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Optimization of degradation conditions for sulfachlorpyridazine by *Bacillus sp.* DLY-11 and analysis of biodegradation mechanisms

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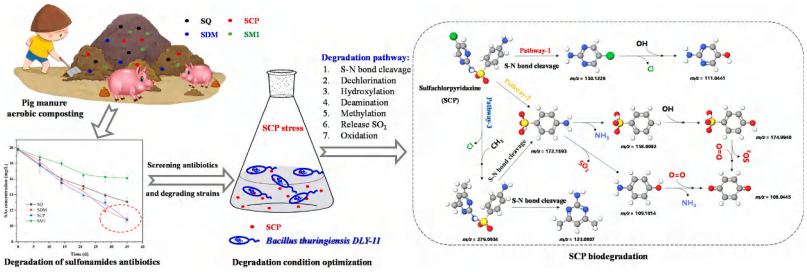
HIGHLIGHTS

- Highly efficient SCP-degrading strains screened from pig manure compost.
- Under optimal conditions, the DLY-11 strain degraded 97.7 % of SCP within 48 h.
- Three potential degradation pathways for SCP were proposed.
- S-N bond cleavage, dechlorination, and hydroxylation are common degradation pathways.

GRAPHICAL ABSTRACT



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ABSTRACT

Sulfachloropyridazine (SCP) is a common sulfonamide antibiotic pollutant found in animal excreta. Finding highly efficient degrading bacterial strains is an important measure to reduce SCP antibiotic pollution. Although some strains with degradation capabilities have been screened, the degradation pathways and biotransformation mechanisms of SCP during bacterial growth are still unclear. In this study, a strain capable of efficiently degrading SCP, named *Bacillus sp. DLY-11*, was isolated from pig manure aerobic compost. Under optimized conditions (5 % Vaccination dose, 51.5 ℃ reaction temperature, pH=7.92 and 0.5 g/L $MgSO_4$), this strain was able to degrade 97.7 % of 20 mg/L SCP within 48 h. Through the analysis of nine possible degradation products (including a new product of 1,4-benzoquinone with increased toxicity), three potential biodegradation pathways were proposed. The biodegradation reactions include S-N bond cleavage, dechlorination, hydroxylation, deamination, methylation, sulfur dioxide release, and oxidation reactions. This discovery not only provides a new efficient SCP-degrading bacterial strain but also expands our understanding of the mechanisms of bacterial degradation of SCP, filling a knowledge gap. It offers important reference for the bioremediation of antibiotic pollutants in livestock and poultry farming.

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1. Introduction

In recent years, the excessive use of antibiotics in livestock, poultry, and aquaculture has led to the emergence of antibiotic-resistant bacteria in the environment and drug residues in food, raising widespread concern [1-3]. China is one of the world's major consumers of veterinary antibiotics. Studies have shown that the total emissions of 80 veterinary antibiotics in China ranged from 23,110 to 40,850 tons per year from 2010 to 2020 [4]. Due to the low metabolic rate of veterinary antibiotics (<10 %), most antibiotics (about 30 %−90 %) are not fully absorbed and metabolized by animals during use [5,6], but are excreted into the environment, posing a long-term threat to human health and ecological balance [7,8]. Sulfonamide antibiotics, being one of the most widely used veterinary drugs, degrade slowly and have long residual times in the environment [9,10], which have been detected in various environmental contexts, including river water [11], domestic sewage[12], groundwater [13], and soil [14]. Their residues not only disrupt natural microbial structures and spread antibiotic resistance genes [15], but also enter the human body through the food chain, potentially inducing aplastic anemia and posing carcinogenic and genotoxic risks, seriously affecting human life [16]. Therefore, exploring effective measures to degrade sulfonamide antibiotics has become a hot topic in environmental research.

Currently, researchers at home and abroad have studied various degradation technologies for sulfonamide antibiotics, including chemical oxidation [17,18], adsorption [19,20], and biological methods [21]. Among these, biological methods have become a research focus for degrading sulfonamide antibiotics due to their strong adaptability, short cycle, low cost, and environmental friendliness [17,22]. Biological degradation technologies mainly include natural treatment, anaerobic treatment, aerobic treatment, and combined treatment [23,24]. Generally, the degradation efficiency of antibiotics follows the order of composting > anaerobic digestion > manure storage > soil [25]. Studies have shown that sulfamethazine (SMZ) and sulfamethoxazole (SMX) are primarily removed through heterotrophic biodegradation during aerobic activated sludge treatment, with degradation efficiencies of 46.0 % and 19.3 %, respectively [26]. Chen et al. [27] used vertical flow constructed wetlands to degrade sulfonamide antibiotics and found that sulfadiazine (SDZ), SMX, and SMZ had high degradation efficiencies (range from 26.4 % to 84.1 %), with microbial degradation being the main pathway for sulfonamide antibiotic removal. Zhang et al. [28] found that 64.7 % of detected antibiotics were removed during the composting of animal manure, showing significant results. However, whether using activated sludge, constructed wetlands, or composting, the degradation of sulfonamide antibiotics mainly relies on microbial degradation pathways, requiring the addition or inherent presence of degrading bacteria to improve the degradation efficiency of sulfonamide antibiotics. Therefore, finding highly efficient degrading bacterial strains is the fundamental solution to sulfonamide antibiotic pollution.

Based on research progress on microbial degradation of sulfonamide antibiotics, it has been found that multiple degradation pathways exist in the degradation process of sulfonamide antibiotics, and the type of degrading bacteria directly affects these pathways. Chen et al. [29] isolated four sulfonamide antibiotic-degrading bacteria from wetland sludge: *Paenarthrobacter*、*Achromobacter*、*Pseudomonas* and *Methylobacterium*, and found that the *SadABC* gene confers strong sulfonamide antibiotic degradation capability to *Paenarthrobacter*. Liu et al. [30] used an anaerobic membrane bioreactor to remove sulfonamide antibiotics from piggery wastewater and found that the degrading bacteria of the genera *Lentimicrobium* and *unidentified_W27* were positively correlated with sulfonamide antibiotics, with removal rates ranging from 32.0 % to 62.0 %. Although researchers at home and abroad have screened strains with sulfonamide antibiotic degradation efficacy from sludge and biofilm reactors, their degradation capabilities vary and have not been practically applied, and their broad-spectrum and stability remain questionable. Moreover, the degradation by-products and pathways of sulfonamide antibiotics are not fully understood. Screening for highly efficient sulfonamide antibiotic-degrading strains in the composting of livestock and poultry waste has not been reported domestically or internationally. Therefore, understanding the metabolic pathways and biotransformation mechanisms of highly efficient degrading bacteria for sulfonamide antibiotics is of significant theoretical importance for understanding their degradation and transformation processes.

Sulfachlorpyridazine is one of the most commonly used sulfonamide antibiotics in livestock and poultry farming, and is mainly used in veterinary medicine to treat infections caused by susceptible bacteria, especially in poultry and pig. Like other sulfonamide drugs, it works by inhibiting the production of folic acid in bacteria, which is crucial for bacterial growth and survival [31,32]. In recent years, researchers have made significant progress in the study of SCP biodegradation. For instance, Yin et al. [33]investigated the degradation capabilities of representative microorganisms (*P. saccharofermentans*, *T. glycolicus*, *C. tyrobutyricum*, *B. caccae*, *P. stercorisuis*, and *M. barkeri*) during anaerobic digestion process, revealing substantial differences in degradation efficiency among different strains. Throughout their growth cycles, these strains exhibited SCP degradation efficiency of 80.9 %, 43.5 %, 56.7 %, 70.8 %, 51.0 %, and 21.4 %, respectively. Additionally, Qi et al. [34] isolated the *Paenarthrobacter sp.* P27 strain from an enrichment culture using SMX as the sole carbon source, which demonstrated significant SCP degradation capacity. However, the commonly used single-bacterium remediation techniques for SCP degradation also face several limitations, such as limited degradation capacity, difficulty in competing with existing microbial communities, and potential production of harmful intermediate metabolites. To overcome these limitations, this study aims to optimize the degradation conditions of a single degrading bacterial strain to enhance its degradation capacity, improve overall remediation efficiency, reduce costs, and minimize the risk of secondary pollution. Regarding the mechanisms of organic pollutant biodegradation, Li et al. [35] and Han et al. [36] elucidated potential degradation pathways of organic pollutants by degrading bacteria using intermediate product analysis and multi-omics approaches. These pathways primarily involve key steps such as hydroxylation, decarboxylation, and ring-opening. These studies have provided valuable insights into understanding the biodegradation processes of complex organic compounds. Despite these important findings, critical research gaps remain. In particular, there is a relative lack of studies on isolating and screening highly efficient SCP-degrading strains from practical production environments, such as aerobic composting of pig manure. Furthermore, a deeper understanding of SCP degradation pathways and biotransformation mechanisms is still needed. Based on these considerations, this study aims to screen and identify novel strains with SCP degradation capabilities from aerobic pig manure compost. We successfully isolated a strain of *Bacillus sp.* DLY-11 and conducted in-depth research on its SCP degradation kinetics and characteristics under various environmental factors. We also optimized the degradation conditions for this strain using response surface methodology. By analyzing intermediate and final products during the degradation process, we preliminarily elucidated the degradation mechanism of SCP by this strain. This study not only provides a new strain for efficient SCP degradation but also reveals the biodegradation pathway of SCP, offering a new perspective on the mechanism of bacterial SCP degradation. These findings provide an important theoretical foundation and practical guidance for future bioremediation of antibiotic pollutants in livestock and poultry farming.

2. Materials and methods

2.1. Experimental materials and chemical reagents

Pig manure was collected from a large piggery in the suburbs of Guangzhou, and wood chips were sourced from furniture processing waste at a factory in Dongguan. Their basic properties are shown in

Table 1
Physical and chemical properties of composting materials.

Material	Total C (%)	Total N (%)	C/N	Moisture content (%)
Pig manure	15.32	0.93	16.47	53.6
Wood chips	50.75	0.84	60.41	4.6

Table 1. The required standard for SCP (>99 %)was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (hereinafter referred to as "Macklin Reagent"). Other chemicals used in the basal medium, such as $CaCl_2$, KH_2PO_4, $MgSO_4$ and $FeSO_4$, were also sourced from Macklin China. Methanol (chromatographic grade), formic acid (98 %), and LiChrolut®EN solid-phase extraction columns were supplied by Merck (Darmstadt, Germany). Milli-Q water (18.2 MΩ.cm) was produced by a Millipore purification system (Billerica, CA, USA).

2.2. Degradation effects of sulfonamide antibiotics during aerobic composting

This experiment used a homemade aerobic composting device (Fig. (B.1)), with device parameters detailed in Appendix D. The experiment was set up with one treatment and three replicates. According to the content of four sulfonamide antibiotics in pig manure (sulfamonomethoxine (SM1), SCP, sulfamethazine (SDM), and sulfaquinoxaline (SQ)), each antibiotic was added exogenously to reach the designed concentration (20 mg/kg). Wood chips were soaked in clean water for 180 min before being mixed with pig manure, adjusting the moisture content to 65.0 %. The total weight of the composting material was 50 kg, with a C/N ratio of 25. Static forced aeration composting was adopted, with intermittent air supply from the bottom at a ventilation rate of 0.050 $m^3/(min{\cdot}m^3)$, blowing for 30 min every five hours. Samples were taken every seven days during composting, with mixed samples taken before composting, in the middle, and at maturity. The contents of SM1, SCP, SDM, and SQ were measured. By comparing the changes in antibiotic content before and after composting, the removal effects were calculated, and the sulfonamide antibiotic with the best degradation effect was selected for subsequent research.

2.3. Isolation and identification of SCP-degrading bacteria

From the results of the pig manure aerobic composting experiment, the best degradation effect was observed for SCP. Therefore, SCP was selected as the research object, and matured compost material was used to screen SCP-degrading bacterial strains. Through enrichment culture, preliminary screening and isolation, acclimatization, and rescreening, highly active dominant strains with strong SCP degradation ability were selected, with detailed steps Appendix E. The selected strains were inoculated onto selective media and incubated at a constant temperature of 30 °C for two days. Gram staining was performed, and bacterial morphology was observed using a scanning electron microscope. According to the "Bergey's Manual of Determinative Bacteriology" [37] and "Manual of Systematic Identification of Common Bacteria"[38], physiological and biochemical experiments were conducted on the selected SCP-degrading strains. The 16S rRNA sequence and genetic analysis of the selected strains were performed to determine specific genetic information. Detailed analysis processes and parameters are shown in Appendix G.

2.4. Biodegradation kinetics of SCP

The selected strain DLY-11 was inoculated into LB medium, incubated in a constant temperature shaker at 30 ℃ and 160 rpm for 12 h to prepare seed liquid, which was then added as needed. The growth curve of the strain was determined at different time points to further understand its growth characteristics and optimize culture conditions. Strain DLY-11 was inoculated into basal medium containing 20 mg/L SCP and pH= 7 at an inoculum amount of 3 %, and incubated at 30 ℃, 150 r/min. Samples were taken at 0 h, 1 h, 3 h, 6 h, 10 h, 16 h, 24 h, and 48 h to measure SCP concentration and cell density (OD_{600}). The nonlinear regression analysis was modelled by first-order kinetics, and the detailed kinetics analysis was according to Terzic et al. [39].

2.5. Factors influencing SCP degradation

Based on the SCP removal kinetics results of strain DLY-11, the following experiments were designed for 48-hour culture. Five single-factor influence experiments were designed in 500 mL flasks containing 200 mL of basal medium (autoclaved at 121 °C for 30 min). Biological degradation experiments were conducted by adjusting temperature (10 ℃, 20 ℃, 30 ℃, 40 ℃), pH(6, 7, 8, 9), inoculum amount (1 %, 2 %, 3 %, 4 %, 5 %), types of metal salts ($MnSO_4$、$MgSO_4$、$FeSO_4$、$CaSO_4$), and metal concentrations (0.1 g/L, 0.2 g/L, 0.3 g/L, 0.4 g/L, 0.5 g/L). The initial SCP concentration was 20 mg/L, and the bacterial solution was added according to the corresponding inoculum amount. The flask was sealed with a rubber stopper and breathable sterile sealing film to ensure no impurities were mixed during the culture. The culture was carried out at 30 ℃ and 160 r/min for 48 h, and the residual SCP concentration was detected after the culture. Before detection, the supernatant was filtered through a 0.22 μm pore-size filter. All experiments were repeated three times.

2.6. Optimization of SCP degradation conditions

This study used response surface methodology (RSM) to design experiments for optimizing the SCP degradation conditions of the optimal strain DLY-11. Temperature (X1), pH (X2), and metal ion concentration (X3) were used as operating parameters, with an initial SCP concentration of 20 mg/L and an inoculum amount of 5 %, in 500 mL Mineral Salts Medium (MSM) observed in a shaking incubator at 120 rpm for 48 h. The detailed design process and parameters of the Box-Behnken are shown in Appendix H. The experimental setup is shown in Table A.3. Response surface analysis and data processing were performed using Design-Expert 13 software. ANOVA was employed to evaluate the significance of the model, while R^2 and R^2adj were used to assess the model fit. A quadratic polynomial regression equation was established to describe the mathematical relationship between the dependent and independent variables. The significance of each factor and their interactions was determined by examining the p-values.

2.7. Degradation mechanism

The degradation bacteria, which have been cultured in ideal conditions, were inoculated into a liquid medium containing SCP and cultured at 30 ℃ for three days. After cultivation, the samples were centrifuged, and the supernatant was analyzed for SCP degradation products using a liquid chromatograph (LC-20AD, Shimadzu, Japan) coupled with a high-resolution hybrid quadrupole time-of-flight mass spectrometer (AB SCIEX X500R Q-TOF, Agilent, USA). Based on the detected degradation products, infer the SCP degradation pathway and elucidate the degradation mechanism of the efficient degradation bacteria. The calculation formula for SCP degradation rate is shown in Eq. 1.

$$\text{Removal rate}(\%) = 100\% \times (C_0\text{-}C_t)/C_0 \quad (1)$$

where C_0 (mg/L) is the initial concentration of SCP, and C_t (mg/L)is the concentration of SCP in the medium at time t.

2.8. Sample pretreatment and instrumental analysis methods

The SCP content in the samples was analyzed using high-performance liquid chromatography (HPLC, Alliance e2695, USA), with sulfachloropyridazine-d_4 as the internal standard. Before analysis,

water samples were filtered through a Millipore filter membrane with a diameter of 11.7 mm and a pore size of 0.22 μm to separate suspended particles. SCP metabolites were detected using a liquid chromatograph (LC-20AD, Shimadzu, Japan) coupled with a high-resolution hybrid quadrupole time-of-flight mass spectrometer (AB SCIEX X500R Q-TOF, Agilent, USA). All samples were stored in the dark at 4 ℃ and subjected to solid-phase extraction (SPE). Before injection, HLB solid-phase extraction columns (6 mL, 200 mg, Waters, USA) were pre-treated with 5 mL methanol and deionized water. The samples were then passed through the HLB column at a flow rate of 3 mL/min, washed with 10 mL water, and dried under gentle airflow. The dried columns were eluted with 5 mL methanol, and the extract was obtained. The extract was evaporated under a gentle stream of nitrogen at 40 °C, subsequently reconstituted in 1 mL of methanol through vortex mixing, and then transferred to liquid chromatography vials for analysis. The content of SCP in the sample was determined using high-performance liquid chromatography (HPLC, Alliance e2695, USA), while the detection of SCP metabolites was conducted with a liquid chromatograph (LC-20AD, Shimadzu, Japan) coupled with a high-resolution hybrid quadrupole time-of-flight mass spectrometer (AB SCIEX X500R Q-TOF, Agilent, USA). Detailed analytical methods and instrument parameters are presented in Appendix I.

3. Results and discussion

3.1. Degradation effects of sulfonamide antibiotics during aerobic composting

As shown in Fig. 1(A), the residual concentrations of the four antibiotics decreased with composting time, with the degradation effect as follows: SCP≈SDM>SQ>SM1, where the degradation rate of SCP reached 45.8 %. During the experiment, monitoring the residual concentrations of antibiotics at different time points revealed that SCP and SDM exhibited relatively better degradation effects, showing faster degradation efficiency and higher degradation degrees. In contrast, SM1showed relatively weaker degradation effects. These results indicate that microbial strains can degrade sulfonamide antibiotics during composting, but there are differences in the degradation effects of different antibiotics. The best degradation effect was observed for SCP, possibly because SCP is more easily decomposed and metabolized by microorganisms in the composting environment. For SM1, its molecular structure or other characteristics may result in a slower degradation rate. Based on these findings, SCP was selected as the research object for further study.

3.2. Strain isolation and identification

Eight strains were isolated from matured compost material, most of which exhibited the ability to reduce SCP concentration in LB liquid medium. Among them, strain DLY-11 showed a strong ability to degrade SCP, so it was further studied. After repeated acclimatization, the SCP degradation rate of strain DLY-11 stabilized at about 90 %. The electron microscope images of the SCP-degrading strain are shown in Fig. 1(B). When cultured on solid MSM medium containing SCP, strain DLY-11 formed colonies. The bacterial cells were rod-shaped, opaque, with a raised center and smooth edges. The cells measured approximately 2–3 μm in length and 0.5–1 μm in diameter. No obvious appendages were visible. Typical endospore morphology could be observed.

Gram staining indicated that DLY-11 was a Gram-positive bacterium, rod-shaped, often arranged in chains, producing spores and associated crystal toxins. Based on the 16S rRNA gene sequence and phylogenetic tree (as shown in Fig. 1(C) and Fig. 1(D)), the 16S rRNA of DLY-11 was 1456 bp long, with a sequence homology of more than 99 % to bacteria of the Bacillus genus, clustering together with *Bacillus sp.* strain. Therefore, strain DLY-11 was identified as *Bacillus sp. Bacillus sp.* is a promising bacterium for degrading emerging pollutants [40]. Studies have shown that *Bacillus thuringiensis* B1(2015b).

Bacillus thuringiensis can degrade ibuprofen in the presence of phenol, benzoates, and 2-chlorophenol, achieving a degradation efficiency of more than 90 % [41].

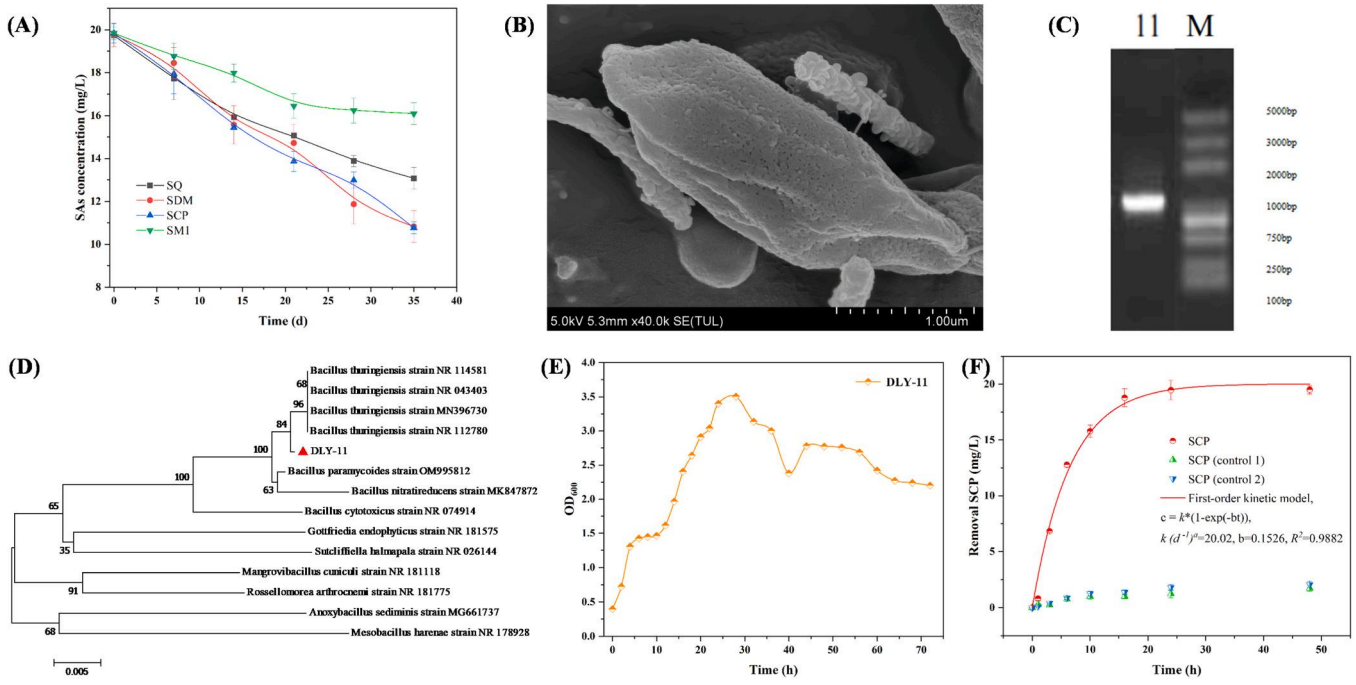


Fig. 1. (A) Residual concentration of SAs after composting. (B) SEM images of SCP-degrading bacterium DLY-11. (C) PCR verification results of 16SrRNA of strain DLY-11. (D) Phylogenetic tree of strain DLY-11. (Note: The phylogenetic tree illustrates the relationships among strains, including DLY-11 and its closely related species, based on the 16S rDNA gene.) (E) Growth curve of strain DLY-11. (F)The change in SCP degradation concentration over time was simulated with a first-order kinetic model, depicting smooth curves. (Note: Control 1: Unvaccinated, Control 2: Sterilized bacterial cells).

3.3. SCP biodegradation kinetics

From 0–2 h, there is a lag phase during which the bacteria adapt to the new environment, resulting in increased cell volume, active metabolism, and the initiation of cell proliferation. Concurrently, appropriate enzymes, energy, and intermediate metabolites accumulate. From 2–28 h, the bacteria enter the logarithmic phase, proliferating rapidly at a geometric rate, reaching an OD_{600} value of 3.5 at 28 h. From 28–32 h, a stationary phase occurs due to the gradual depletion of nutrients and the accumulation of toxic metabolic products, leading to a deceleration in the proliferation trend. After 36 h, the bacteria enter a decline phase, where the rate of cell autolysis and death exceeds the rate of reproduction. By studying the degradation kinetics of SCP by strain DLY-11, we determined that its biodegradation kinetics model aligns with a first-order kinetics model (see Fig. 1(F)). The correlation coefficient R^2= 0.9882, and the rate constant k = 20.02 mg/L·d^{-1} (see Fig. 1(F)). Related research indicates that the biodegradation kinetics of various organic pollutants by bacteria conform to the first-order decay model [42]. For example, Peng et al. [43] reported that the biodegradation kinetics of tetracycline at 1–50 mg/L by microbial communities fit the

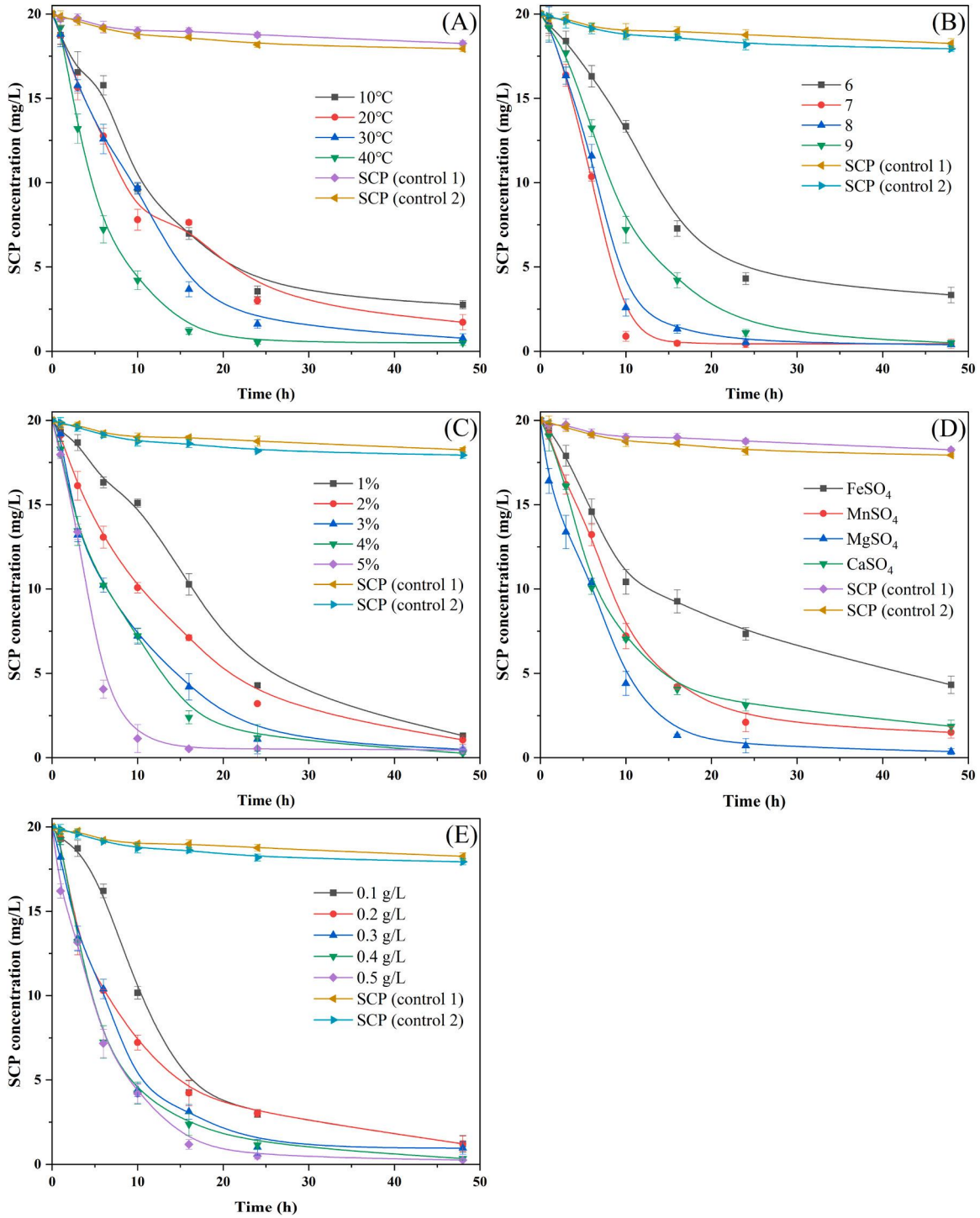


Fig. 2. The effect of different treatment conditions on the degradation of SCP by DLY-11. (A) Temperature. (B) pH value. (C) Inoculum size. (D) Metallic salt. (E) Metal ion concentration. Note: Control 1: Unvaccinated, Control 2: Sterilized bacterial cells.

first-order model, with a correlation coefficient $R^2 > 0.98$. The heavy metal-resistant bacterium *Novphingobium panipatense* P5:ABC can utilize aromatic hydrocarbons and crude oil as the sole carbon source for growth, and its biodegradation kinetics also conform to the first-order kinetics model [44].

3.4. Single-factor influence on SCP degradation by strain DLY-11

To optimize the SCP degradation efficiency of the DLY-11 strain, we investigated the impact of crucial environmental factors, including temperature, pH, inoculum size, metal salt types, and metal ion concentrations. As illustrated in Fig. 2(A), the degradation efficiency of SCP by strain DLY-11 increased with rising temperatures, reaching its peak at 40 ℃. This finding aligns with previous research suggesting that heat treatment effectively removes antibiotics in pig manure [45]. At temperatures between 10–20 ℃, DLY-11 required 48 h to achieve a 90 % degradation rate of SCP. In contrast, at 40 ℃, DLY-11 attained over 90 % SCP degradation within 24 h, with the degradation rate continuing to increase over time. This observation indicates that 40 ℃ likely approximates the optimal temperature for SCP degradation by DLY-11. Nevertheless, DLY-11 still demonstrated the ability to eliminate a significant portion of SCP at lower temperatures (10–20 ℃), albeit potentially requiring an extended period to achieve complete removal.

pH is a critical factor influencing microbial growth and metabolism, not only directly affecting bacterial proliferation but also impacting enzyme activity within their decomposition metabolic systems [46]. Consequently, we investigated the effect of pH on the efficiency of SCP degradation by the DLY-11 strain. As illustrated in Fig. 2(B), when the pH values were 7 and 8, DLY-11 was able to elevate the removal rate of SCP to 97.7 % and 93.4 %, respectively, within 16 h. In contrast, at pH 6 and 9, the degradation efficiency of SCP were merely 63.6 % and 79.0 %. Overall, as the pH value increased, the removal rate exhibited a trend of initial increase followed by a subsequent decrease. These findings indicate that neutral and slightly alkaline environments are more conducive to SCP degradation by DLY-11. This discovery aligns with other studies, such as Shewanella bacteria demonstrating the highest SMX transformation efficiency at pH 7.0 and 8.0 [47]. Excessively acidic or alkaline conditions were found to be unfavorable for the biodegradation of SCP by DLY-11, possibly due to the inhibition of DLY-11 growth under extreme pH values, thereby reducing the production of its degradative enzymes [48]. In conclusion, we determined that a pH range of 7–8 is optimal for SCP degradation by DLY-11. This discovery provides valuable insight for optimizing the degradation efficiency of DLY-11 in practical applications. Our findings also corroborate with the research of Wang and Wang [49], who observed that *Acinetobacter sp*.exhibited higher degradation efficiency of SMX under neutral conditions compared to acidic environments.

Inoculation amount is a crucial metric in practical applications [50], directly affecting the growth rate and metabolic efficiency of microorganisms. As shown in Fig. 2(C), during the 24-hour degradation process, SCP exhibited significant degradation effects when the inoculation amount was between 3 % and 5 %. Specifically, when the inoculation amount reached 5 %, the removal rate of SCP within 16 h reached 97.4 %, almost achieving complete degradation. These results indicate that a higher inoculation amount enables strain DLY-11 to achieve a stable growth state more quickly. This might be because a larger initial bacterial population results in the production of more degradative enzymes within the cells, thus accelerating the SCP degradation process. This finding is consistent with previous studies that suggest appropriately increasing the inoculation amount helps shorten the lag phase and promotes the microorganisms' rapid entry into the logarithmic growth phase [51]. In summary, we found that a 5 % inoculation amount was most effective for DLY-11 in degrading SCP, providing an important reference for optimizing treatment efficiency in practical applications. Our findings have also been validated in other studies. For instance, Yan et al. [52] found that the biodegradation efficiency of sulfonamide sulfamethoxazole by *Proteus mirabilis sp*. ZXY4 increased with the inoculation amount, further confirming the important role of inoculation amount in the microbial degradation process.

To explore how to further enhance the SCP degradation efficiency of strain DLY-11, we studied the effects of different metal ions on the degradation process. This is based on the theory that metal ions complexing with antibiotics may accelerate antibiotic oxidation [53]. As shown in Fig. 2(D), all four tested metal ions promoted SCP degradation to some extent, but with varying effectiveness. Adding $MgSO_4$ to the medium significantly promoted the degradation of SCP by strain DLY-11, with a removal rate of 96.4 % within 24 h. This may be because Mg^{2+} ions, as essential cofactors for many enzymes, play a crucial role in microbial metabolism [54]. Additionally, $MnSO_4$, another inorganic salt metal ion, also exhibited significant promoting effects, achieving an SCP degradation rate of 95.0 %. This is consistent with the findings of Huang et al. [55], who found that Mn^{2+} ions play an important role in the metabolism of many microorganisms, promoting the degradation of organic matter. Although $FeSO_4$ also promoted SCP degradation, its effect was slightly weaker compared to Mg^{2+} and Mn^{2+}. This could be due to the different mechanisms of action of Fe^{2+} in microorganisms, or its effect might be influenced by other factors such as microbial species and environmental conditions. Despite Fe^{2+} being an essential trace element for many organisms, its specific role in the degradation of SCP by DLY-11 requires further investigation.

Based on the aforementioned experimental results, we determined that $MgSO_4$ had the best effect on SCP degradation by strain DLY-11. To optimize the use of $MgSO_4$, we further explored the effect of different concentrations of $MgSO_4$ on SCP degradation efficiency by DLY-11 (Fig. 2(E)). As depicted in Fig. 2(E), an augmentation in the concentration of $MgSO_4$ within the gamut of 0.1–0.3 g/L was inextricably linked to a conspicuous escalation in the efficacy of SCP degradation, surging from 84.9 % to 94.9 % over the course of 24 h. However, when the $MgSO_4$ concentration increased from 0.3 g/L to 0.5 g/L, the increase in SCP degradation efficiency was not substantial, only rising from 94.9 % to 97.6 %. This suggests that the promoting effect of $MgSO_4$ concentration on SCP degradation efficiency might reach a saturation point. This phenomenon might be because, within the lower concentration range, Mg^{2+} as enzyme cofactors can significantly enhance the activity of related degradative enzymes. However, when the Mg^{2+} concentration reaches a certain level, the enzyme activity may have already reached its maximum, so further increasing the Mg^{2+} concentration does not bring significant efficiency improvements.

3.5. Optimization of degradation conditions for SCP-degrading strains

3.5.1. Optimization of degradation conditions

The results obtained from the SCP biodegradation experiments conducted with the combined factor levels for each experimental group are presented in Table A.3, and the ANOVA analysis of the model is shown in Table 2. SCP degradation is a dependent variable. The mathematical relationship between the responses of the three variables was evaluated using a quadratic polynomial equation (Eq. (2)).

$$Y_1 = -90{\cdot}18 + 1{\cdot}97A + 33{\cdot}38B + 13{\cdot}05\ C - 0{\cdot}0240AB - 0{\cdot}1485AC - 0{\cdot}6000BC - 0{\cdot}0165A^2 - 2{\cdot}01B^2 + 4{\cdot}55\ C^2 \quad (2)$$

In the formula, Y_1 represents the response for SCP degradation. The coded factor equation can be used to predict responses for given factor levels. By default, high levels of factors are coded as + 1, and low levels as −1. The coded equation facilitates the determination of the relative impact of factors by comparing factor coefficients.

As evident from Table 2, the overall model's F-value of 20.57 and p-value of 0.0003 < 0.01 (model p-value < 0.05 is considered significant) indicate that the model is statistically significant, suggesting that the experimental model effectively explains the variation in the response variable. The R^2 (goodness of fit) value of 0.9636 , and R^2adj (adjusted

Table 2
Analysis of variance for SCP degradation.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	35.85	9	3.98	20.57	0.0003	significant
A-X1	3.26	1	3.26	16.83	0.0046	
B-X2	0.4513	1	0.4513	2.33	0.0171	
C-X3	1.59	1	1.59	8.21	0.0242	
AB	0.2304	1	0.2304	1.19	0.3115	
AC	0.0882	1	0.0882	0.4555	0.5214	
BC	0.0144	1	0.0144	0.0744	0.7930	
A^2	11.53	1	11.53	59.52	0.0001	
B^2	17.03	1	17.03	87.93	< 0.0001	
C^2	0.0087	1	0.0087	0.0449	0.8382	
Residual	1.36	7	0.1937			
Lack of Fit	0.8258	3	0.2753	2.08	0.2458	not significant
Pure Error	0.5298	4	0.1324			
Cor Total	37.21	16				

$R^2 = 0.9636$; $R^2adj = 0.9567$

goodness of fit) of 0.9567 further corroborate the model's reliability. Among the various factors, factors A (X1), B (X2), and C (X3) have significant impacts on the response variable, being key factors. Their p-values are all less than 0.05, statistically significant. This indicates that factors A (X1), B (X2), and C (X3) play important roles in the variation of the response variable. Additionally, the interaction effects of AB, AC, and BC are not statistically significant, with p-values greater than the significance level of 0.05. Further analysis shows that the model performs well in explaining the total variance. The p-value of the lack-of-fit test is 0.2458, indicating that the difference between the model's residuals and the model variance is not significant. This means that the experimental model may have certain errors in predicting the response variable in specific regions, but overall, the model fits well.

3.5.2. Response surface analysis of SCP

The response surface graphs depicting the influence of the interaction term AB on the response value (Fig. 3(A) and (B)) show that when the $MgSO_4$ concentration is fixed at 0.4 g/L, pH and temperature have substantial effects on the results, though their interaction is not significant. Under constant temperature conditions, as pH increases, the SCP degradation rate first increases and then decreases, with the surface graph exhibiting a general shape of low edges and high center, and the curve's center point representing the highest point of the response surface. In Fig. 3(C) and (D), the surface graph of the AC interaction term generally assumes a half-saddle shape. At a pH of 8, with a fixed $MgSO_4$ concentration, increasing experimental temperature levels initially positively influences the response value, followed by an inhibition of the degradation process. When temperature is constant, $MgSO_4$ concentration in the range of 0.3–0.5 g/L has minimal impact on the response value. In Fig. 3(E) and (F), the influence of the BC interaction term on the response value is similar to that of the AC interaction, primarily reflecting the significant impact of pH on SCP biodegradation. According to the optimal solution provided by the steepness graph shown in Fig. (B.2), the optimal conditions are: temperature 51.5 ℃, pH 7.92, and $MgSO_4$ concentration 0.5 g/L, predicting a maximum SCP degradation rate of 97.5 %. A validation experiment conducted under these conditions yielded an actual degradation rate of 97.7 %. The minimal discrepancy between the experimental and predicted values confirms the model's reliability. Multiple studies have also demonstrated that *Bacillus sp.* exhibits significant degradation capabilities for antibiotics. The strain *Bacillus sp.* GIMCC1.817, isolated by Zhou et al. [56], effectively removed 77 % of 1 μM erythromycin within 24 h, with 53 % being completely degraded. Wen et al. [57] transformed the *Bacillus sp.* strain H38 into immobilized bacteria and found that it could achieve a removal rate of 98 % for sulfamethazine (SM2) within 36 h. In comparison, our isolated strain, *Bacillus sp.* DLY-11, effectively removed 97.7 % of 20 mg/L SCP within 48 h, similarly demonstrating excellent antibiotic degradation capabilities. These research findings collectively

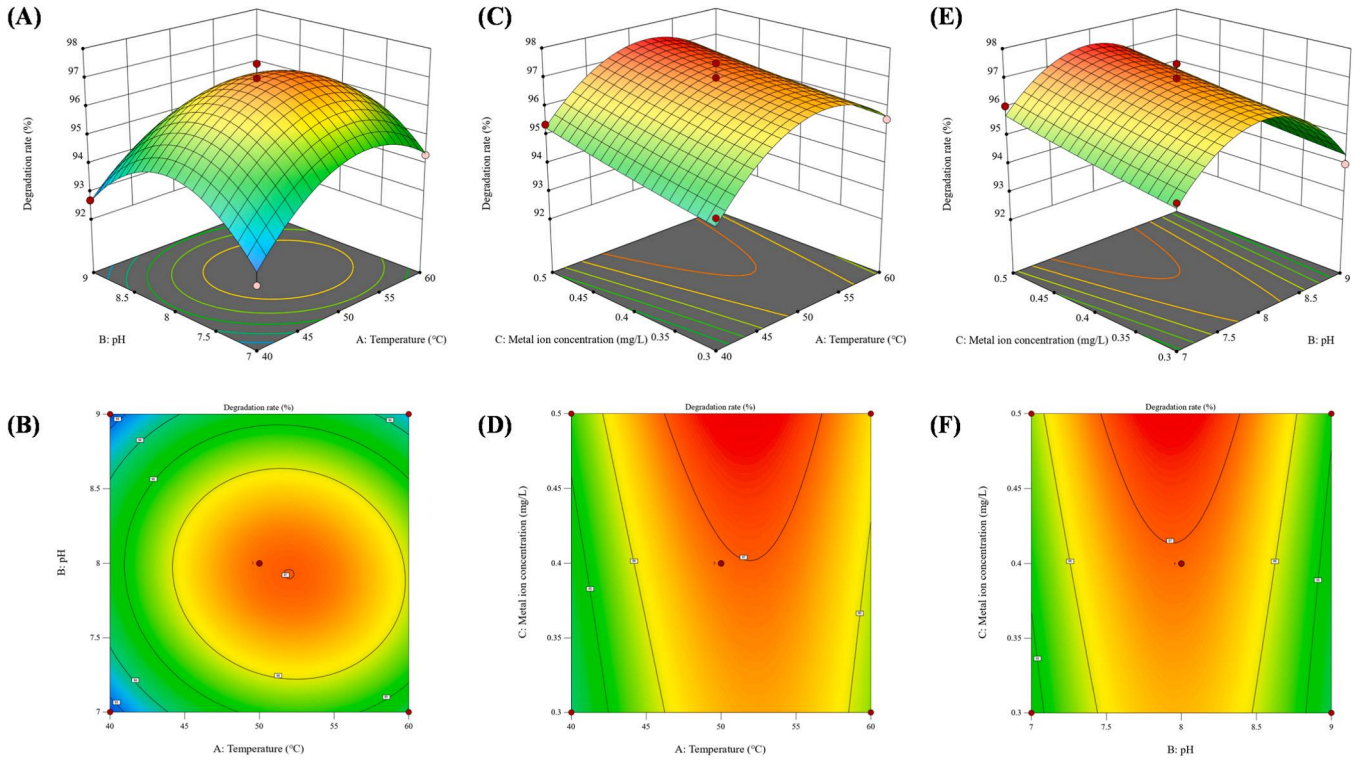


Fig. 3. Response surface and contour plots of SCP degradation rates. Note: Surface plot (A) and contour plot (B) of SCP degradation rate versus temperature (X1) and pH (X2); Surface plot (C) and contour plot (D) of SCP degradation rate in relation to temperature (X1) and metal ion concentration (X3); Surface plot (E) and contour plot (F) of SCP degradation rate versus pH (X2) and metal ion concentration (X3).

confirm the immense potential of *Bacillus thuringiensis* in antibiotic degradation. Despite the low solubility of SCP in water (0.14 g/L), which might limit its bioavailability, *Bacillus sp.* DLY-11 still demonstrated efficient degradation capabilities, suggesting that this strain may possess special absorption mechanisms. The optimized pH value of 7.92 that we selected is close to neutral and higher than the *pKa* value of SCP (5.5)[58], allowing SCP to exist primarily in its ionic form, which may facilitate its interaction with the bacterial cell surface. This neutral to slightly alkaline environment may also activate certain degradation enzymes in *Bacillus sp.* DLY-11, further promoting SCP degradation. The multiple degradation pathways we observed (such as S-N bond cleavage, dechlorination, etc.) demonstrate the strain's powerful degradation capability to overcome the relative stability of SCP. Notably, the DLY-11 strain maintains good degradation activity over a wide range of pH and temperature, exhibiting strong environmental adaptability. As a Bacillus species, its ability to produce spores enhances its survival in adverse environments, which is crucial for practical applications. Given the efficient SCP degradation capability and excellent environmental adaptability of *Bacillus sp.* DLY-11, we propose several potential application scenarios: a) Preparation of single or composite bacterial agents for aerobic composting treatment of livestock and poultry manure. b) Treatment of wastewater from farms and slaughterhouses containing SCP residues. c) Processing of industrial and medical wastewater generated during SCP production. d) Remediation of agricultural soils and soils surrounding farms contaminated with SCP. In general, our optimized conditions strike a good balance between the physicochemical properties of SCP and the physiological requirements of *Bacillus sp.* DLY-11, achieving efficient degradation and providing a powerful tool for SCP contamination remediation.

3.6. Possible degradation pathways of SCP by strain DLY-11

As illustrated in Fig. 4 and Table 3, the characteristic peaks of compounds with different molecular weights are distinctly visible, representing the products generated from the degradation of SCP by strain DLY-11. Based on the retention time and mass-to-charge ratio of the fragment ions, nine intermediate products of SCP biodegradation were detected, with *m/z* values of 279.0934、174.9940、172.1693、158.0082、130.1228、123.0807、111.0441、109.1014和108.0445. Based on the biodegradable properties of SCP and the detected intermediates, this study proposes three possible degradation pathways (as illustrated in Fig. 5).

Pathway 1: 2-Amino-5-chloropyrimidine ($m/z = 130.1228$) is generated through the cleavage of the S-N bond in SCP, a process frequently observed during the microbial metabolism of SCP [59], and is a common pathway for SCP biodegradation [60]. Subsequently, 2-amino-5-chloropyrimidine undergoes hydrolytic dechlorination to form 2-amino-5-hydroxypyrimidine ($m/z = 111.0441$). During the biodegradation of SCP, the disappearance of the chlorine atom is particularly notable, indicating that dechlorination is a critical step in its degradation [61]. This process may occur through hydrolysis, reduction, or enzymatic reactions, resulting in dechlorinated products with reduced toxicity and environmental risks. Studies indicate that during aerobic biodegradation, compounds containing conjugated π bonds and high electron cloud density are prone to hydroxylation reactions [24]. The hydroxylation reaction results in the substitution of chlorine atoms on the pyrimidine ring with hydroxyl groups.

Pathway 2: Similarly begins with the S-N bond cleavage of SCP, producing p-aminobenzenesulfonic acid ($m/z = 172.1693$). The degradation of p-aminobenzenesulfonic acidproceeds in two directions: (i) Initially, deamination yields benzenesulfonic acid ($m/z = 158.0082$), which undergoes hydroxylation to form p-hydroxybenzenesulfonic acid ($m/z = 174.9940$), and finally, oxidation and SO_2 release produce 1,4-benzoquinone ($m/z = 108.0455$). (ii) Alternatively, SO_2 release first results in para-aminophenol ($m/z = 109.1014$), which then forms 1,4-benzoquinone ($m/z = 108.0455$) directly through deamination and oxidation. 1,4-benzoquinone is a newly discovered product, not previously reported in earlier studies [62]。.

Pathway 3: The dechlorination and methylation of the chlorine atom on the pyrimidine ring in SCP result in the emergence of 2-(para-aminobenzenesulfonamide)−4,6-dimethylpyrimidine ($m/z = 279.0934$) [63]. This compound undergoes S-N bond cleavage to form 2-amino-4,

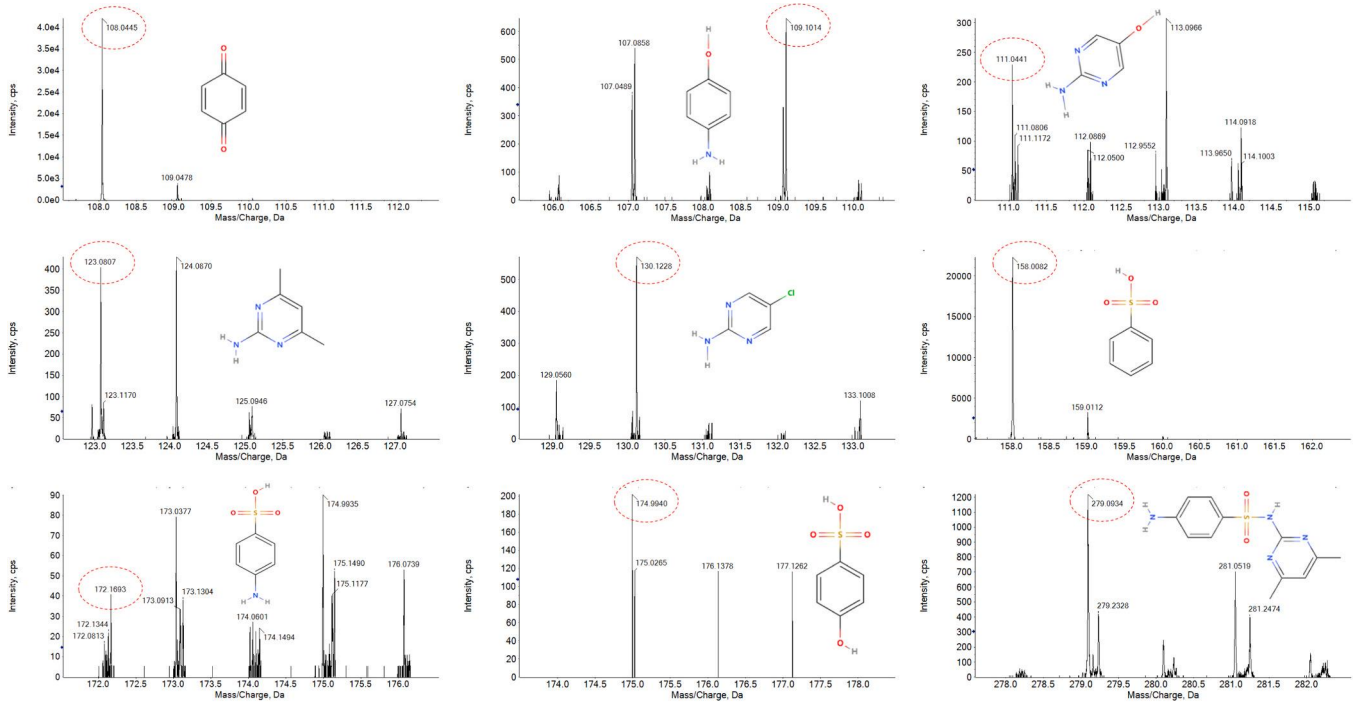
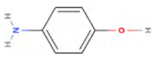
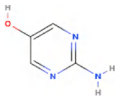
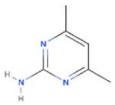
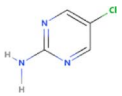
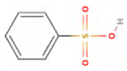
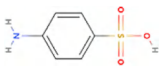
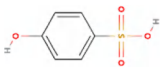
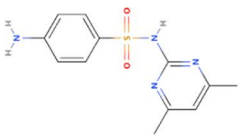


Fig. 4. Mass spectrometry of SCP biotransformation products.

Table 3
Biodegradation products of SCP.

No.	Molecular formula	*m/z*	Mass Error (ppm)	Retention time (min)	Structure	Name
1	$C_6O_2H_4$	108.0445	−4.1	14.929		1,4-benzoquinone (PBQ)
2	C_6H_7ON	109.1014	−4.5	14.932		Para-aminophenol
3	$C_4N_3H_5O$	111.0441	7.5	6.464		2-amino−5-hydroxypyrimidine
4	$C_6N_3H_9$	123.0807	5.5	33.864		2-amino−4,6-dimethylpyrimidine
5	$C_4N_3H_4Cl$	130.1228	0.6	19.896		2-amino−5-chloropyrimidine
6	$C_6H_6O_3S$	158.0082	−6.4	14.962		Benzenesulfonic acid
7	$C_6H_8O_2N_2S$	172.1693	0.9	8.514		Para-aminobenzenesulfonic acid
8	$C_6H_6O_4S$	174.9940	3.6	15.078		Para-hydroxybenzenesulfonic acid
9	$C_{12}H_{14}N_4O_2S$	279.0934	8.8	20.860		2-(para-aminobenzenesulfonamide)−4,6-dimethylpyrimidine

6-dimethylpyrimidine ($m/z = 123.0807$) and para-aminobenzenesulfonic acid ($m/z = 172.1693$)) from pathway 2, the latter ultimately forming 1,4-benzoquinone.

These three pathways indicate that the main reactions likely occurring during the degradation of SCP by strain DLY-11 include S-N bond cleavage, dechlorination, hydroxylation, deamination, methylation, sulfur dioxide release, and oxidation. Among these, S-N bond cleavage and dechlorination may be crucial initial steps. Based on the degradation reactions we observed (such as S-N bond cleavage, dechlorination, hydroxylation, etc.), we speculate that the following classes of enzymes may play important roles in the degradation process of SCP: a) Monooxygenases or dioxygenases [17]: Potentially involved in the initial hydroxylation reactions of the SCP molecule. b) Dehalogenases [64]: Possibly responsible for the removal of chlorine atoms from the SCP molecule. c) Deaminases [65]: May participate in the deamination process. d) Sulfur transferases [66]: Potentially involved in the cleavage of S-N bonds and the transfer of sulfur atoms. These enzymes may exist as intracellular enzymes, periplasmic enzymes, or extracellular enzymes, collectively participating in the degradation process of SCP. Although we have not yet conducted gene-level studies, based on the observed degradation reactions and existing literature, we speculate that the following classes of genes may play important roles in the degradation process of SCP: a) Genes related to nitrogen metabolism [67]: Such as genes involved in deamination. b) Genes related to sulfur metabolism[67]: Such as genes involved in S-N bond cleavage and sulfur atom transfer. c) Genes encoding the aforementioned key enzymes: Such as genes encoding monooxygenases, dehalogenases, etc. Although dechlorination reduces the toxicity and environmental risk of some products, SCP degradation also generates 1,4-benzoquinone, which is more toxic to humans. Since this study did not measure the concentration of each degradation product, it is uncertain whether the overall toxicity to humans or the environment is reduced after SCP biodegradation by strain DLY-11. This requires further in-depth analysis in our future research.

4. Conclusion

A strain of *Bacillus sp.* DLY-11 capable of degrading SCP was isolated from mature compost produced by aerobic composting of pig manure. Under optimized conditions (5 % Vaccination dose, 51.5 ℃ reaction temperature, pH=7.92 and 0.5 g/L $MgSO_4$), this strain was able to degrade 97.7 % of 20 mg/L SCP within 48 h. By analyzing nine possible biodegradation products, including a new product of 1,4-benzoquinone with increased toxicity, the study proposed three possible biodegradation pathways for SCP. These pathways involve S-N bond cleavage, dechlorination, hydroxylation, deamination, methylation, sulfur dioxide release, and oxidation reactions. This finding provides a new effective strain for the efficient degradation of SCP, filling the gap in our understanding of bacterial degradation pathways of SCP, and offers a scientific basis for further application in the remediation of antibiotic pollutants in livestock and poultry farming. Future research can build upon this foundation to further explore the molecular mechanisms of SCP degradation by *Bacillus sp.* DLY-11, including the identification of key enzymes and the determination of crucial functional genes, thereby providing a more comprehensive understanding of the microbial degradation process of SCP.

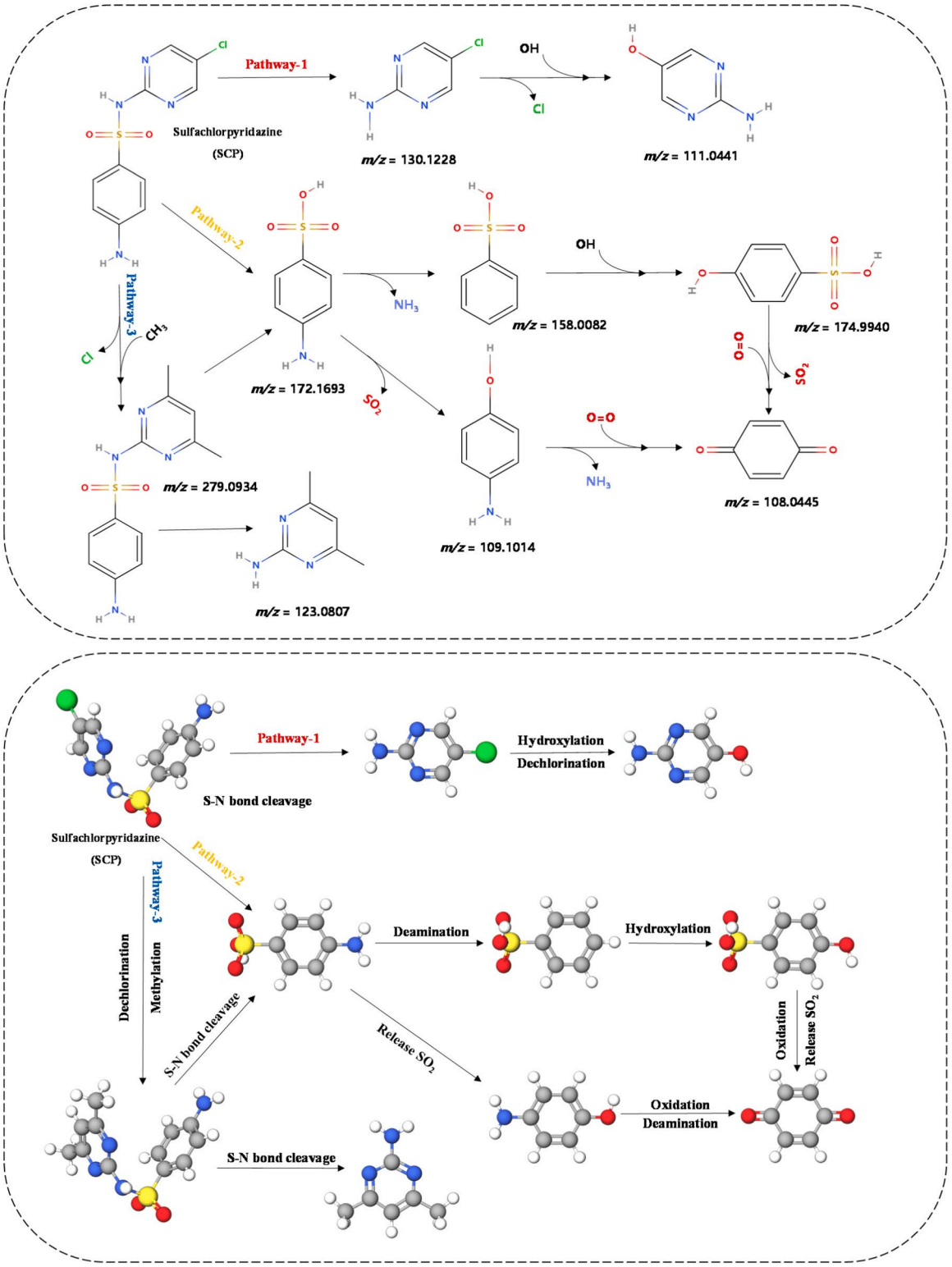


Fig. 5. Biodegradation pathway of SCP by *Bacillus sp.* DLY-11.

CRediT authorship contribution statement

Jingtong Li: Visualization, Investigation. **Zifeng Luo:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Xiujuan Wang:** Visualization, Investigation. **Xue Xiao:** Visualization, Investigation. **Hongxing Tu:** Investigation. **Jingwen Zeng:** Validation. **Jun Zhang:** Visualization, Investigation. **Xiaojun Lin:** Writing – original draft, Formal analysis, Data curation. **Jinrong Qiu:** Writing – review & editing, Methodology, Conceptualization. **Qianyi Cai:** Validation. **Junshi Tao:** Validation. **Weida Yu:** Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Environmental Implication

The discovery of Bacillus cereus DLY-11, a bacterial strain from pig manure compost, offers significant environmental benefits. This strain efficiently degrades 97.7 % of 20 mg/L SCP within 48 h, revealing three potential biodegradation pathways. The study introduces a new tool for reducing SCP antibiotic pollution in livestock waste and enhances our understanding of SCP biotransformation mechanisms. This knowledge is crucial for developing targeted bioremediation strategies, improving environmental health and sustainability in agriculture. The research highlights the importance of microbial solutions in addressing antibiotic contamination and promoting cleaner agricultural practices.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.135755.

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