Journal of Functional Materials and Applied Engineering

Research Paper

Study on Strains Degrading Veterinary Antibiotics in Activated Sludge

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Abstract. Currently, veterinary antibiotics are widely used in China's aquaculture industry and play an important role in animal husbandry. However, due to insufficient supervision, the abuse of veterinary antibiotics is relatively severe. Particularly concerning the production of veterinary antibiotics and the treatment of animal husbandry wastewater, clear discharge limits have not yet been established, making the treatment of antibiotic wastewater extremely urgent. An SPE–HPLC protocol was developed for the first simultaneous quantification of tylosin (TYL) and tylvalosin (TAT) in wastewater matrices. The calibration curves were linear over 0.5–1000 mg L^{-1} ($R^2 \geqslant 0.9995$), delivering LODs of 0.20 mg L^{-1}(TYL) and 0.15 mg L^{-1}(TAT) and corresponding LOQs of 0.50 mg $L^{-1}$and 0.42 mg L^{-1}. Spiked real-wastewater recoveries spanned 74.3–97.3 % with RSDs below 8.8 %, confirming reliable accuracy and precision. Subsequently, six strains capable of degrading TYL and TAT were isolated from activated sludge. Among them, Providencia vermicolastrain CT1 showed the best degradation effect on TYL and TAT. At 30 °C, pH 6.0 and 50 mL inoculum, the isolate removed 90.7 % of 200 mg L^{-1} TYL and 94.8 % of 300 mg L^{-1} TAT within 48 h. GC-MS analysis was employed to characterize TAT metabolites, enabling reconstruction of the microbial catabolic pathway. Strain CT1 showed significant treatment effects on actual wastewater containing TYL and TAT, achieving degradation rates of 95.3% for TYL and 93.7% for TAT, along with degradation rates of 97.8% for NH_3-N, 92.9% for COD, and 80.7% for BOD_5.

Keywords: *Advanced Oxidation Technology; Tylosin; Tylvalosin; Pharmaceutical Wastewater*

Received on 02 July 2025, Accepted on 05 Nov 2025, Published on 15 Dec 2025

1 Introduction

China's pharmaceutical industry has shown rapid development in recent years, with its market scale jumping to the second largest globally. According to the latest statistical data, China's pharmaceutical market size reached 1.8 trillion RMB in 2022, accounting for about 12%~15% of the global market share, with an average annual compound growth rate stable at 5%~7%. As the world's largest producer and exporter of active pharmaceutical ingredients (APIs), China holds over 40% of the global API market share, with API export value exceeding 50 billion USD in 2022 [1]. However, with the continuous expansion of application scope, the problem of antibiotic abuse has become increasingly prominent, especially in the field of veterinary antibiotics. Due to relatively lax regulation and their multiple functions in disease prevention and growth promotion, the abuse of veterinary antibiotics is particularly severe [2]. Literature indicates that roughly 6,000 tonnes of veterinary antibiotics are employed annually as feed supplements for China's livestock and poultry industries, constituting approximately 50% of worldwide consumption patterns [3]. While the European Union has prohibited antibiotics as growth promoters, prophylactic antibiotic application persists among certain Chinese farming operations [4]. This not only exacerbates the problem of antibiotic abuse but is also more likely to lead to the generation and spread of resistance genes [5-7], posing a potential threat to public health security [8]. However, the rapid development of the pharmaceutical industry also brings severe environmental challenges. Although its output value only accounts for 2.1% of the national industrial output value, its wastewater discharge volume is as high as 3% of the total national industrial wastewater discharge. This type of wastewater is characterized by complex composition

and high pollutant concentration, with COD values typically ranging from 5000~80000 mg/L, and some antibiotic wastewater even reaching up to 100000 mg/L, making treatment extremely difficult [9]. Untreated pharmaceutical wastewater contains a large amount of antibiotic residues, which enter the environment through surface runoff, groundwater infiltration, and other pathways, polluting water bodies, sediments, and soil [10], disrupting ecological balance, adversely affecting the growth and development of animals and plants, and ultimately threatening human health [11]. It is noteworthy that antibiotics exhibit "pseudo-persistence" in the environment, and traditional water treatment processes are difficult to remove them completely, making antibiotic wastewater treatment an important research topic in the current environmental field.

Effluent from antibiotic manufacturing plants constitutes a grave risk to both ecosystems and public health. Among its primary dangers, antibiotic residues in these discharges can drive the dissemination of resistance genes within environmental compartments. Studies have shown that the abundance of resistance genes in water bodies around pharmaceutical factories is much higher than in ordinary water bodies [12], which will accelerate the emergence of "superbugs," seriously threatening public health security. Second, antibiotics have significant toxic effects on aquatic ecosystems, disrupting the ecological balance of water bodies. Furthermore, antibiotics can inhibit the activity of microorganisms in traditional activated sludge treatment systems, severely affecting wastewater treatment efficiency. Additionally, the persistent existence of antibiotics in the environment allows them to accumulate through the food chain [13], ultimately affecting human health, such as causing intestinal flora disorders and immunosuppression. Antibiotic pharmaceutical wastewater has a complex composition and contains biologically inhibitory substances. Direct discharge will severely pollute water bodies. The residual antibiotics and their metabolites can cause severe acute toxicity and genotoxicity to aquatic organisms and also promote the spread of resistance genes. Moreover, antibiotics in wastewater can exert stress on aquatic microorganisms, leading to the emergence of antibiotic-resistant bacteria [14]. These resistant bacteria continuously release resistance genes through metabolic activities, making water bodies reservoirs and diffusion media for resistance genes. The problem of antibiotic wastewater pollution has attracted much attention, but traditional treatment methods are difficult to effectively block the spread of resistance genes. This type of wastewater has poor biodegradability, and antibiotics and their metabolites often remain in the effluent of sewage treatment plants, causing accumulation in water bodies and inducing bacterial resistance, ultimately threatening human health [15]. Furthermore, antibiotic abuse not only weakens their therapeutic effect but also increases the variety and complexity of antibiotics in water bodies, exacerbating treatment difficulty and accelerating the development of resistance [16], forming a vicious cycle. To address these issues, comprehensive measures such as source control, specialized treatment technologies (such as advanced oxidation coupled with biotechnology), and strict supervision are needed to reduce the environmental and health risks posed by antibiotic pharmaceutical wastewater.

Biological treatment is a common technology that utilizes microbial metabolism to remove pollutants from wastewater [17]. Common biological treatment methods include phytodegradation and microbial degradation [18]. Phytodegradation refers to the removal of pollutants through the absorption, volatilization, rhizofiltration, and degradation actions of plants and their rhizosphere microorganisms [19]. This method utilizes the natural purification capacity of plants, combined with the metabolic activities of rhizosphere microorganisms, to effectively reduce pollutant concentrations in wastewater. Microbial degradation refers to the stepwise degradation of large molecules into harmless small molecules such as water and carbon dioxide through assimilation, altering the molecular structure and physicochemical properties of antibiotics, with resistant bacteria playing a key role [20]. Enzymes secreted by resistant bacteria hydrolyze chemically labile bonds in antibiotics, modifying their physicochemical characteristics and leading to reduced or eliminated antimicrobial activity. Feng et al. [21] isolated Bacillus brevisfrom tylosin fermentation products; inoculating 7% bacteria at 35°C could degrade 75% of tylosin after 7 days. Wang Qianfeng et al. [22] screened four strains of fungi from heavy metal-contaminated soil that can degrade sulfamethazine, norfloxacin, oxytetracycline, and other antibiotics. Biological treatment is usually achieved through the activated sludge process, specifically divided into aerobic activated sludge anaerobic activated sludge [23]. These methods effectively degrade pollutants by precisely controlling the dissolved oxygen concentration in the reaction tank to create aerobic or anaerobic environments, respectively. (1) Aerobic biological treatment. Aerobic biological treatment is a technical method that degrades organic matter through microbial metabolic activities under dissolved oxygen conditions [24]. Currently, in China, common aerobic biological treatment technologies for antibiotic pharmaceutical wastewater mainly include contact oxidation process, oxidation ditch process, deep shaft aeration process, sequencing batch

reactor (SBR) and its improved processes, and membrane bioreactor (MBR). (2) Anaerobic biological treatment. Anaerobic biological treatment of wastewater is conducted under an oxygen-free environment where dissolved oxygen concentration is below 0.2 mg/L, by creating conditions suitable for the survival of anaerobic bacteria and utilizing their metabolic activities to decompose and degrade organic matter in wastewater [25]. Widely adopted anaerobic technologies today include the up-flow anaerobic sludge blanket (UASB), anaerobic hybrid bed (UFB), anaerobic baffled reactor (ABR), and internal-circulation (IC) anaerobic reactor. Biological processes offer clear strengths: modest capital outlay, minimal energy demand, and a low-carbon, eco-friendly footprint. However, this method usually requires cultivating dominant strains for specific pollutants, and there are problems such as long strain cultivation cycles, limited application scope, and low treatment efficiency, and its operational effect is greatly affected by environmental temperature. Gobe et al. [26] studied the degradation effect of traditional primary and secondary treatment in sewage treatment plants on wastewater containing macrolides, sulfonamides, and trimethoprim matrices. The results showed that the pollutant removal rate was very limited, only about 20%.

Given that the ultimate goal of the research is to apply the treatment process to actual wastewater degradation, it is necessary to conduct in-depth research on integrated treatment processes to maximize treatment benefits. In the biodegradation process, antibiotics are toxic to microorganisms. Advanced oxidation technology is usually used as a pretreatment step to reduce pollutant toxicity and avoid fatal effects on microorganisms in subsequent biological treatment [27]. Furthermore, before advanced oxidation treatment, physical or chemical methods are usually used to remove most insoluble impurities. From the perspective of practicality and effectiveness, combined processes are expected to become the best solution for treating antibiotic-containing wastewater. However, existing combination strategies still have problems such as single technology and high treatment cost. For example, Silva et al. [38] used a biological method - photo/Fenton oxidation - biological method process to treat landfill leachate, with a treatment capacity of 100 m^3/day and a cost of 6.8~11.3 €/m^3. This cost is still higher than practical application requirements. Torres et al. [29] used physical flocculation pretreatment + solar/Fenton oxidation + biological post-treatment process to treat landfill leachate, with a treatment capacity of 40 m^3/day. The biodegradability of the waste liquid after solar/Fenton oxidation exceeded 70%, and biological post-treatment could completely remove pollutants, with a total cost of about 4.3 €/m^3, of which H_2O_2 consumption cost accounted for 72%.

This study selected Tylosin Tartrate (TYL) and Tylvalosin Tartrate (TAT), two widely used macrolide veterinary antibiotics in the breeding and animal husbandry industry, as target compounds, aiming to study the removal effect of advanced oxidation technology combined with biofilm method on antibiotic residues and other pollutants in veterinary antibiotic pharmaceutical wastewater.

2 Experimental Process

2.1 Water Sample Collection

Actual wastewater was collected from a veterinary antibiotic pharmaceutical enterprise in Hohhot, Inner Mongolia. 1L was collected, contained in brown glass containers, sealed, stored at 4°C away from light for later use. Water samples were analyzed within 3 days.

2.2 Liquid Chromatography Analysis

An Agilent 1260 Infinity series liquid chromatograph was used for analysis, mainly equipped with four modules: an autosampler (G7129A), a quaternary pump (G1311B), a column oven, and a DAD detector (G1315D). An Agilent 5 TC-C18(2) chromatographic column (250mm x 4.6mm, 5μm) was used to separate the two antibiotics. Column temperature = 35°C, wavelength = 290nm, sample injection volume = 10μL. The mobile phase consisted of acetonitrile (A) and 0.1 % formic acid in water (B), delivered under the following gradient: 0–4 min, 50 % B; 4–6 min, 40 % B; 6–12 min, 50 % B; 12–15 min, 50–40 % B. The eluent was delivered at 1 mL min^{-1}, followed by a 5-min post-run equilibration.

2.3 Domestication, Enrichment, and Screening of Tolerant Strains

In brief, 5.0g of sludge sample was weighed and inoculated into 100mL of sterilized saline. The mixture was shaken at 180 rpm and 30 °C for 2 h, then allowed to settle for 30 min to obtain the bacterial suspension. Three millilitres of the supernatant were transferred into LB medium containing 50 mg L^{-1} TYL and incubated at 30 °C, 180 rpm for 72 h (first acclimation cycle). Using the same method, gradient domestication was continued by inoculating into LB TYL media with concentrations of 100, 150, 200, 250, and 300 mg/L, and cultured continuously for 6 cycles to complete the domestication of TYL-degrading bacteria. Following the sixth enrichment cycle, the culture was serially diluted; 100 µL aliquots were spread on LB agar and incubated at 30 °C for 48 h. Distinct colonies differing in morphology, size and colour were selected and purified by streak-plate isolation. The obtained pure strains were transferred to slants for preservation, and the strain names were marked. TAT-tolerant strains were domesticated, enriched, and isolated using the same method described above.

2.4 Strain Identification

Isolates were streaked onto solid medium, inverted, and incubated at 30 °C for 24 h until colonies appeared; colonial traits were recorded and cells were Gram-stained for light-microscopy examination. For tentative identification, isolates were subjected—each in triplicate—to the suite of physiological and biochemical assays prescribed in Bergey's Manual: V-P, citrate utilisation, starch hydrolysis, methyl red, oxidase, mannitol fermentation, indole and catalase. Genomic DNA of each isolate was amplified with universal bacterial 16S primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CTACGGCTACCTTGTTACGA-3'). The PCR amplification reaction system was: genomic DNA 1µL, 10x Buffer 5µL, extaq 0.25µL, dNTP 2µL, upstream and downstream primers 1µL each, ddH_2O 39.75µL. Thermocycling conditions: 98 °C 30 s pre-denaturation; 30 cycles of 98 °C 10 s, 55 °C 30 s, 72 °C 90 s; final extension 72 °C 5 min. PCR products were verified using 1.5% agarose gel electrophoresis, and the gel was recovered and purified. The target fragments were observed under blue light. PCR products were sequenced using an ABI3730-XL sequencer, and then the obtained sequence files were compared with the NCBI 16S database using the BLAST program. Sequences were aligned with ClustalW against selected GenBank entries from the same family, and a neighbor-joining phylogenetic tree was constructed with MEGA 11 (USA).

2.5 Degradation Characteristics Study

(1) Activation of Degrading Strains

In a sterile environment, a small amount of slant culture was picked with an inoculation loop and inoculated into LB medium, then incubated in a constant temperature shaker at 30°C, 180 rpm for 24 hours to obtain the seed liquid of the degrading strain.

(2) Drawing of Growth Curve

After strain activation, when the culture reached OD600 ≈ 0.5, the bacterial solution was used as seed liquid and inoculated at a 1% volume ratio into fresh LB medium. Cultures were incubated at 30 °C, 180 rpm, and OD600 was read every hour with a spectrophotometer; time was plotted as the abscissa and OD600 as the ordinate to construct the growth curve.

(3) Influence of Different Culture Conditions on Antibiotic Degradation by the Strain

To pinpoint the best antibiotic-degrading conditions, we varied initial TYL/TAT concentration (100–500 mg L^{-1}), temperature (20–40 °C), pH (3–11) and inoculum volume (50–150 mL). All runs were performed in 250 mL Erlenmeyer flasks at 180 rpm; one factor was altered at a time. Samples withdrawn every 12 h were filtered and analysed for residual antibiotic to compute degradation rates.

(4) Degradation Kinetics

A first-order kinetic model was built to probe degrading-bacteria behaviour at 100–500 mg L^{-1} antibiotic under optimal culture (30 °C, pH 6, 50 mL bacterial suspension), keeping the word count virtually unchanged.

(5) Degradation of Antibiotics by Intracellular and Extracellular Enzymes

The degrading strain was cultivated in LB broth (30 °C, 180 rpm, 24 h), then centrifuged at 10 000 rpm for 10 min; the resulting supernatant served as the extracellular enzyme source. The pelleted cells were resuspended in PBS and re-centrifuged at 10 000 rpm for 10 min; the wash was repeated twice and the final pellet was retained. Then, the precipitate was subjected to ultrasonic cell disruption to break the cell wall to obtain the intracellular enzyme. Main parameter settings: power 300W, interval 2s, pulse 4s, for a total of 300 cycles. Under the optimal degradation conditions, intracellular enzymes and extracellular enzymes were added to PBS solutions containing 50 mg/L TYL and TAT, respectively, and the residual antibiotic concentration was determined by HPLC.

(6) Degradation Product Analysis

The degrading strain was inoculated into LB medium spiked with 300 mg L^{-1} TAT and cultivated under optimal conditions; culture broth was collected every 12 h and concentrated by rotary evaporation. The concentrates were redissolved in methanol, filtered through a 0.22 μm nylon membrane, and the degradation products were identified by GC-MS (Agilent 7890B-7000C, USA). An Agilent HP-5MS column (30 m × 0.25 mm, 0.25 μm) resolved the degradation products; 3 μL was injected in split mode (30:1). High-purity helium flowed at 1 mL min^{-1}. The oven began at 80 °C, ramped to 200 °C at 5 °C min^{-1} (20 min hold), then to 250 °C at 5 °C min^{-1} (10 min hold). Full-scan spectra were collected at 70 eV over m/z 40–700.

(7) Treatment of Actual Wastewater by the Degrading Strain

The field wastewater was fully characterised for antibiotic content, COD, BOD_5 and NH_3-N. The authentic effluent was transferred into conical flasks, inoculated with the degrader under its optimum conditions, and periodically sampled to track antibiotic concentration, COD, BOD_5 and NH_3-N, thereby gauging field performance.

3 Results and Discussion

3.1 Detection of Water Sample

Selecting an appropriate water sample pH is beneficial for converting impurities into unadsorbed ionized forms during the solid-phase extraction process, improving the extraction efficiency of the target compounds. This experiment compared the recovery rates of the target compounds under different water sample pH values (3, 5, 7, 9, and 11). The results are shown in Figure 1a. When pH=3, the recovery rates of TYL and TAT were the highest, at 87.14% and 93.9%, respectively. Furthermore, as the pH increased, the extraction efficiency of TYL and TAT showed a downward trend. The reason is that tartrate is weakly acidic and easily decomposes in an alkaline environment, while protein impurities in pharmaceutical wastewater are more likely to precipitate under acidic conditions. Therefore, before SPE treatment, the water sample was adjusted to pH=3.

Separation performance of the Agilent ZORBAX SB-C18 (4.6 mm × 150 mm, 5 μm) and Agilent 5 TC-C18(2) (250 mm × 4.6 mm, 5 μm) columns was also compared. As shown in Figure 1b, when the ZORBAX SB-C18 column was selected for analysis, the separation effect of TYL and TAT from impurities was poor. Therefore, the longer Agilent 5 TC-C18(2) column was chosen to obtain higher resolution. This column showed better separation for TYL and TAT, with higher chromatographic peak response values and no tailing phenomenon. Therefore, the Agilent 5 TC-C18(2) column was selected for HPLC analysis.

This experiment also investigated the analytical effects under different mobile phase compositions. Eluent 1: neat acetonitrile (ACN) / 0.01 M $NH_4H_2PO_4$ (pH 2.65); eluent 2: ACN / 0.1 % formic acid in water. With ADP as the low-polar phase, Figure 1c shows that raising ACN content eroded TYL resolution, whereas lowering ACN broadened the TAT peak and depressed its signal. Furthermore, under this mobile phase analysis, the TAT chromatographic peak showed varying degrees of pre-delay peaks. As shown in Figure 1d, when 0.1% FA was used as the weak mobile phase, the separation of TYL and TAT was insufficient during isocratic elution, and interference peaks appeared. Gradient elution resulted in higher response values and better separation. Therefore, this experiment selected ACN-FA (0.1%) as the mobile phase, using a gradient elution method.

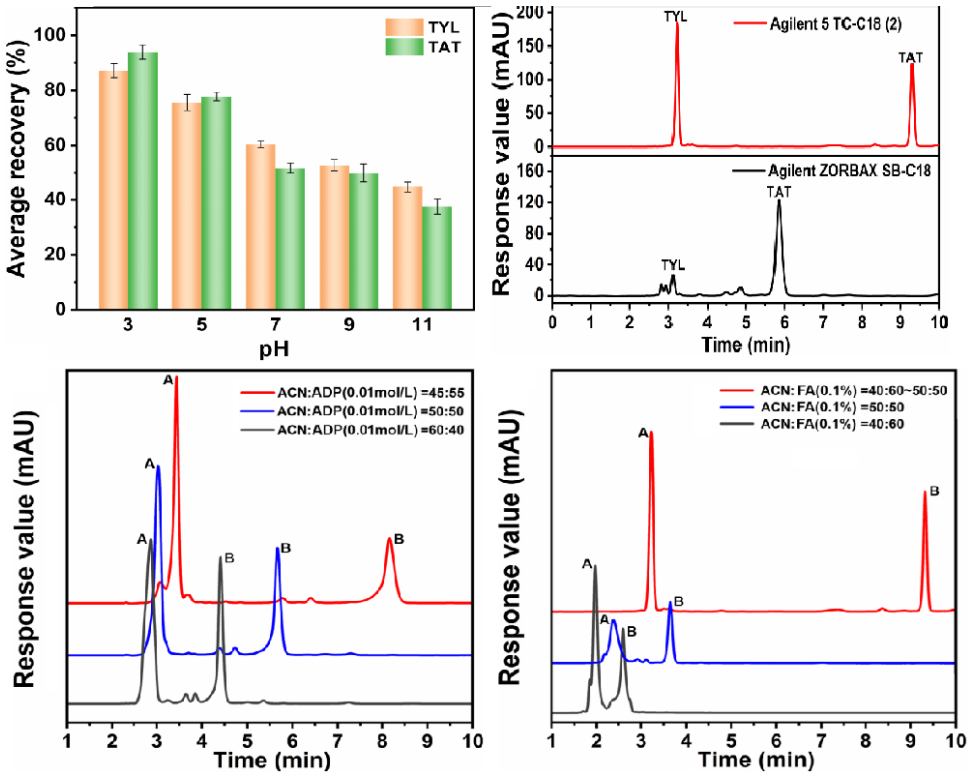

Figure 1 Average recoveries of two antibiotics treated with different pH (a); Chromatograms of mixed standard samples under different HPLC columns (b); Chromatograms of mixed standard samples in different mobile phase, with a and b representing the TYL and TAT chromatographic peaks (c, d), respectively.

3.2 Screening of Degrading Strains

Through gradient domestication and screening of the sludge sample, a total of 6 strains of TYL-tolerant bacteria were obtained, see Figure 2a; 9 strains of TAT-tolerant bacteria were obtained, see Figure 2b. All tolerant strains were respectively inoculated into mixed LB liquid medium containing both TYL and TAT at 100 mg/L. After 120 hours of treatment, the antibiotic content in the culture medium was detected to observe the degradation effect of the strains on TYL and TAT. Among them, CT1 showed the best degradation effect on both antibiotics, with degradation rates of 78.41% for TYL and 85.04% for TAT. Therefore, strain CT1 was selected for subsequent antibiotic degradation research.

Figure 2 TYL tolerant strain (a); TAT tolerant strain (b).

The 16S rRNA gene of strain CT1 was amplified, sequenced, and matched against the NCBI 16S database; a phylogenetic tree was then generated with MEGA (Fig. 3). Comparative analysis revealed that strain CT1 is phylogenetically closest to *Providencia vermicola* strain OP1, sharing 99.6 % 16S rRNA sequence identity. Based on morphological, physiological-biochemical and 16S rRNA data, strain CT1 was identified as *Providencia vermicola* and designated *Providencia vermicola* CT1.

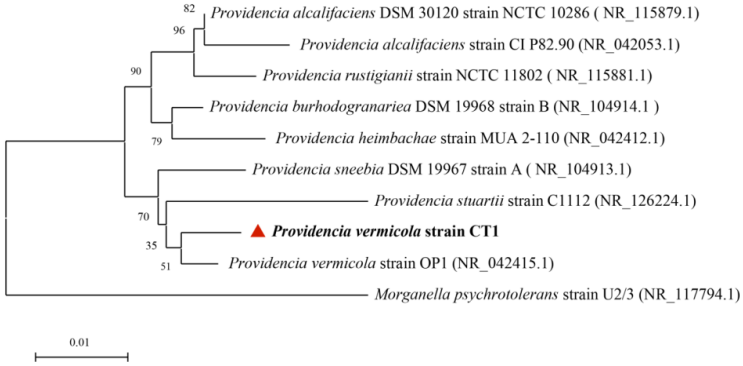

Figure 3 Phylogenetic tree of strain CT1.

3.3 Influence of Different Culture Conditions on Antibiotic Degradation by the Strain

(1) Influence of Different Temperatures on Antibiotic Degradation by Strain CT1

Because enzymes that drive most biochemical reactions exhibit strict thermal optima, temperature strongly shapes bacterial metabolism and catalytic rates [30,31]. Figures 4a–b show temperature-dependent growth and degradation: raising the temperature from 20 °C to 30 °C lifted 48 h TAT removal by CT1 from 68.8 % to 83.3 % and TYL removal from 48.3 % to 77.9 %. From the OD600 values, it can be seen that strain CT1 grew slowly at 20°C. This is because the temperature was too low and did not provide the necessary environmental conditions for microbial proliferation, thus hindering microbial growth. Yet, at 40 °C the same rates dropped to 52.3 % (TYL) and 69.9 % (TAT). This is because excessively high temperatures had a negative impact on the metabolic process of strain CT1, leading to a decrease in its enzyme activity, which in turn affected the degradation efficiency. Overall, 30 °C provided the optimum balance between CT1 growth and its capacity to degrade both TYL and TAT.

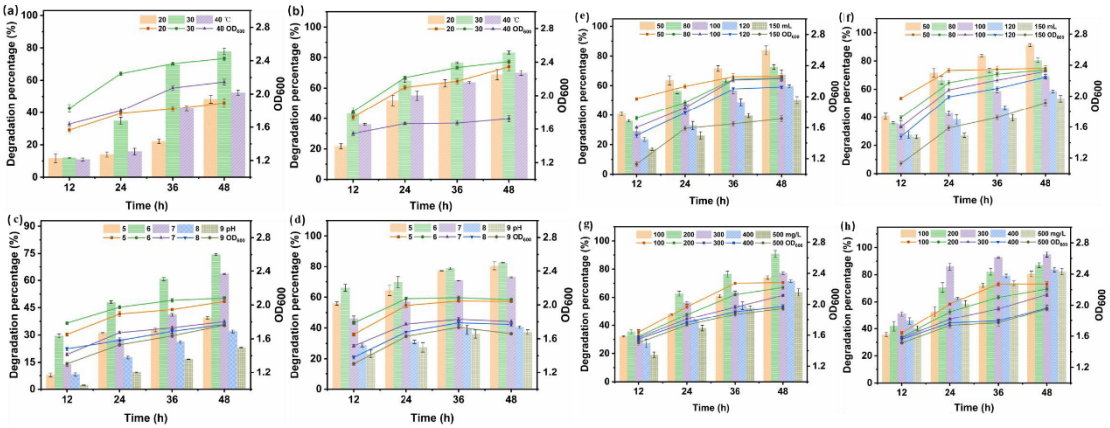

Figure 4 Effect of temperature on degradation of TYL (a) and TAT (b) by strain CT1; Effect of pH on degradation of TYL (c) and TAT (d) by strain CT1; Effect of bacterial solution volume on degradation of TYL (e) and TAT (f) by strain CT1; Effect of Initial antibiotic concentration on degradation of TYL (g) and TAT (h) by strain CT1.

(2) Influence of Different pH on Antibiotic Degradation by Strain CT1

pH shifts modulate membrane charge and enzyme conformation, altering nutrient uptake and metabolic flux;

each microbe therefore operates within a characteristic pH optimum [32]. At pH 8, for instance, a community containing Providencia vermicola degraded gentamicin in sewage far more effectively than at any other pH tested [33]. From Figures 4c and 4d, it can be seen that during the 48-hour treatment of the two antibiotics using strain CT1, the degradation rate first increased and then decreased as the pH value increased, reaching peaks at pH=6 for both TYL and TAT, with degradation rates of 74.39% and 82.69%, respectively. Likewise, pH 6 delivered the best CT1 growth: rapid early proliferation and entry into stationary phase after 24 h, confirming that mild acidity favors the strain's metabolism and degradative activity over alkaline conditions.

(3) Influence of Different Bacterial Solution Volumes on Antibiotic Degradation by Strain CT1

The dissolved oxygen in the microbial fermentation broth can directly affect the activity of microbial enzymes and metabolic pathways, as well as microbial growth and the accumulation of metabolites. This experiment introduced strain CT1 into a 250-mL Erlenmeyer flask for culture. Varying the liquid volume in the flask modulated dissolved oxygen: raising the volume from 50 mL to 150 mL dropped CT1 degradation from 83.8 % to 50.2 % for TYL and from 91.3 % to 53.2 % for TAT (Figs. 4e–f). OD600 tracking showed that 50 mL gave the highest cell density and, correspondingly, the fastest removal of both antibiotics. Although dissolved oxygen was virtually exhausted within the first 5 h, CT1 kept growing, indicating facultative anaerobic metabolism that sustains activity under hypoxia.

(4) Influence of Different Initial Antibiotic Concentrations on Antibiotic Degradation by Strain CT1

Initial antibiotic levels directly govern both strain growth and degradation efficiency. Figures 4g–h reveal that raising the initial antibiotic concentration lifted the degradation rate to a maximum, after which further increases caused a decline. The degradation rate was highest when the initial TYL concentration was 200 mg/L, at 90.73%, and highest when the initial TAT concentration was 300 mg/L, at 94.84%. When the antibiotic concentration is low, the degradation mechanism of strain CT1 may not be fully induced, and the potential substrate concentration threshold required to activate its biodegradation ability may not be reached. When the antibiotic concentration is too high, the antibacterial function of the antibiotics comes into play. Concurrent build-up of metabolic intermediates progressively impairs CT1 viability and metabolism, curbing its degradative capacity. OD600 tracking shows pronounced growth suppression of CT1 once antibiotic levels exceed the tolerance threshold. Hence, 200 mg L^{-1} TYL and 300 mg L^{-1} TAT represent the optimal substrate thresholds, at which CT1 delivers its highest removal efficiency.

3.4 Degradation of Antibiotics by Intracellular and Extracellular Enzymes

To clarify the degradation mechanism of strain CT1 against antibiotics, this study compared and evaluated the degradation effects of intracellular and extracellular enzymes. The results are shown in Figure 5.

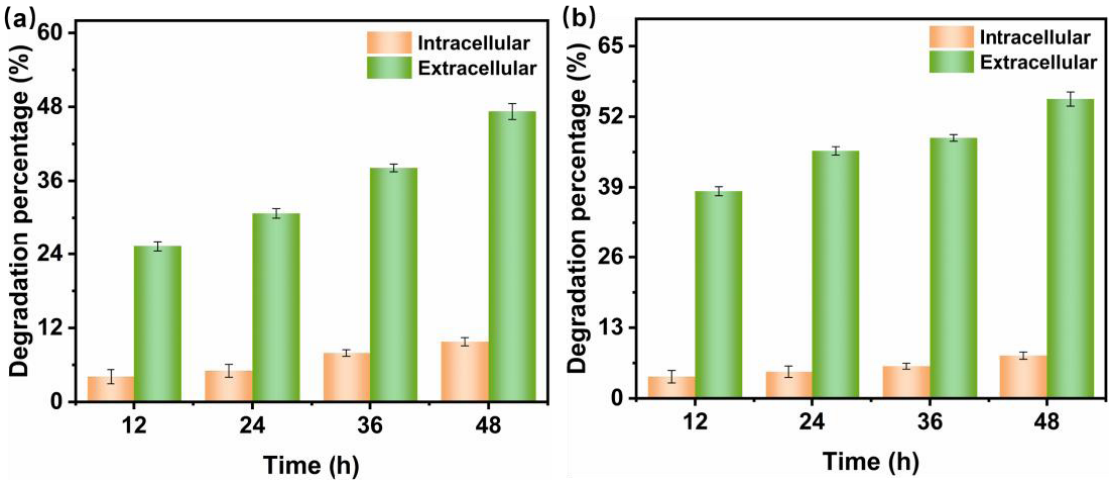

Figure 5 Degradation mechanism of strain CT1 against antibiotics

The degradation effects of TYL and TAT in the extracellular enzymes of strain CT1 were significantly higher than those in the intracellular enzymes. After 48 hours of treatment, the degradation rates of TYL and TAT in the extracellular enzymes reached 47.27% and 55.27%, respectively, while the degradation rates of the intracellular enzymes were relatively low, at 9.77% and 7.88%. These results indicate that the degradation of TYL and TAT by strain CT1 mainly relies on the catalytic action of its secreted extracellular enzymes. This finding is of great significance for understanding the degradation mechanism of strain CT1 and also provides key clues for subsequent research on degradation pathways.

3.5 Inference of TAT Degradation Pathway by Strain CT1

Elucidating biodegradation hinges on mapping products and pathways [34]; microbes achieve this by deploying enzymes that remodel or hydrolyse antibiotic scaffolds [35].

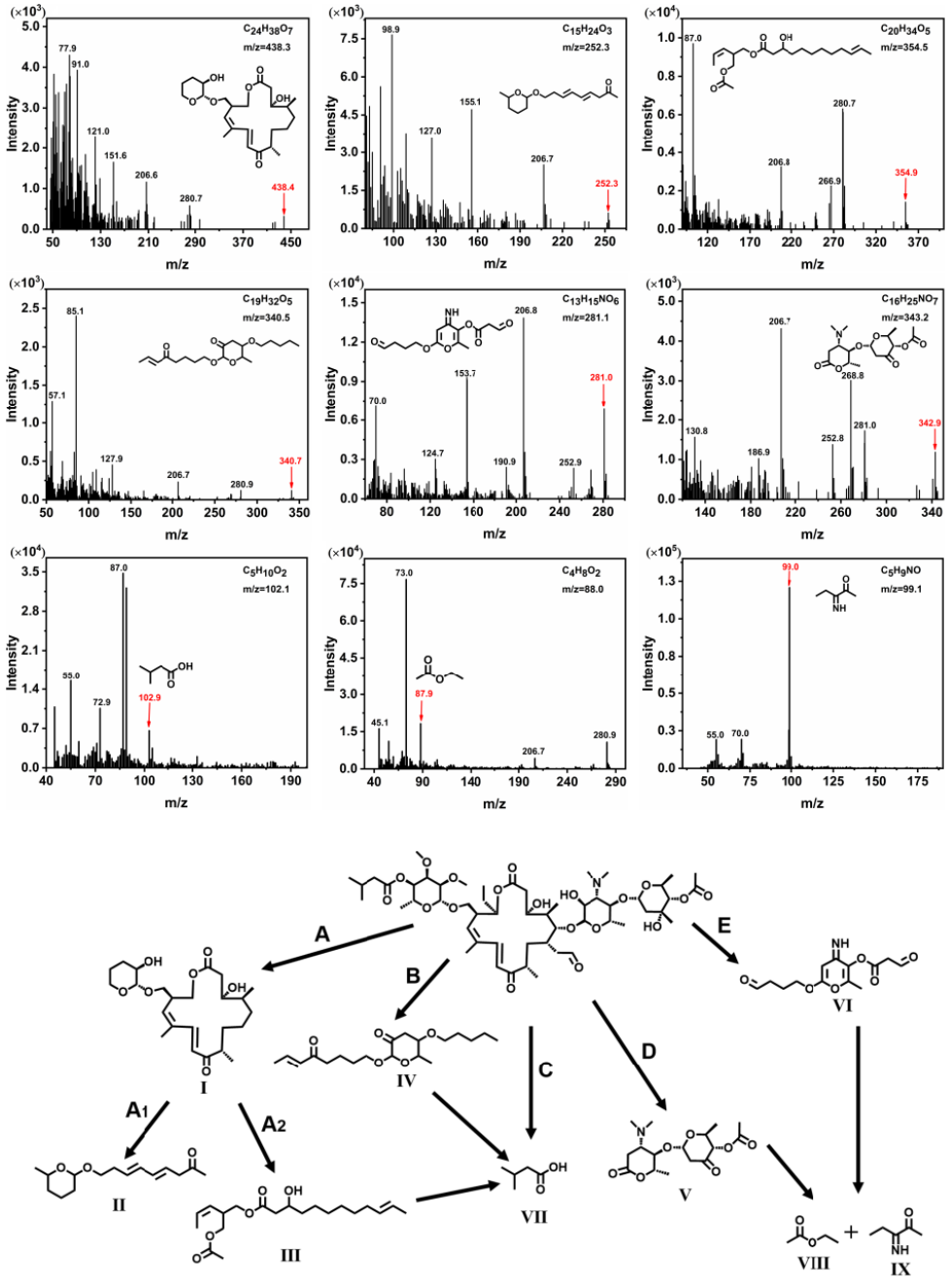

Figure 6 Possible biodegradation pathways of TAT degraded by strain CT1.

Since the degradation products and pathways of TYL (tylosin) have been analyzed by several researchers, for example, Tao Zhang et al. [36] screened a strain of Klebsiella oxytocaTYL-T1 and proposed a new TYL degradation pathway, which includes macrolide lactone bond cleavage, redox reactions, and loss of mycarose. Furthermore, Wang Yan et al. [3] studied the possible pathway of TYL degradation by Burkholderia vietnamiensisstrain, suggesting that Tylosin A (TYL-A) first loses carbon mycarose to convert to Tylosin B (TYL-B), and then the lactone bond and aldehyde group in the molecule undergo hydrolysis and reduction to generate two new degradation products. This study referenced the above related research results and used TAT as a representative to study the degradation products during the degradation process by strain CT1 and infer the degradation pathway.

Potential CT1-mediated TAT metabolites were profiled by GC-MS and authenticated against the NIST library (Fig. 6). Guided by TAT biodegradation behaviour and the detected intermediates, five plausible catabolic routes were proposed (Fig. 6). In route A, sequential loss of two mycarose units and 3-methylbutanoic acid yields by-product I. The sixteen-membered lactone of I is then hydrolyzed at alternative positions, producing ring-opened II and III. In pathway B, the resulting lactone fragments recombine with sugar moieties to afford by-product IV. Routes B and C continue with oxidative and hydrolytic steps that release small molecules such as 3-methylbutanoic acid. In route D, successive hydrolyses and redox events cleave the lactone ring, furnishing by-product V that retains only the two linked mycarose units. In route E, hydrolytic scission of one mycarose ring gives by-product VI; both V and VI are subsequently reshaped through further hydrolysis and reduction, finally yielding VIII and IX. Finally, strain CT1 degrades TAT through a series of biochemical reactions such as hydrolysis and oxidation, generating various small molecular compounds including 3-methylbutanoic acid, ethyl acetate, 3-iminopentanoic acid-2-1, etc.

3.6 Treatment of Actual Wastewater by Strain CT1

Strain CT1 was challenged with authentic wastewater whose pH was mildly acidic and contained 2.60 mg L^{-1} TYL and 4.37 mg L^{-1} TAT (Table 1). NH_3-N, COD and BOD all lay above the discharge limits for fermentation effluent [37]; because the initial TAT level was low, removal was complete within 2 h, giving a large measurement uncertainty. To enhance the intuitiveness of the experimental data, TYL and TAT standard solutions were added to the actual wastewater to obtain the final actual wastewater spiked solution. The concentrations of TYL and TAT after spiking were 180 mg/L and 220 mg/L, respectively. By analyzing the wastewater before and after treatment, it was confirmed that strain CT1 had a significant degradation effect on TYL and TAT. After 60 h of CT1 treatment, TYL and TAT in the real wastewater fell from 180 mg L^{-1} and 220 mg L^{-1} to 11.4 mg L^{-1} and 10.4 mg L^{-1}, respectively, achieving a 95 % removal rate (Fig. 7a). NH_3-N dropped from 42.8 mg L^{-1} to 0.96 mg L^{-1}. Likewise, COD and BOD_5 fell from 10 992 mg L^{-1} and 2 904 mg L^{-1} to 781 mg L^{-1} and 561 mg L^{-1}, pushing the BOD_5/COD ratio from 0.26 to 0.72 and markedly enhancing wastewater biodegradability (Fig. 7b).

Table 1 Various water quality parameters in actual wastewater.

	Limitation	Value
COD (mg/L)	4	10992
BOD_5 (mg/L)	0.5	2904
NH_3-N (mg/L)	0.025	42.8
TYL (mg/L)	0.2	180
TAT (mg/L)	0.15	220

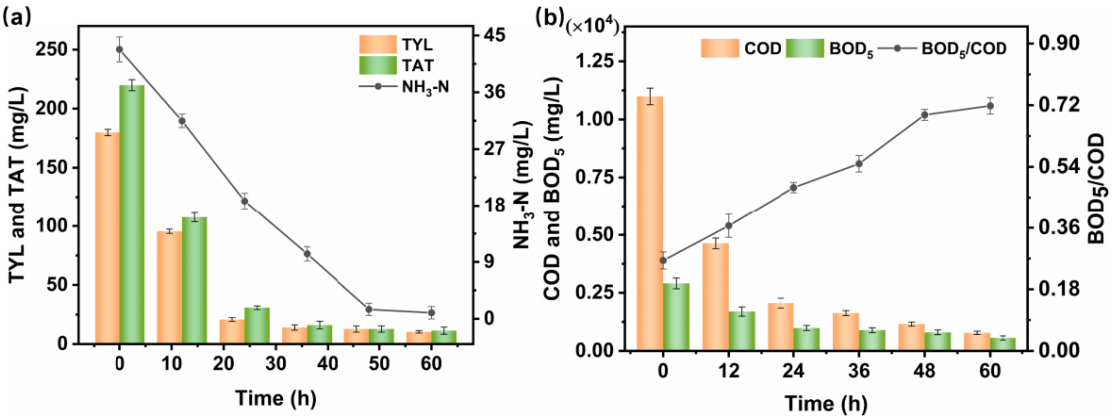

Figure 7 TYL, TAT and NH3-N concentration (a) and BOD5, COD and BOD5/COD concentration (b) at different treatment times.

4 Conclusion

An SPE–HPLC-DAD protocol was developed for the first simultaneous quantification of TYL and TAT in wastewater matrices. The specific research contents are as follows: (1) The chromatographic separation conditions and pretreatment process were optimized. The results showed that the following conditions yielded the best pretreatment effect for wastewater: using 30% zinc sulfate and 20% potassium ferrocyanide as protein precipitants, extracting the target compounds using an HLB solid-phase extraction column at pH=3, and using methanol and a 5% methanol solution as the eluent and washing solution, respectively. Chromatographic conditions: Agilent 5 TC-C18(2) column, acetonitrile-0.1% formic acid gradient elution to screen TYL and TAT degrading bacteria, laying the foundation for the subsequent construction of biofilms and practical production applications. (2) From the activated sludge of a veterinary antibiotic pharmaceutical enterprise, six strains with degradation effects on both Tylosin (TYL) and Tylvalosin (TAT) were successfully screened. Among them, one strain achieved degradation rates of 78.41% for TYL and 85.04% for TAT. On the basis of 16S rRNA sequencing, physiological-biochemical profiling and morphological examination, the isolate was assigned to *Providencia vermicola* and designated strain CT1. (3) The conditions for the degradation of TAT and TYL by strain CT1 were systematically optimized. Under the optimal degradation conditions of 30°C, pH 6.0, and a bacterial solution volume of 50 mL, strain CT1 could degrade 90.73% of 200 mg/L TYL and 94.84% of 300 mg/L TAT within 48 hours. (4) In the study of the degradation mechanism of strain CT1, it was found that the degradation rates of TYL and TAT under the action of extracellular enzymes reached 47.27% and 55.27%, respectively, while the degradation rates of intracellular enzymes were only 9.77% and 7.88%. This result indicates that the degradation of TYL and TAT by strain CT1 mainly relies on the catalytic action of its secreted extracellular enzymes. (5) GC-MS profiling identified nine TAT metabolites generated by CT1—among them 3-methylbutanoic acid, ethyl acetate and 3-iminopentanoic acid-2-1—allowing five plausible degradation routes to be proposed. (6) The degradation effect of strain CT1 on actual wastewater was studied. The results showed that within 60 hours, strain CT1 could efficiently degrade multiple pollutants simultaneously. Specifically, the removal rates for TYL and TAT reached 93.7% and 95.3%, respectively, and the removal rate for NH_3-N reached 97.8%. COD and BOD_5 levels dropped from 10 992 mg L^{-1} and 2 904 mg L^{-1} to 781 mg L^{-1} and 561 mg L^{-1}, respectively, raising the BOD_5/COD ratio from 0.26 to 0.72 and markedly enhancing wastewater biodegradability.

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