4,6-trichlorophenol.

3.6.2. Analysis of 2,4,6-TCP Functional Gene Abundance and Distribution

In Figure 9, the abundance of functional genes and their distribution proportions among different genera are depicted. At the genetic level, there were a total of six functional genes that were associated with 2,4,6-trichlorophenol metabolism, namely, *PcpA*, *pcaF*, *pcaI*, *MaI-r*, *chqB*, and *fadA*, which was consistent with previous research [9]. The abundance of functional genes in the sludge samples was 52,104 hits, significantly higher than when domestic wastewater was used as a carbon source [9]. This difference in gene abundance may have explained why the highest 2,4,6-trichlorophenol degradation concentration was achieved using sludge fermentation liquid rather than domestic wastewater as the carbon source.

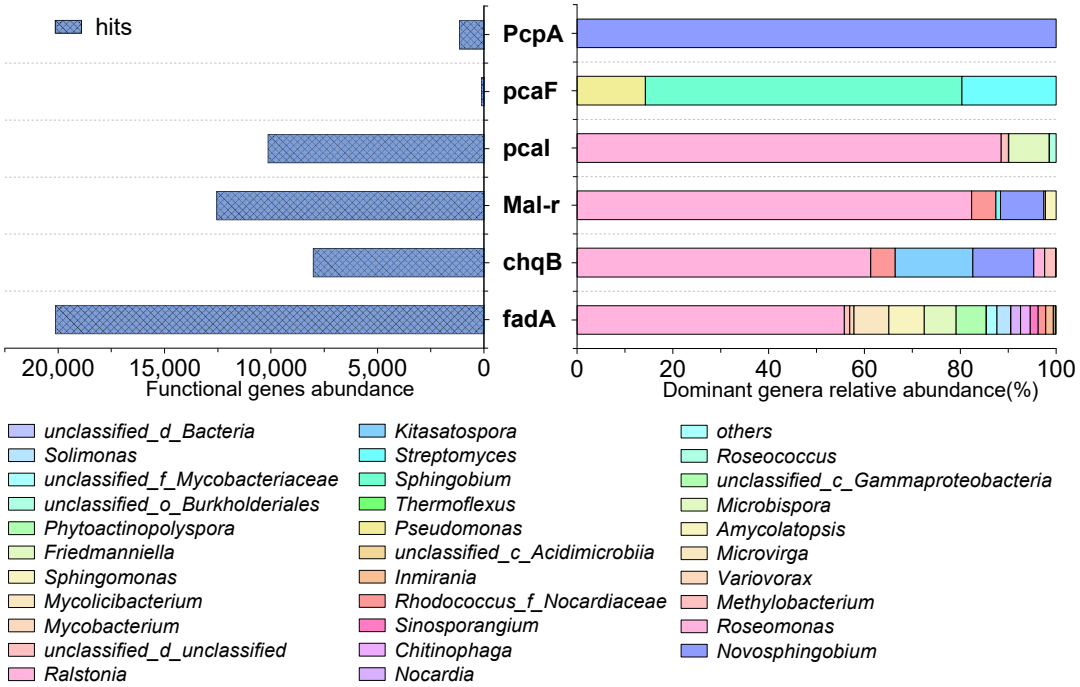

Figure 9. Abundance of 2,4,6-TCP functional genes and the contribution of dominant genera to functional genes.

The individual gene abundances were as follows: 1152 hits (*PcpA*), 112 hits (*pcaF*), 10,144 hits (*pcaI*), 12,552 hits (*Mal-r*), 8022 hits (*chqB*), and 20,122 hits (*fadA*). Although *pcaF* had a relatively low abundance of only 112 hits, it is worth noting that *pcaF* is positioned at the end of the 2,4,6-trichlorophenol metabolic pathway and is responsible for degrading less toxic by-products. This lower abundance of *pcaF* did not impact the overall effectiveness of 2,4,6-trichlorophenol degradation. In contrast, the functional genes at the metabolic front end had relatively high abundances.

PcpA was exclusively contributed by the *Novosphingobium* genus, and the content of *Novosphingobium* had a direct relationship with the removal efficiency of 2,4,6-trichlorophenol. *PcaF* was contributed by the *Pseudomonas* (contribution ratio: 14.29%), *Sphingobium* (66.07%), and *Streptomyces* (19.64%) genera. Multiple genera contributed to *pcaI*, *Mal-r*, *chqB*, and *fadA*, with *Ralstonia* being the most prominent contributor for each, with contribution ratios of 87.54%, 82.12%, 60.91%, and 52.64%, respectively. *Ralstonia* was also the most abundant genus in the sludge used for 2,4,6-trichlorophenol degradation. Therefore, the abundance of *Novosphingobium*, *Pseudomonas*, *Sphingobium*, *Streptomyces*, and *Ralstonia* genera directly influences the metabolic characteristics of 2,4,6-trichlorophenol when using sludge fermentation liquid as a co-metabolic carbon source.

4. Conclusions

Compared with the carbon sources documented in the literature, sludge fermentation broth has demonstrated the ability to enhance the proliferation of functional microorganisms and facilitate the degradation of 2,4,6-TCP. The fermentation liquid derived from sludge exhibits the potential to completely replace other commercially available carbon sources, thereby resulting in a significant reduction in operational investments for practical wastewater treatment. However, the presence of propionic acid components in sludge fermentation broth can partially inhibit the degradation of 2,4,6-TCP. Therefore, during the operation of the coupled fermentation and acid production process, it is necessary to control the level of propionic acid at a lower concentration. Pilot-scale experiments have confirmed that metabolic competition still exists, and the addition of fermentation broth at excessively high concentrations can inhibit the metabolism of 2,4,6-TCP. The analysis of the metabolic

genes of functional bacterial strains revealed the presence of six chlorophenol degradation genes, with the following abundances: 1152 hits (*PcpA*), 112 hits (*pcaF*), 10,144 hits (*pcaI*), 12,552 hits (*Mal-r*), 8022 hits (*chqB*), and 20,122 hits (*fadA*). This study has successfully demonstrated the feasibility of using sludge fermentation broth as a co-metabolic carbon source for the degradation of 2,4,6-TCP.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w15244279/s1: Figure S1: 2-4 Liquid chromatographic peak of 2,4,6-TCP; Figure S2: General process of high-throughput sequencing. References [19,46–48] are cited in the Supplementary Materials.

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