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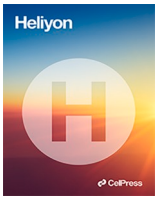

Research article

Isolation, identification, and optimization of conditions for the degradation of four sulfonamide antibiotics and their metabolic pathways in *Pseudomonas stutzeri strain* DLY-21

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ABSTRACT

Overuse of sulfonamides in aquaculture and agriculture leads to residual drugs that cause serious pollution of the environment. However, the residues of sulfonamides in the environment are not unique, and the existing microbial degradation technology has a relatively low degradation rate of sulfonamides. Therefore, in this study, a *Pseudomonas stutzeri* strain (DLY-21) with the ability to degrade four common SAs was screened and isolated from aerobic compost. Under optimal conditions, the DLY-21 strain degraded four sulfonamides simultaneously within 48 h, and the degradation rates were all over 90%, with the average degradation rates of SAs being sulfoxide (SDM) ≈ sulfachloropyridazine (SCP) > sulfa quinoxaline (SQ) > sulfadiazine (SQ). In addition, the main compounds of the strain DLY-21-degrading SAs were identified by LC-MS analysis. On this basis, four detailed reaction pathways for SA degradation were deduced. This is the first report of the use of a *P. stutzeri* strain to degrade four sulfonamide antibiotics (SQ, SDM, SCP, and SM1), which can improve the removal efficiency of sulfonamide antibiotic pollutants and thus ameliorate environmental pollution. The results showed that DLY-21 had a good degradation effect on four SAs (SQ, SDM, SCP, and SM1).

1. Introduction

Sulfonamides used in aquaculture and agriculture contaminate the environment with residual drugs. These antibiotics are primarily used in veterinary medicine and enter the soil through animal feces used as fertilizer. Due to their low absorption and high mobility, sulfonamide antibiotics spread widely in the soil. The degree of sulfa antibiotic contamination varies depending on the animal excretion rate. They constitute a significant portion (25–87%) of total excretory pollutants. Livestock farm manure and slurries often contain primary antibiotics at detectable levels. Concentrations vary based on livestock species, antibiotic type, location, and farm type, ranging from a few μg/kg to hundreds of mg/kg. The highest levels of antibiotic residues have been found in the manure of

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modern industrial-scale farms compared to average family farms [1,2]. In addition, antibiotics detected in swine manure are usually detected in high concentrations, including sulfonamides, penicillins, tetracyclines, and fluoroquinolones, with sulfonamide antibiotics being the most abundant. This is followed by poultry and cattle [3] and mainly results from the relatively higher and more frequent doses of antibiotic drugs administered to pigs. In swine manure, different types of SA are usually detected, and the higher concentrations detected contain sulfadiazine (91 mg/kg) and sulfadiazine ethyl pyrimidine (20 mg/kg) [4]. As demonstrated by Spiel Meyer [5], the treatment of manure, either by storage or using different processes (e.g., composting or aerobic digestion), may partially eliminate residual antibiotics, and the microorganisms present in manure degrade a wide range of antibiotics. These microorganisms degrade antibiotic molecules through metabolism, degradation, and transformation to reduce the extent of their contamination in the environment. The effectiveness of residual antibiotic degradation depends on different conditions and may be influenced by environmental factors such as degradation time, temperature, humidity, and oxygen concentration. Microbial degradation of antibiotics is more efficient under the right conditions. For example, in the storage treatment of manure, the efficiency of antibiotic degradation depends on the time spent storing the manure and the type of antibiotic. In this case, the fermentation time for liquid and solid manure is generally 9 and 6 months, respectively [6]. The effectiveness of antibiotic removal during the composting process also depends on different factors, such as temperature, water content, other pollutants, the initial concentration of the antibiotic present, the duration of composting, the mixture in the pile, and the physicochemical characteristics of the antibiotic [7]. Overall, the composting process is capable of degrading SAs in manure with relatively high removal rates of 70–99% [7]. Although it is possible to reduce the concentration of antibiotic residues in manure using existing technologies, in most cases, animal manure goes directly to the farmland without any special treatment, as there are no regulations regarding the need to treat manure and slurry before application [8]. Moreover, there are no established limits or corresponding quality standards for the concentration of antibiotic residues in the environment [3]. Therefore, most antibiotics are released into the environment, causing environmental pollution [9].

The degradation of sulfonamide antibiotics in the actual environment is influenced by several factors, including environmental conditions, microbial activity, and chemical properties, and the slow rate of degradation in water and soil may lead to their residue accumulation in the environment. Sulfonamides are resistant to hydrolysis and are fairly stable at acidic pH values. Sorensen [10] showed that sulfonamides are not readily biodegradable, and Baozi's et al. [11] studied the biodegradation and abiotic degradation of SDZ and SMT and confirmed that these compounds are biodegradation resistant. In recent years, some authors have studied the biodegradation of sulfonamides [12,13]. It is important to note that the biodegradation of sulfonamides sometimes is slow to begin as shown by Adamski et al., which studied the ability of microorganisms to degrade sulfonamides. The degradation of sulfonamide antibiotics by microbes in the environment is a complex and variable process that involves multiple microbial species and degradation mechanisms. There are two main stages of degradation: enzymatic and metabolic pathways [14]. Enzymatic degradation involves the hydrolysis of the amide bond of sulfonamide antibiotics by specific enzymes, resulting in the production of inactive metabolites. Sulfonamide hydrolyzing enzymes are primarily found in certain bacteria, including *Acinetobacter* spp*., Pseudomonas* spp*., and Klebsiella* spp. [15,16]. These microorganisms break down sulfonamide antibiotics into simpler compounds, such as glycosides and nitro metabolites, as part of their metabolic pathway. The specific form and yield of the degradation products depend on the microbial species and environmental conditions. The efficiency and capacity of the degradation process are influenced by the diversity and functional characteristics of the microbial community [17]. Key players in the degradation process include sulfonamide antibiotic tolerant bacteria such as Pseudomonas spp., Acinetobacter spp., and Klebsiella spp. Environmental factors, such as pH, temperature, and nutrients [18,19], also play a role in the degradation of sulfonamide antibiotics. Researchers have already succeeded in identifying several potentially degrading strains. For example, *Spinozism* sp. Strain SMX completely degrades sulfamethoxazole within 7 days [20], whereas *Pseudomonas* sp. Strain SS degrades a variety of sulfonamide antibiotics, including SMX, SMZ, and SDM. Three kinds of SAs were removed from a sludge and a sludge with added strains. The removal rate of SAs from the sludge with added strains was significantly higher than that without added strains. Within 12 days, SMX and SDM are completely removed, and 98% of SMZ is degraded [21]. These results demonstrate the potential and efficacy of microbial strains in degrading sulfonamide antibiotics. However, it should be noted that under different environmental conditions, microbial strains may show different degradability. In an actual environment, veterinary drug residue antibiotics usually exist in the form of a mixture of multiple antibiotics. In addition, the complete degradation of sulfonamide antibiotics takes a long time, and the degradation rate is relatively low. In addition, the by-products and degradation pathways of degradation products are not fully understood, and these factors may have unknown effects on the environment. Therefore, in the study of the microbial degradation of sulfonamide antibiotics, more strains need to be screened to identify new strains with high degradability. Future research should focus on increasing the rate of degradation, the safety of degradation products, and exploring new antibiotic treatments to mitigate the environmental and human health impacts of sulfonamide antibiotics. Microbial degradation of sulfonamide antibiotics is a promising area of research; however, several issues still need to be addressed, including the search for more efficient microbial degradation strains, in-depth studies on the environmental safety of the degradation products, optimization of the degradation conditions, and consideration of the practical application and sustainability of the technology, to achieve a more effective and sustainable antibiotic treatment. The feasibility of this technology has been confirmed by a review of the relevant literature, which has shown that microorganisms degrade sulfonamide antibiotics [22].

In summary, sulfonamide antibiotics are commonly used in agriculture, animal husbandry, and farming, and can enter the environment through soil, water, and plant pathways. Their long-term presence in the environment may pose a potential threat to human health. Previous studies have shown that microbial degradation can remove sulfonamide antibiotics. Therefore, further research can be conducted on these commonly used antibiotics. To better simulate real-world conditions, we selected experimental subjects and tested the effects of antibiotic degradation on pig manure and wood chip compost.

The purpose of this study was to find a sustainable and effective treatment method that can degrade four sulfonamide antibiotics (SQ, SDM, SCP, and SM1) at the same time and to develop microorganisms that can convert sulfonamide antibiotics into harmless

substances and reduce their concentration in the environment by screening and identifying strains with degradability, thereby reducing the potential impacts on ecosystems and human health. An in-depth understanding of the metabolic pathways and degradation mechanisms of degraded strains may provide a basis for further improvement and optimization of antibiotic treatment methods. This study provides new ideas and methods to solve the actual pollution problem of sulfonamide antibiotics to mitigate the negative impact of antibiotics on the environment.

2. Materials and methods

2.1. Chemicals and reagents

The target SAs of analytical grade standards (>99%), including SQ, SDM, SCP, and SM1, were purchased from Shanghai McLean Biochemical Technology (referred to as "McLean Reagent"). Other chemicals used in the base medium, such as calcium chloride ($CaCl_2$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), and iron sulfate ($FeSO_4$), are sourced from McLean China. Methanol (chromatographic grade), formic acid (98%), and a LiChrolut® EN solid-phase extraction column were purchased from Merck (Darmstadt, Germany). Milli-Q water (18.2 MΩ cm) is produced by the Millipore Purification System (Billerica, CA, USA).

2.2. Isolation and identification of the DLY-21 strain

Aerobic compost piles of pig manure and wood chips (see text S1 for details) were subjected to inoculant enrichment cultures. A total of 10 g of fresh compost was inoculated in a sterilized triangle bottle, sterilized distilled water was added, and subjected to oscillating suspension for approximately 5 min, followed by natural precipitation for 1 h. A 1 mL aliquot of supernatant was taken and coated on the enriched medium containing SM1, SCP, SDM, and SQ antibiotics, and cultured at 30 °C. Colonies of the same color and size were selected from the enriched medium for multiple isolations and purifications until pure species were observed under the microscope [23]. The screened bacteria were inoculated into the screening medium containing the four sulfonamides, SM1, SCP, SDM, and SQ, and cultured in an oscillating incubator with an initial concentration of 100 μg/mL. Increases of 100 μg/mL were performed as a gradient, and the inoculation amount was 10%. Under the oscillatory culture conditions of 30 °C and 80 rpm, the acclimation was carried out every 7 days, and the antibiotic concentration was gradually increased for four cycles. A certain amount of acclimation solution was inoculated into the screening medium and cultured under the oscillatory culture conditions of 30 °C and 80 rpm. At the same time, the culture medium without bacteria was set as a blank control to exclude the influence of hydrolysis and other factors on the reduction of sulfonamide antibiotics. The content of sulfanilamide antibiotics was determined by regular sampling and the degradation rate was calculated, from which the high-activity dominant strains with strong degradation ability were screened (the above operations were performed under aseptic conditions).

The selected SA-degrading bacterium DLY-21 was identified by its physical and biochemical characteristics and 16S rRNA gene sequence. The degrading bacteria were inoculated on the screening medium plate, kept at a constant temperature of 30 °C for 2 days, stained with Gram, and then observed with an optical microscope and a scanning electron microscope. Physiological and biochemical experiments were carried out on the selected sulfonamide antibiotic-degrading strains. The detailed analysis process and parameters are shown in text S2 and Table S1. The growth of the strain at different time points was determined according to the growth curve of the strain, to further evaluate its growth characteristics and optimize the culture conditions. The detailed analysis process and the curve are shown in text S4 and Fig. S1.

2.3. Effects of single factors on the degradation of sulfonamide antibiotics

To assess the effects of different single factors (temperature, pH, inoculum size, metal salt species, and metal ion concentration [24]) on the degradation efficiency of sulfonamide antibiotics, the metal salt species for testing are essential trace elements for microbial growth and metabolism and play a crucial role in catalyzing metabolic reactions, maintaining enzyme activity, and cell structure function. Experiments to detect the single-factor degradation of SAs were carried out in 500-mL flasks containing 200 mL of basal medium (MSM) (autoclaved at 121 °C for 30 min) with temperature (10 °C, 20 °C, 30 °C, and 40 °C), pH (5, 6, 7, 8, and 9), inoculations (1%, 2%, 3%, 4%, and 5%), type of metal salts ($MnSO_4$, $MgSO_4$, $FeSO_4$, and $CaSO_4$), and concentration of metal (1 g/L, 2 g/L, 3 g/L, 4 g/L, and 5 g/L) adjusted to the required values before the biodegradation experiments. Four sulfonamide antibiotics, SM1, SCP, SDM, and SQ, were added to the basal medium at a concentration of 20 mg/L, and the bacterial solution was added according to the corresponding inoculum. The bottles were sealed with rubber stoppers and breathable aseptic sealing films under aseptic conditions to ensure that there was no mixing of heterogeneous bacteria during the incubation process and incubated at 30 °C, 160 r/min, with a degradation time of 48 h, and pipetting at a specific time. Samples (3 mL) of the supernatant were used to determine the concentration of the four sulfonamide antibiotics: SM1, SCP, SDM, and SQ. The supernatant was filtered through a membrane with a pore size of 0.22 μm before the assays. Experiments were performed in triplicate.

2.4. Antibiotic analysis

The target sulfonamides in the sample were analyzed using high-performance liquid chromatography (HPLC, Alliance e2695, Americas). The chromatographic column was equipped with a Luna C18 column (5 μm, 2.0 mm × 150 mm) with 0.01 mol/L mobile-phase oxalic acid solution and methanol. The flow rate was 1.0 mL/min, the sample volume was 5 μL, the column temperature was

30 °C, and the detection wavelength was 268 nm. Sulfa quinoxaline (D4), sulfoxide (D4), sulfochlorinated pyridazine (D4), and sulfadiazine (D4) were used as internal standards. Before analysis, water samples were passed through a membrane with a 0.22-μm aperture (diameter 11.7 mm; Millipore) filtration to remove suspended particles in the sample. A liquid chromatogram (LC-20AD, Shimadzu)-high-resolution hybrid quadrupole time-of-flight mass spectrometer (AB SCIEX X500R QTOF) was used to detect SA metabolites. Detailed analysis procedures and parameters are described in the text.

3. Results and discussion

3.1. Identification of a SA-degrading bacterium

After enrichment and screening, strain DLY-21 had the best degradation rate among all strains and was selected for subsequent experiments because it used SAs as the only carbon and energy source. When cultured on solid mineral salt broth media (MSM) supplemented with SAs, strain DLY-21 colonies were rod-shaped, 1–3 m long, and 0.5 m wide, with a unipolar flagellum and raised granules on the surface (Fig. 1). Under certain conditions, one or two lateral flagella of shorter wavelengths were produced. The phenotypic features include Gram-negative staining and strict respiratory metabolism. DLY-21 was analyzed based on the 16S ribosomal DNA (rRNA) (GenBank accession number OQ996972.1). A phylogenetic tree was constructed using the Mega7.0 software. The maximum composite likelihood method was used with 1000 bootstrap replicates. A strain phylogenetic tree was constructed by comparing the 16S rDNA gene sequences with the EzBioCloud database using Mega.7.0 software and the maximum likelihood (ML) method. Fig. 2 shows that the 16SrRNA sequences of strain DLY-21 and the bacteria of the genus Pseudomonas clustered together, with over 98% homology, and the strain DLY-21 clustered with the *Pseudomonas stutzeri* strains. The combination of morphological, physiological, and biochemical characteristics with the results of 16S rRNA genetic analysis identified DLY-21 as *P. stutzeri*. Before the use of genomic approaches to identify bacteria, *Pseudomonas* strains were often misidentified as other bacterial species. Of these, *P. stutzeri* was often confused with other *Pseudomonas* species (*Pseudomonas medicine*, *Pseudoalcaligenes pseudomonas,* and *Pseudomonas putida*). In some collections, *Pseudomonas syringe* cultures were labeled as *Pseudomonas saccharophilus*.

The newly isolated colonies were adherent and had a typical pleated appearance, being reddish-brown, not simply yellow. Generally, they were hard, dry, and continuous. Removing an entire colony from the surface of a solid medium was relatively easy. After purification in laboratory media and repeated transfers, the colonies that emerged became smooth and white. Similar to most Pseudomonas species, *P. syringe* strains can be grown in basal media with ammonium ions or nitrate and a single organic molecule as the sole source of carbon and energy without the addition of additional growth factors. In a recent publication on *Pseudomonas chemotaxis*, Perales et al. showed that “all Pseudomonas bacteria are highly chemotactic, moving through one or more polar flagella” [25], and *P. stutzeri* was no exception. *Pseudomonas* are ubiquitous bacteria with a high degree of physiological and genetic adaptation. They are present in a large number of different natural environments. Similar to other pseudomonads (e.g., *Pseudomonas malodor*) and *P. stutzeri*, environmentally important metabolic activities are involved. *P. syringe* was one of the first microorganisms identified to degrade alkanes [26], and its main function was the degradation of metal cycles and bio isosteric compounds (petroleum derivatives, aromatic and non-aromatic hydrocarbons, and biocides). There are few reports on the degradation of crude oil, petroleum derivatives, and aliphatic hydrocarbons by *P. syringe* [27–30]. In a wastewater treatment study, 297 species of gasoline-degrading bacteria were isolated and identified from contaminated aquifers [31]. Among them, *Pseudomonas* spp. was dominant, accounting for 86.9% of all screened strains. *Pseudomonas* spp. was the third most common in this study, accounting for 7.4% of all screened strains and 10.2% of *Pseudomonas* spp. *P. syringe* has been less studied than other species of *Pseudomonas*, such as *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas oleophilic,* and *Pseudomonas malodorous* [32].

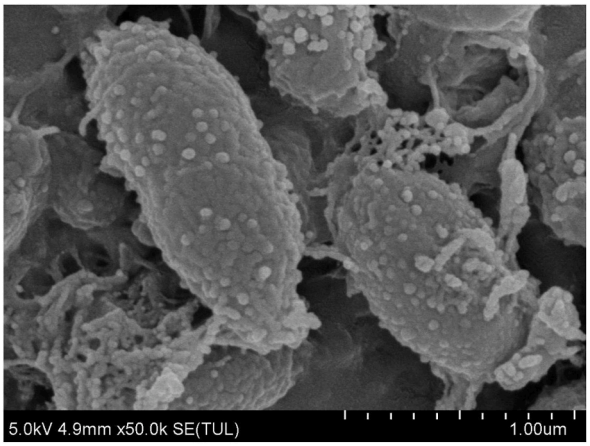

Fig. 1. Scanning electron micrograph of DLY-21.

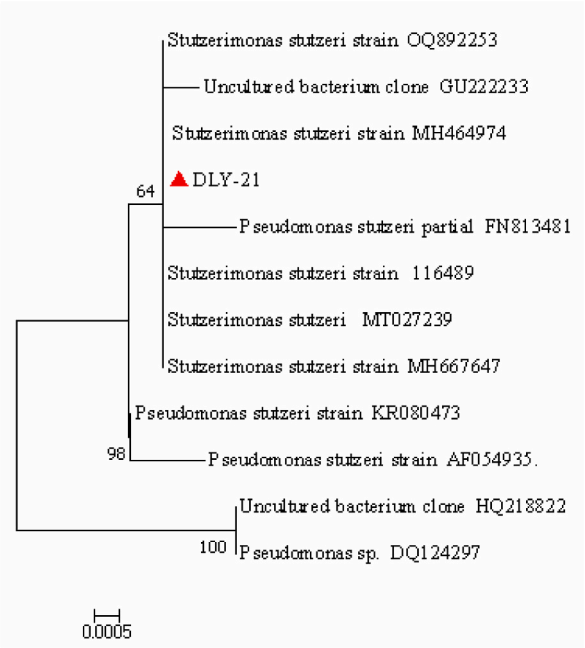

Fig. 2. Phylogenetic tree showing the relationship between strains. DLY-21 and its close relatives based on 16SrDNA gene.

3.1.1. Effects of temperature on the degradation of sulfonamide antibiotics

The main physicochemical factor affecting biodegradation was the temperature of the growth medium, as shown in Fig. 3. The experiment was set up to observe the effect of different temperatures on the biodegradation of SAs by *Pseudomonas stutzeri strain* DLY-21 (the adsorption of SAs by the strain was negligible in the two groups of control experiments, compare1 and compare2), and it was found that the optimal temperatures for the degradation of the strain were 30 and 40 °C; the low temperatures of 10 and 20 °C were not ideal degradation temperatures, preventing bacterial metabolism. According to a previous study, lower temperatures decrease the degradation rate of antibiotics [33]. The degradation rates of the four SAs at different temperatures (from 10 °C to 40 °C) were approximately the same for strain DLY-21. The four antibiotics were mostly eliminated at all temperatures; however, their biodegradation kinetics varied more significantly with temperature. As shown in Figs. 3a and 78.50% of SDM was eliminated by *P. stutzeri* DLY-21 degradation at 48 h at 10 °C. When the incubation temperature was increased to 40 °C, DLY-21 degraded 96.51% of SDM after 48 h. As shown in Fig. 3d, similar to the biodegradation of SDM, when the incubation temperatures were as low as 10 °C and 20 °C, *P. stutzeri* DLY-21's SQ degradation was only 77.43%, whereas when the incubation temperature reached 40 °C, *P. stutzeri* DLY-21 degraded 91.55% of SQ within 48 h, and the degradation rate increased with time. The concentrations of SCP (Fig. 3b) were degraded to 95.21% at temperatures up to 30°C-40 °C, and efficient degradation was not achieved at low temperatures (10 °C). As shown in Fig. 3c, temperature did not greatly affect the degradation of SM1, which was 70.50% at 48 h, whereas *P. stutzeri* DLY-21 degraded SM1 up to 75.00% when the temperature was increased to 40 °C. This may be because the degradation products generated during the degradation process affect the SAs. Based on the results of the biodegradation of SAs at different temperatures, 30°C-40 °C may be very close to the optimal temperature for the degradation of SAs by strain DLY-21. The order of degradation of the four antibiotics was SDM ≈ SCP > SQ > SM1. Nevertheless, *P. stutzeri* DLY-21 degraded most of the SAs at 10 and 20 °C, and the complete removal of SAs may take a longer time. The total SA concentration decreased with increasing temperature and reached a minimum at 40 °C, which validates the idea that antibiotics in swine manure can be effectively removed after heat-resistant treatment [34].

3.1.2. Effects of pH on SA biodegradation

Another physicochemical factor affecting biodegradation is the pH of the growth medium. The variation in SAs concentration and its removal are shown in Fig. 4. In this experiment, the effect on the biodegradation of four SAs (SM1, SCP, SDM, and SQ) was investigated under different initial pH conditions. The growth of microorganisms is usually sensitive to changes in pH, and the concentrations of SM1, SCP, SDM, and SQ changed under different pH conditions. As shown in Fig. 4a, when the pH was 5–6, the highest removal of SQ was 91.52% within 48 h. In contrast, at pH 9, the degradation rate of SQ was only 70.00% at 48 h. SDM, SCP, and SQ were degraded under similar conditions, as shown in Fig. 4b and c. When the pH was acidic, SA degradation was the most prominent at 48 h (<90%) for all of them; the degradation effect was slightly weaker when the pH was alkaline. As shown in Fig. 4d, when the pH was neutral, the degradation rate of SM1 varied steadily with an increase in degradation time and reached 77.50% in 48 h, whereas the degradation effect was only ~70.00% under alkaline conditions. Finally, it was concluded that the acidic and neutral pH conditions were suitable for the biodegradation of SAs by *P. stutzeri* DLY-21, whereas the alkaline pH condition slightly delayed the

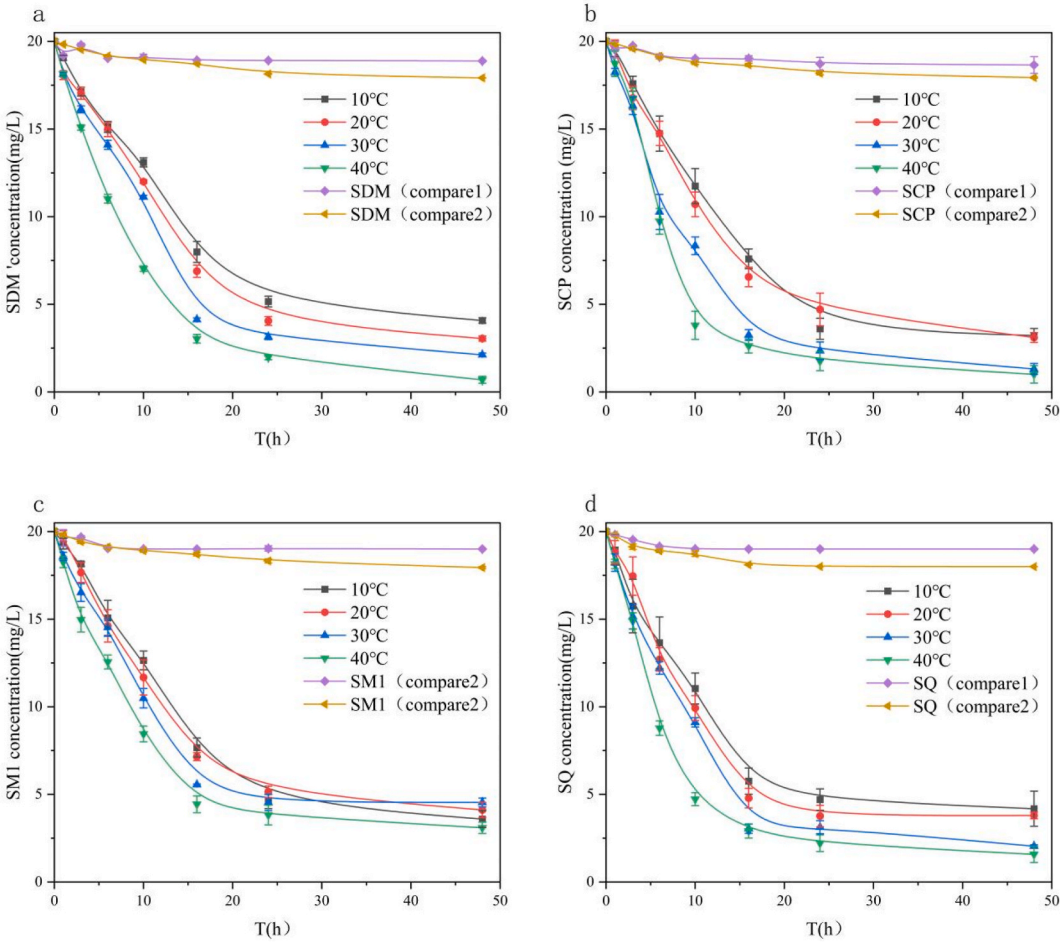

Fig. 3. Effect of different temperatures on the degradation of sulfonamide antibiotics by DLY-21, where the pH was 7, the inoculum was 3%, and the concentration of $MnSO_4$ was 3 g/L (a) sulfoxide, (b) sulfachloropyridazine, (c) sulfadiazine, (d) sulfa quinoxaline. The control under the same conditions, compare1:uninoculated, compare2:sterilized bacterial cells.

biodegradation of SAs, which may result from the fact that this pH condition is more suitable for the growth and metabolism of the microorganisms, which have higher biological activity. These results are in line with those of Roth et al., who verified the affinity sequence of SAs under acidic conditions [8,35,36].

3.1.3. Effect of inoculum size on SA biodegradation

The biodegradation activity of *P. stutzeri* DLY-21 on SAs at different inoculum concentrations is shown in Fig. 5. The strain was added to a medium containing SAs at a concentration of 20 mg/L at inoculum levels of 1%, 2%, 3%, 4%, and 5% and incubated at 30 °C for 48 h. At 24 h of degradation, at an inoculum level of 5%, SQ, SDM, and SCP showed significant degradation(Fig. 5a、5b、5c). Especially at the 5% inoculum level, the removal of SQ (Fig. 5a) at 48 h of degradation was already near completion with 97.02% degradation. SM1 (Fig. 5d) showed 84.73% removal at 48 h of degradation. Compared with the effect of pH, the effect of the inoculum amount on the degradation of SAs was relatively small, and the optimal inoculum amount was 5%.

3.1.4. Effects of metal ions on SA biodegradation

The complexation of some metal ions with antibiotics can accelerate the oxidation of antibiotics; the electrons of the complex formed by the two are transferred within the molecule, and a certain amount of oxidative degradation of antibiotics can be achieved under natural environmental conditions. In this study, four different metal ions were chosen to observe the biodegradation ability of *P. stutzeri* DLY-21 on four SAs (SM1, SCP, SDM, and SQ). Fig. 6 shows that all four metal ions had a certain promotion effect. When the metal ion $FeSO_4$ was added to the medium, SQ degradation (Fig. 6a) was close to complete at 48 h, the complete degradation of SDM (Fig. 6b) was achieved, and the removal of SCP(Fig. 6c) and SM1(Fig. 6d) was slightly weaker, although the degradation rates reached 95.11% and 84.74%, respectively. The next most effective degradation promoter was the addition of the inorganic salt metal ion, $MgSO_4$, where the degradation rates of SQ, SDM, and SCP reached 90.00%. The degradation of SAs was also promoted by the addition of $MnSO_4$ when used as an energy source for *P. stutzeri* DLY-21, and the optimal metal ion was judged to be $FeSO_4$, which verified the

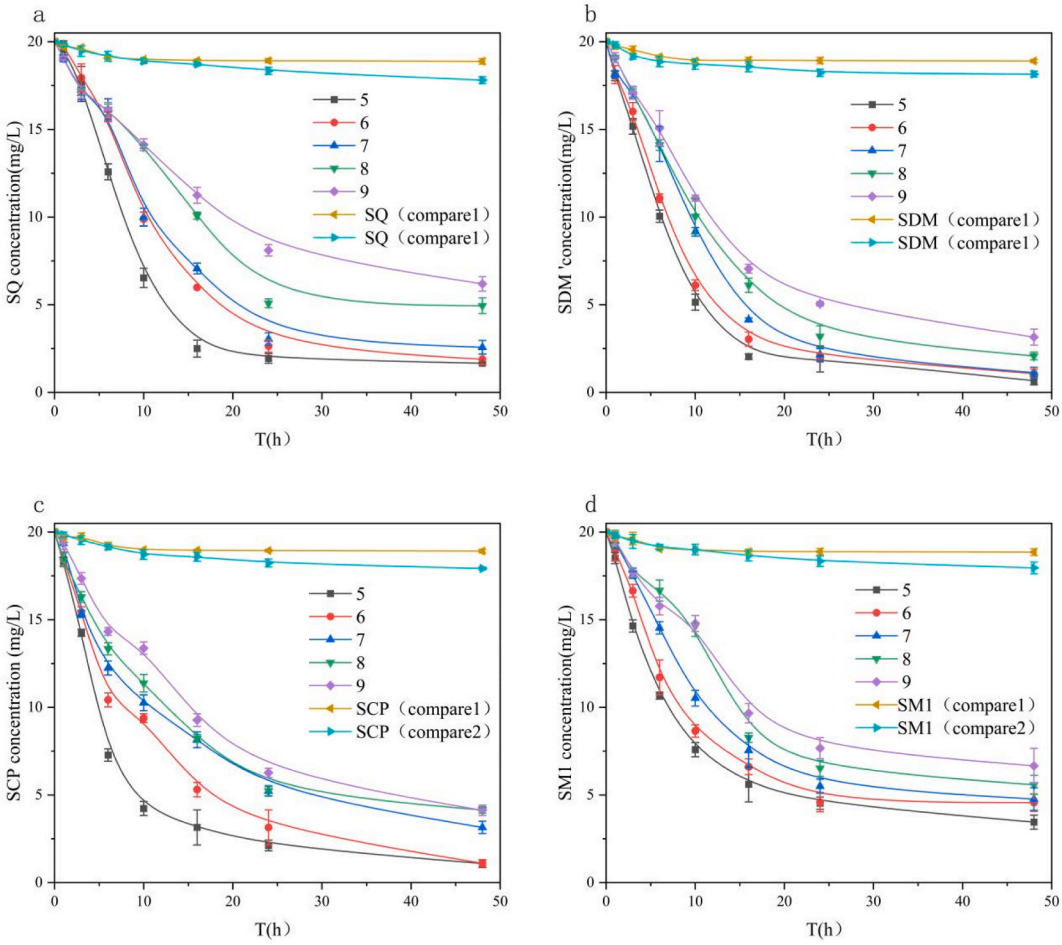

Fig. 4. Effect of different pH on the degradation of sulfonamide antibiotics by DLY-21, where the temperature was 30 °C, the inoculum was 3%, and the concentration of $MnSO_4$ was 3 g/L (a) sulfa quinoxaline, (b) sulfoxide, (c) sulfachloropyridazine, (d) sulfadiazine. The control under the same conditions, compare1:uninoculated, compare2:sterilized bacterial cells.

claim that pyoverdine is an efficient iron carrier, which is mainly found in microorganisms (bacteria and fungi). From taxonomic and physiological points of view, microorganisms need iron to synthesize cytochromes and enzymes. Their production is enhanced under iron-deficient conditions. Although they do not contain pigments, some strains can synthesize iron carriers. Microorganisms secrete iron carriers when small amounts of iron are present in the medium. Once the iron carriers come into contact with the iron carrier receptor proteins on the cell membrane, either the iron atoms are released directly into the cytoplasm or the entire iron carrier complex enters the cell via ABC transport (the ATP-binding cassette transporter protein system). Iron enters the cytoplasm and is reduced to divalent iron (ferrous form), which dissociates from the iron carrier because the affinity of the iron carrier for trivalent iron is higher than that for divalent iron. Iron is important for microorganisms, and there is more than one pathway for iron uptake, thus enhancing microbial degradation. *P. stutzeri* ATCC17588 produces deferoxamine E and D2 [37,38]. Another strain (RC7) produces catechol-like iron carriers [39].

3.1.5. Effects of different metal ion concentrations on the degradation of sulfonamide antibiotics by degrading bacterial strains

Based on the analysis and comparison of the results of the previous phase of experiments, when the metal ion $FeSO_4$ was added to the culture medium as an energy source for *P. stutzeri* DLY-21, it most efficiently degraded the four SAs. In previous studies, KC was found to be the best iron carrier [40]. It is likely that the secondary iron carrier in this strain [41] (*P. stutzeri* KC) can degrade carbon tetrachloride (CT) to carbon dioxide, chloride ions, and other nonvolatile compounds as for mate. Chloroform is not produced during this process. To use *P. stutzeri* DLY-21 as an efficient iron carrier, we then added different concentrations of $FeSO_4$ and observed its effects (Fig. 7). The degradation effect of *P. stutzeri* DLY-21 on the four antibiotics was enhanced with an increase in incubation time in the medium and the addition of different concentrations of the metal ion $FeSO_4$. When the concentration of $FeSO_4$ was 5 g/L, the degradation of SQ (Fig. 7a), SDM (Fig. 7b), and SCP (Fig. 7c) was almost complete after 48 h, and the degradation rate exceeded 95% in all cases. Fig. 7b、7c、7d shows that the higher the concentration of the added metal ions, the higher the removal efficiency. Although metals are essential nutrients, excess metals can cause toxicity. Furthermore, some metals are toxic and have no beneficial effects.

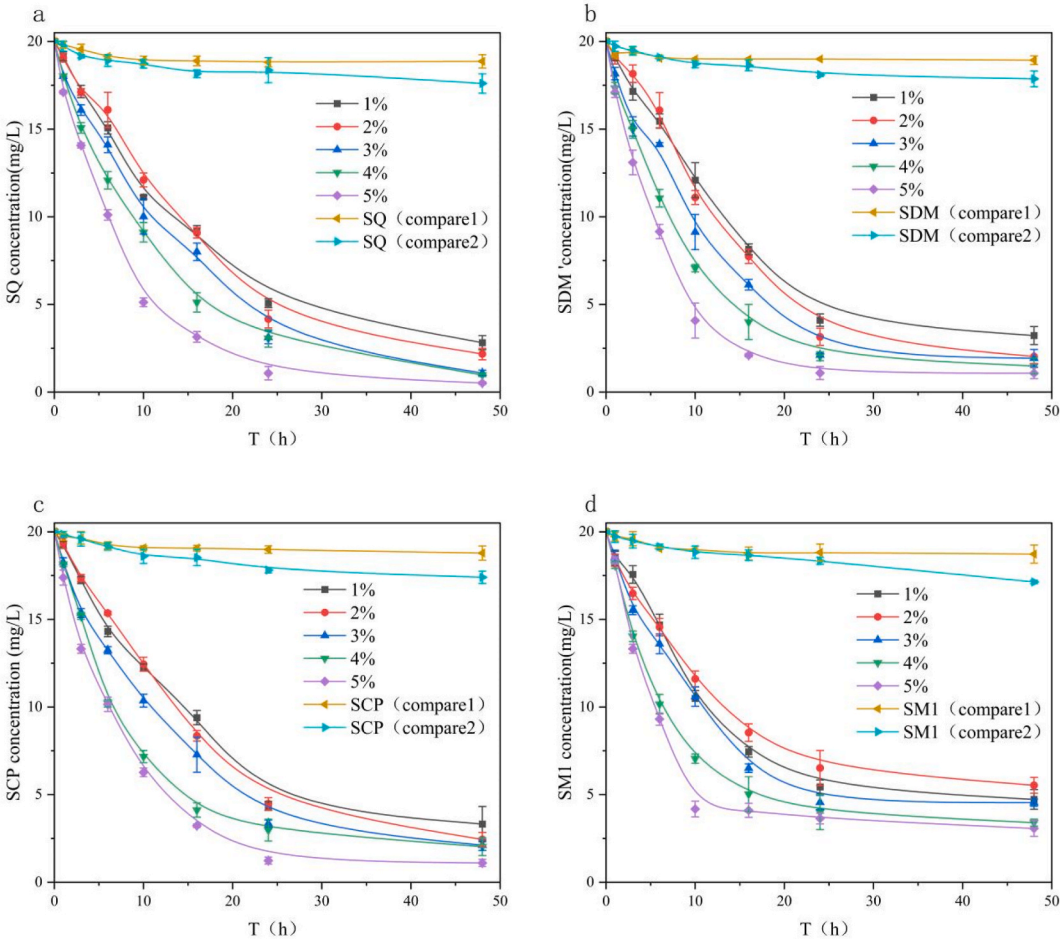

Fig. 5. Effect of different inoculum amounts on the degradation of sulfonamide antibiotics by DLY-21, where the temperature was 30 °C, pH was 7, and the concentration of $MnSO_4$ was 3 g/L (a) sulfa quinoxaline, (b) sulfoxide, (c) sulfachloropyridazine, (d) sulfadiazine. The control under the same conditions , compare1 : uninoculated , compare2 : sterilized bacterial cells.

However, bacteria have developed systems to ensure the availability of essential metals, and metal toxicity can be addressed. *P. stutzeri* is no exception. Three different types of iron carriers (nicardipine, an arginine conjugate of 2,3-dihydroxybenzoic acid, and pyridine 2, 6-dithiocarboxylic acid) have been demonstrated [39].

3.2. Biodegradation pathways of SAs in MSM

Few studies on SA metabolites and degradation products have been published, and most of them lack data on lipoamide biodegradation; however, it has been shown that wherever SAs are detected, metabolites are often present, sometimes at concentrations higher than those of the parent drug. The four SAs in this study all have different azaheterocycle structures, and to better understand the biodegradation process of SAs, the degradation products of SAs were detected using LC-MS. Based on the degradation products identified in the experiments (Tables S3–S6 and Figs. S2–S14), the pathways of biodegradation of the four SAs by in addition DLY-21 were inferred, as shown in Figs. 8–11.

3.2.1. Degradation pathways of SQ

The degradation products of SQ are shown in Table S3, and the degradation pathways are shown in Fig. 8. Overall, 13 products were detected, and their *m/z* values were 317, 268, 237, 237, 210, 186, 173, 156, 146, 131, 108, 94, and 81. Among them, the oxidative reduction of the sulfite group of SQ produced the *m/z* of 237 of the 4-(quinazolin-2-yl) aniline product (Pathway-1), followed by a direct attack by radical OH to produce a product with an *m/z* of 237. During aerobic biodegradation, the amino groups at the N4 and N1 positions are readily substituted with nitro groups, resulting in the formation of N4–OH-SQ or N1–OH-SQ with *m/z* 268. In contrast, the N1 position of SQ was substituted with an acetyl group to produce the product N-(4-(N-(quinoxalin-5-yl) subfamily) phenyl) acetamide with an *m/z* of 317 (Pathway-2), wherein the breaking of the N–C bond of N4 may produce 4-aminobenzenesulfonamide (Pathway-2-1) and quinoxalin-2-amine (Pathway-2-2) at *m/z* 146. After acetylation, the N–C bond in the SA molecule is more

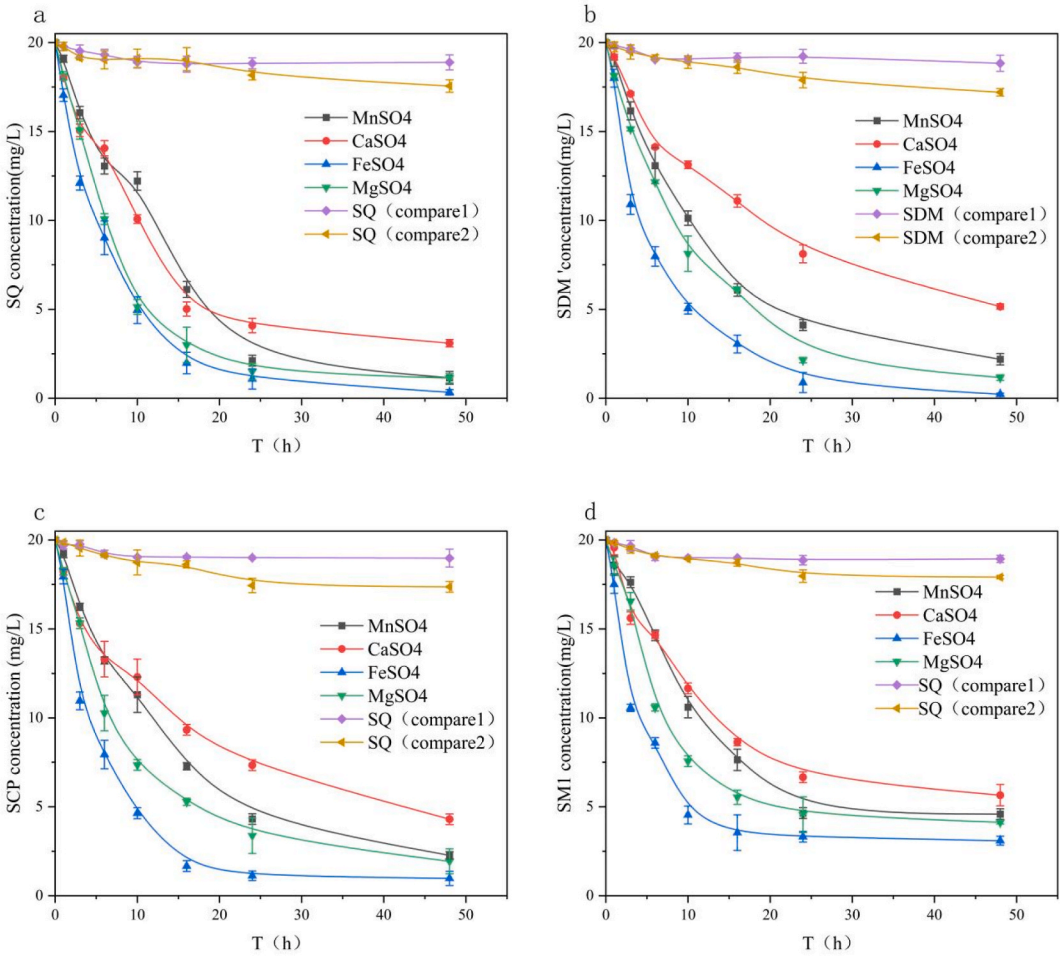

Fig. 6. Effect of different metal ions on the degradation of sulfonamide antibiotics by DLY-21. where the temperature was 30 °C, pH 7 and inoculum amount was 3%. (a) sulfa quinoxaline, (b)sulfoxide, (c)sulfachloropyridazine, (d)sulfadiazine. The control under the same conditions，compare1：uninoculated，compare2：sterilized bacterial cells.

susceptible to breakage at N4 in the amino group. This proved to be an effective way to reduce the molecular weight of SAs [42]. SQ has a specific nitrogen-containing heterocyclic functional group on its quinoline moiety. It is degraded by microorganisms under both aerobic and anaerobic conditions [43], and part of its quinoline moiety is transformed during sulfate reduction, denitrification, and methane production [13]. A common degradation product of the quinoline moiety was quinoxaline, with an *m/z* of 131. Similar to the quinoxaline degradation pathway, cleavage centered on the S–N and S–C bonds results in the formation of products with smaller molecular weights (*m/z* 156 and 108), which are subsequently degraded to aniline with an *m/z* of 94 and benzene with an *m/z* of 81 (pathway 2-1) [43]. Methylation and hydroxylation are effective methods to degrade quinoline moieties. Compounds with conjugated π-bonds and high electron cloud densities are more prone to substitution reactions, and hydroxyl substitution is more likely to occur on the benzene ring since the C–N bond reduces the electron cloud density of the ring and can increase the electron density on the benzene ring [43]. This is immediately followed by conversion via pathway 2-2 to products with *m/z* values of 186 and 210.

3.2.2. Biodegradation pathways of SDM

SDM biodegradation can be inferred through two pathways (Fig. 9). Similar to other types of compounds, reactions such as substitution, bond cleavage, and ring opening occur [44]. Ten products were detected during SDM degradation (S4), including compounds with molecular ion peaks (*m/z*) at 486, 323, 276, 270, 166, 156, 149, 108, 94, and 81. We speculate that the substitution of the N1 or N4 amino groups by hydroxyl, propenyl, and glucuronic acids generated the products (*m/z* 323), (*m/z* 486), and (*m/z* 276) (Pathway 1), followed by cleavage centered on the S–N bond to form the product with $m/z = 166$. Furthermore, the N–C bond of (4-hydrosulfonylphenyl) acetamide (*m/z* 166) could be broken, and the acetyl group substituted by the N1 amino group was removed to form an aminobenzene sulfonamide. In the presence of aerobic microorganisms, it can be further degraded to produce products of lower molecular weights (*m/z* 156, 108, 94, and 81) by further breaking-S of N and S–C bonds (pathway 1-1) [45]. Meanwhile, methoxy pyrimidines are likely to be demethylated because of the protonation of nitrogen, which promotes a nucleophilic attack on the methyl

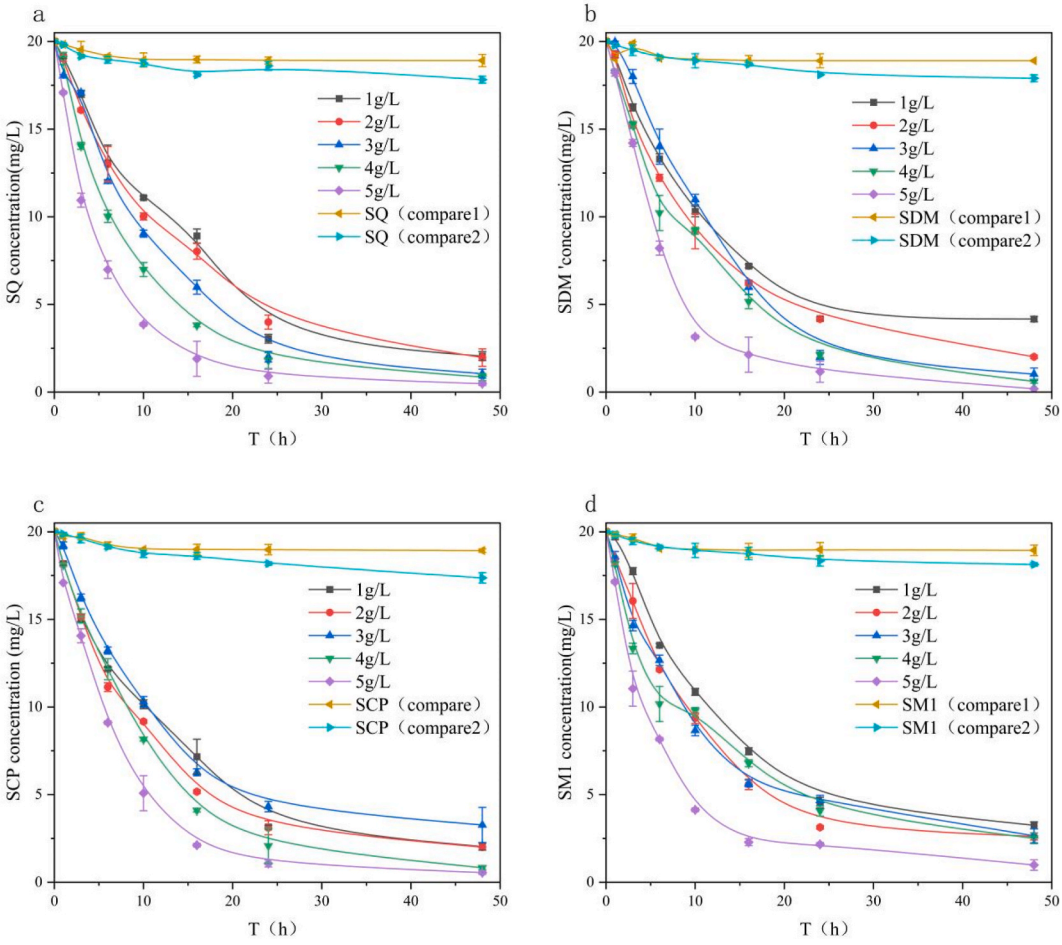

Fig. 7. Effect of different concentrations of optimal metal ions on the degradation of sulfonamide antibiotics by DLY-21. where the temperature was 30 °C, pH 7 and inoculum amount was 3%. (a) sulfa quinoxaline, (b)sulfoxide, (c)sulfachloropyridazine, (d)sulfadiazine. The control under the same conditions , compare1 : uninoculated , compare2 : sterilized bacterial cells.

group. The 5-hydroxyl group between the pyrimidine ring and the nitrogen atom, on the other hand, is suitable for coupling reactions. The presence of the sulfate hydroxyl metabolite allowed the generation of 4-amino-N-(5-hydroxypyrimidin-2-yl) benzene sulfonamide (*m/z* 270) via a second pathway. Similar results have been reported in a previous study [46]. Pyrimidine is a common azahetero cyclic moiety that is similarly degraded by microorganisms [45]. Methoxy pyrimidines can be hydroxylated to produce hydroxy methoxy pyrimidines (*m/z* 127) [47], which are then generated by N–C bond breakage to produce a product with an *m/z* of 149 (pathways 1–2) [48]. Similar degradation pathways have been reported previously. In summary, the degradation pathway of SDM is similar to that of SMX and involves an advanced oxidation process [49].

3.2.3. Biodegradation pathways of SCP

The SCP degradation pathway was hypothesized to be based on the main intermediate product (S5). Fig. 10 demonstrates the two SCP decomposition pathways. Thirteen intermediates were detected with *m/z* values of 297, 279, 202, 176, 174, 158, 157, 145, 124, 113, 109, 109, and 108. The degradation pathways of hydroxylation, C–N bond cleavage, SO_2 release, and nitrification typically occur throughout the growth cycle of the strain. This phenomenon may be related to the biodegradation of SCP. Compounds containing conjugated π-bonds with high electron cloud density are prone to hydroxylation reactions during aerobic biodegradation [50]. Hydroxylation leads to the substitution of the amino group on the benzene ring by the hydroxyl group. When the hydroxyl group attacks the molecular group, the SCP is hydroxylated in the presence of dichlorination. The addition of a hydroxyl group to the aniline ring results in the loss of chlorine and conversion of SCP to SCP ($m/z = 279$), which is then attacked by the free radical OH to produce a product with an *m/z* of 297, whose C–N bond breakage yields the intermediate 4-aminobenzenesulfonamide ($m/z = 174$). The presence of the C–N bond decreased the electron cloud density on the ring and increased the electron density on the benzene ring. As a result, hydroxyl substitution on the benzene ring was more likely to occur, resulting in the formation of two hydroxylated products, 4-hydroxybenzenesulfonic acid ($m/z = 176$), and an oxidation reaction to produce a product with $m/z = 202$ (Pathway 1-1). Further, 4-hydroxybenzenesulfonic acid undergoes a dehydration reaction, losing its hydroxyl group to form benzenesulfonic acid ($m/z = 158$),

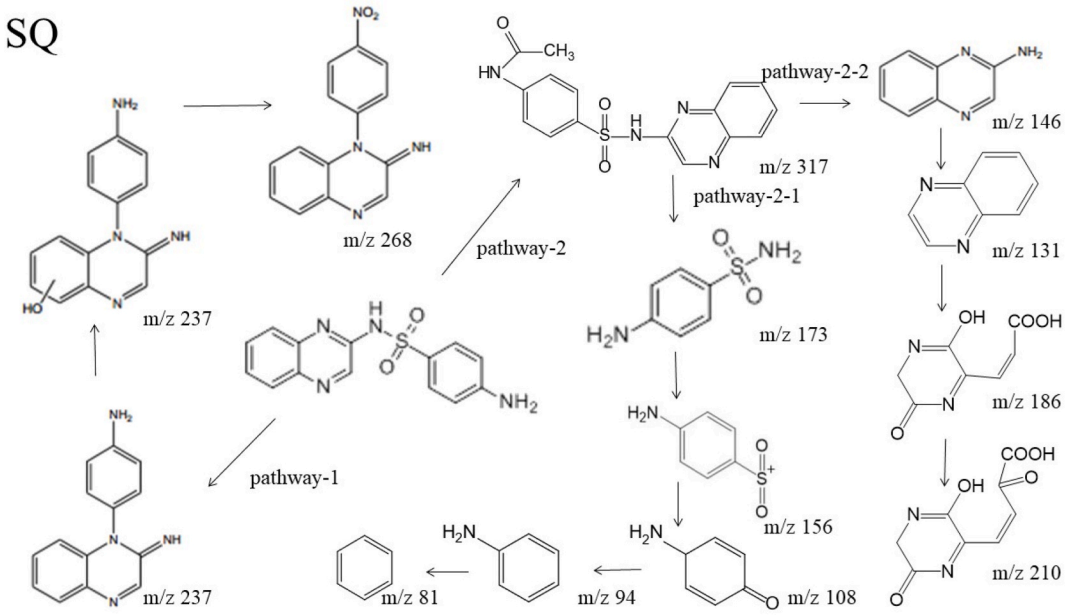

Fig. 8. Biodegradation pathways of SQ by *Pseudomonas stutzeri strain*DLY-21.

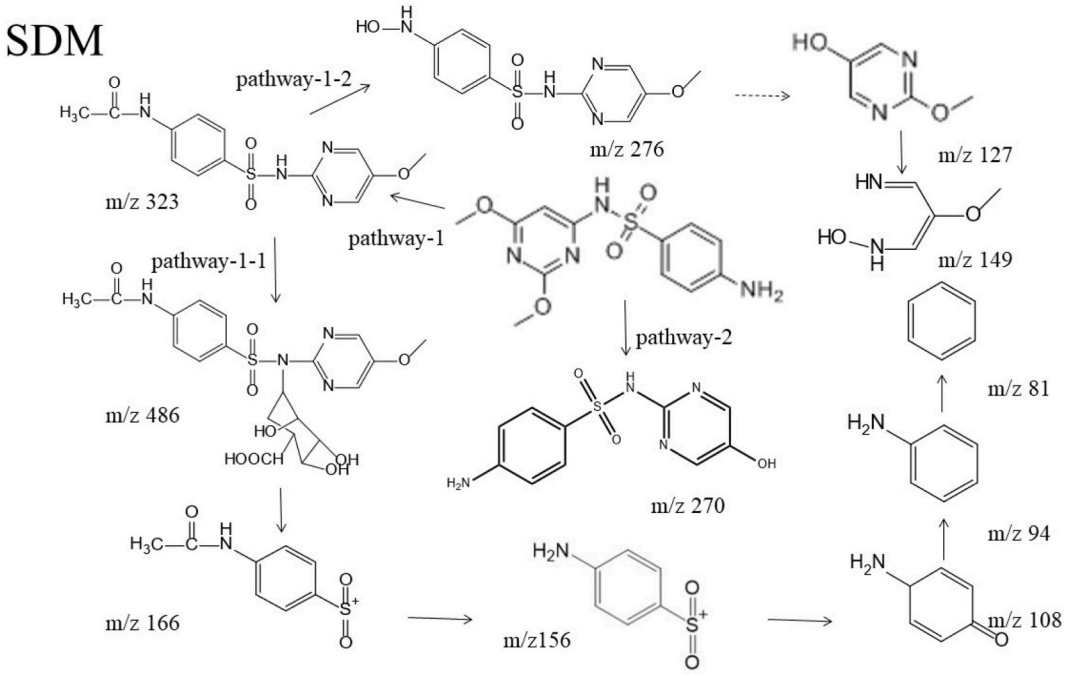

Fig. 9. Biodegradation pathways of SDM by *Pseudomonas stutzeri strain*DLY-21.

which then undergoes oxidative reduction to form a product with $m/z = 145$ (pathways 1–2). The hydroxyl group of benzenesulfonic acid was replaced with an amino group to form a product with $m/z = 157$, which lost SO_2 to form a compound with $m/z = 109$ (pathways 1–3). Similar reactions have been observed in the degradation of other SAs [13,50,51]. Gauthier et al. showed that hydroxylation occurs during the degradation of sulfamethoxazole by a strain of Robodoc's rhodochrosites in pure cultures [52]. The N-bond breaking resulted in the SCP molecule becoming divided into two parts, and it was hypothesized that one of the parts was the m/z of 130, the substance 3-amino-6-chloropyridazine. However, sulfamic acid was not detected in other parts of the product, presumably because sulfamic acid is more water-soluble and not retained by the reversed-phase chromatographic column, and therefore

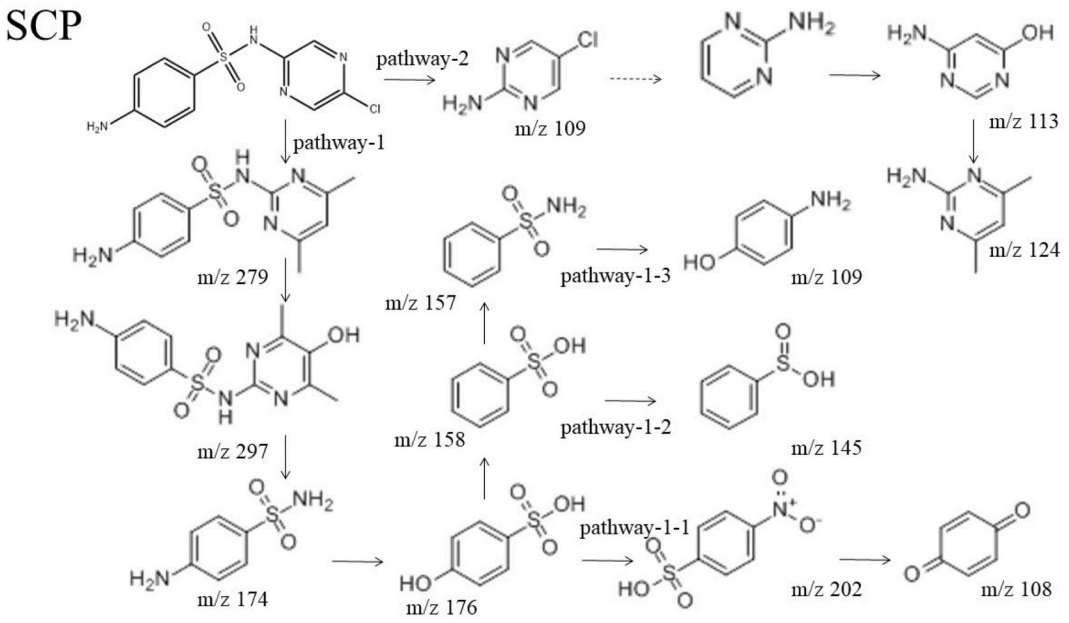

Fig. 10. Biodegradation pathway of SCP by *Pseudomonas stutzeri strain*DLY-21.

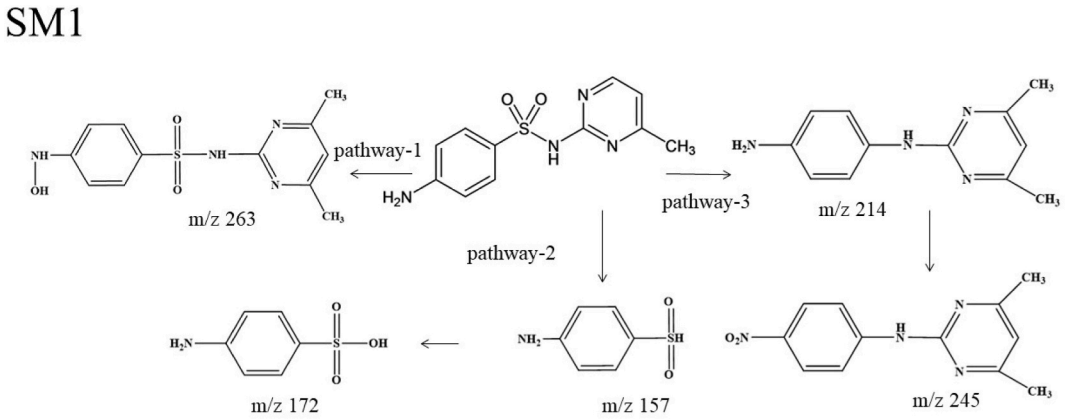

Fig. 11. Biodegradation pathways of SM1 by *Pseudomonas stutzeri strain*DLY-21.

was not detected [53]. The pyridazine ring is a six-membered heterocyclic compound containing two nitrogen heteroatoms at positions 1 and 2, isomeric to the pyrimidine ring, and breakage of the S–N bond of the SCP results in the transformation of the SCP molecule into 3-amino-6-chloropyridazine ($m/z = 109$). The addition reaction at the N1 position of the amine-containing pyridazine ring produced a product with an m/z of 113, which was presumed to be 3-nitro-6-hydroxypyridazine. However, the associated mass-to-charge ratio of the pyridazine ring was detected at the end of the reaction, and the probable reason for this is that the system produced a pyridazine ring that was rapidly converted by addition into a 3-nitro-6hydroxypyridazine, which loses its hydroxyl group to form a compound with an m/z of 124 (pathway 2).

3.2.4. Biodegradation pathways of SM1

The biodegradation pathway of SM1 is shown in Fig. 11. Five transformation products (S6) of SM1 were detected with m/z values of 263, 245, 214, 172, and 157. Among them, the specific functional group of SM1 is the pyrimidine ring, but the relevant mass-to-charge ratios could not be detected in the experiments, which may be attributed to the fact that it is very difficult for the pyrimidine ring to undergo electrophilic substitution reactions. Pyrimidines and their homologs, nitro and halogenated derivatives, are aromatic and oxidative, and result from electrophilic substitution reactions. Nucleophilic reactions are not important in this context and sulfamic acid is more water-soluble and cannot be retained by the reversed-phase columns, and therefore was not detected. Only 4-methylpyrimidines can react with sodium carbamate to form 2- or 4-substituted aminopyrimidines [54]. Based on the major products detected, three possible transformation pathways of SM1 were deduced. First, the N7 or N9 sites of SM1 are directly attacked by OH radicals to

generate N-(4,6-dimethylpyrimidin-2-yl)-4-(hydroxyamino)benzenesulfonamidem/z 263 (pathway 1) [55]. Second, the S8–N9 bond of SM1 is broken to produce a substance with an *m/z* of 157, and the S8 site is attacked by OH radicals to produce 4-aminobenzenesulfonic acid with an *m/z* of 172 (pathway 2) [56]. SM1 then loses SO_2 to generate N1-(4,6-dimethylpyrimidin-2-yl)benzene-1, 4-diamine with *m/z* 214, and the N7 site is readily oxidized to generate a product with *m/z* 245 (pathway 3) [56].

In summary, the results of this study improve our understanding of SA degradation pathways in aerobic processes. In future studies, chromatography and stable isotope analysis of antibiotics should be performed to elucidate the degradation mechanisms of different strains.

4. Conclusions

DLY-21, a strain with the ability to degrade four SAs (SQ, SDM, SCP, and SM1), was isolated from the aerobic compost of swine manure and wood chips by selective enrichment, screening, and purification and was identified as *P. stutzeri* based on 16S rRNA sequence analysis and biological methods. Previously, *P. stutzeri* strain P16, isolated from non-enriched cultures of creosote-contaminated soil, and *P. stutzeri* strain AN10, isolated from contaminated marine sediments in the western Mediterranean Sea, have been described as PAH-degrading [57] and naphthalene-degrading [58] bacteria, respectively. Strain P16 grows on salicylate, using phenanthrene (tricyclic), fluorene (bicyclic), naphthalene, and methylnaphthalene (bicyclic) as the sole carbon and energy sources [57,59]. Strain AN10 can use naphthalene, 2-methylnaphthalene, and salicylate as the sole carbon and energy sources [60]. However, this aspect of the degradation of SAs has not been studied extensively, and the present study confirmed the biodegradation activity of strain DLY-21 on lipoamide antibiotics.

The one-way experiment revealed that the residual concentration values of the four different SAs decreased with time, and the highest removal rate was achieved at 48 h, indicating that *P. stutzeri* DLY-21 utilized the four SAs (SQ, SDM, SCP, and SM1) as a carbon source to synthesize biomass. According to the graph, the decrease in the concentration of different SAs under all conditions indicated that biodegradation played a major role in the removal of the four SAs. The change in antibiotics adsorbed by higher densities of organisms over time was small to negligible, and in this case, the half-life was even shorter. In the absence of nutrients, microorganisms can only utilize SAs as carbon and nitrogen sources.

Strain DLY-21 exhibited the highest degradation activity for all four sulfonamide antibiotics at a temperature of 40 °C. Variations in the removal efficiencies of different antibiotics within the tested temperature range were attributed to the temperature's effect on enzymes responsible for the biodegradation of sulfonamide transformation products. An optimum pH range of 5.0–6.0 was determined for the degradation of the antibiotics. Acidic conditions were found to facilitate the hydrolysis of the antibiotics. The pH-induced increase in the Pseudomonas genus was associated with enhanced sulfonamide removal [8,61]. The inoculum amount showed a relatively weak effect on degradation, with a 5% inoculum selected as optimal. The addition of FeSO4 as a metal ion enhanced the degradation rates, with approximately 95.00% removal for three antibiotics and 90.00% removal for SM1 at a concentration of 5 g/L [62].

In summary, the optimal conditions for the degradation of SAs by strain DLY-21 were a pH of 5.0–6.0, an incubation temperature of 40 °C, a 5% inoculum, and a 5 g/L metal ion $FeSO_4$ concentration. *P. stutzeri* DLY-21 showed relatively high removal of all four SAs (SQ, SDM, SCP, and SM1).

All the four SAs exhibited similar degradation pathways. Aminobenzene sulfonamide is the basic functional group of sulfamate, and the S–N, S–C, and C–N bonds are broken and decomposed sequentially to form aminobenzene sulfonamide, aminobenzene, and aniline, respectively. The substitution reaction usually occurs on the N1 or N4 amino groups of SQ, and the degradation pathways of specific functional groups of SAs are as follows: the quinoline functional group of SQ is partially substituted by a hydroxyl group, the C–N bond of the pyrimidine ring of SDM is substituted by a hydroxyl group, and the ring-opening reaction occurs; the S–N bond of the pyridazine ring of SCP is broken, which results in the transformation of the SCP molecule into two parts: the specific functional group of SM1 is a pyrimidine ring. After the ring opening of specific functional groups, all of them were further substituted and decomposed into small-molecule products. This study provides a theoretical basis for SA degradation during aerobic composting. In addition, different degradation pathways are used for sulfonamide antibiotics in the environment, which may result from differences in their molecular structures, chemical properties, and biological activities [63].

After aerobic composting screening, *P. stutzeri* was isolated, with a degradation effect on four different SAs. In summary, this strain has the advantages of high efficiency, versatility, and tolerance in degrading SAs, which makes it potentially applicable in the fields of environmental management and resource utilization. Based on the above study, further in-depth research can be carried out to investigate the degradation mechanism of SAs by the strain DLY-21 in the aerobic composting process, and microbial agents can be prepared according to the optimal degradation conditions of the strain to be utilized in practice to explore their potential practical application.

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Data availability statement

The datasets generated or analyzed during this study areavailable from the corresponding author on reasonable request.

CRediT authorship contribution statement

Yaxin Li: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Ting Yang:** Investigation, Formal analysis, Conceptualization. **Xiaojun Lin:** Writing – review & editing, Project administration. **Jianfeng Huang:** Project administration. **Jingwen Zeng:** Investigation. **Qianyi Cai:** Investigation. **Yuanling Zhang:** Investigation. **Jinnan Rong:** Investigation. **Weida Yu:** Investigation. **Jinrong Qiu:** Supervision, Funding acquisition. **Yuwan Pang:** Resources. **Jianli Zhou:** Validation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yaxin Li, Ting Yang, Xiaojun Lin, Jingwen Zeng, Qianyi Cai, Yuanling Zhang, Jinnan Rong, Weida Yu, Jinrong Qiu has patent #2023116547703 pending to South China Institute of Environmental Sciences, Ministry of Ecology and Environment (MEE), Guangzhou Shangran Environmental Technology Co., Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29123.

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