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# Isolation and degradation characterization of a cyproconazole-degrading bacterial strain

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***Abstract:*** *A strain TF-1 capable of utilizing cyproconazole (CZ) as sole carbon and nitrogen source was isolated from the activated sludge of a domesticated sewage treatment plant. The strain was identified as Halalkalibacterium halodurans by morphological observation, physiological and biochemical experiments, 16S rRNA gene sequencing and phylogenetic tree analysis. It was named as Halalkalibacterium halodurans strain TF-1. The biodegradation experiment of CZ by TF-1 strain showed that the strain reached the optimum degradation conditions when the temperature reached 30 ℃ and the pH was 9. When the initial concentration of CZ increased from 20 to 80 mg $L^{-1}$, the maximum volume degradation rate also increases, indicating that TF-1 has strong tolerance and excellent degradation performance for refractory CZ. The addition of appropriate amount of external carbon source will accelerate the biodegradation of CZ, but the excessive organic carbon source will delay the degradation. The addition of appropriate amount of $NH_4^+$ and $NO_3^-$ contributes to the biodegradation of CZ, while the addition of $NO_2^-$ will significantly inhibit the degradation. Seventeen CZ degradation intermediates were detected by LC/MS and GC/MS, and three CZ degradation pathways were obtained, including the reaction of oxidation, hydrolysis ring opening, dehydroxylation, deamination, decarboxylation, dechlorination, hydroxylation, hydration and final mineralization. In the further wastewater small-scale experiments, the degradation efficiency of CZ in the activated sludge reactor inoculated with TF-1 strain increased from about 10% to more than 98%, and the reduction of COD and TOC in the reactor increased by 20 percentage points, which shows that inoculation of microorganisms can effectively improve the removal effect of activated sludge on CZ and reduce the biological toxicity of wastewater. The feasibility of bio-enhanced degradation of CZ wastewater by TF-1 strain was verified to a certain extent.*

***Keywords:*** *Cyproconazole, Biodegradation, Bacillus sp., Degradation pathway*

## 1. Introduction

Cyproconazole(2-(4-chlorophenyl)-3-cyclopropyl-1-(1,2,4-triazol-1-yl)butan-2-ol, hereinafter referred to as CZ) is a nitrogen-containing heterocyclic compound, which is mainly used as a fungicide. Its mechanism of action is to inhibit the demethylation of sterols and has outstanding preventive and therapeutic effects[1]. CZ can inhibit the synthesis of 1,8-dihydroxynaphthalene melanin, which is the pathogenic factor of rice blast[2]. It also has significant effects on the prevention and control of peanut smut[3], coffee bean rust and chocolate spot[4], soybean spot and purple spot[5]. In addition, CZ can also be used in non-agricultural fields such as horticulture, forests, wood preservation, and has a broad market prospect.

Previous studies have shown that the half-life of CZ in field-conditioned soils is close to 600 days[6], indicating that CZ has strong stability in soil systems. Diana et al.[7] studied the effects of triazole fungicides on soil biological activity and soil enzymes. Studies have shown that high doses of CZ reduce soil biodiversity and enzyme activity, which is due to the fact that the energy consumed by microorganisms in the detoxification process is much greater than the consumption of their normal life activities, resulting in a decrease in microbial biomass. Most importantly, the high toxicity, teratogenicity and carcinogenicity of CZ even at low concentrations post an obvious threat to the health of animals and humans. When studying the carcinogenicity of CZ, Peffer et al.[8] found that，compared to control group, the incidence of cell adenoma and cancer increased significantly when the mice were exposed to CZ through diet for more than seven days. Cao et al.[9] found that the hatching rate of zebrafish embryos was significantly reduced and the deformity rate was significantly increased when exposed to CZ for a long time. In addition, Dajana et al.[10] found that CZ can induce the accumulation of triglycerides in animals and humans, causing a series of health problems, including hypertension, coronary heart disease and acute pancreatitis. In the study of the effect of CZ on human health, Ma et al.[11] found that the severity of fatty liver was closely related to the content of CZ. At present, triazole fungicides based on CZ account for nearly 20% of the fungicide-market share, and are widely used in many fields such as agriculture, forestry and gardening, leading to its large accumulation in the environment. Due to the wide application of CZ and its high stability, fluidity and adsorption, it accumulates in the ecological environment, especially in the water environment and soil environment for a long time, posing a threat to the ecosystem and affecting the balance of the ecosystem[12-14]. Therefore, it is particularly important to find scientific and reasonable means to effectively degrade CZ in environmental media, especially in water environment.

In recent years, the research on the degradation of triazole fungicides such as CZ has mainly focused on physical and chemical methods. Wang et al.[15] prepared β-cyclodextrin-polyacrylamide/covalent organic framework hydrogels at room temperature for efficient removal of triazole fungicides. Subsequent studies have shown that the prepared hydrogel has a removal rate of 79.4%-99.0% for triazole fungicides in water, but the preparation cost of the hydrogel is much higher than that of ordinary adsorption materials. After adsorption, the hydrogel has difficulties in subsequent treatment and cannot be reused. Souhila et al.[16] studied the combined removal of CZ in pure water by photocatalysis and Pseudomonas fluorescens. After 255 min of irradiation, the removal rate of CZ in water reached 85.8%, the mineralization rate was 38.5%, and the oxidation rate was 51.6%. Lhomme et al.[17] studied the excellent effect of photocatalytic treatment of CZ aqueous solution under $TiO_2$ dielectric coating. However, such chemical methods still have problems such as high treatment cost and high toxicity of by-products[18-19], which seriously restrict the development and promotion of related technical methods. Compared with traditional physicochemical methods, biological methods, as a low-cost and environmentally friendly pollutant degradation method, have received extensive attention in recent years. Existing research has proved the outstanding potential of biological methods in the treatment of refractory wastewater to a considerable extent[20-21]. However, due to the toxicity, drug resistance and stability of CZ, there is no report on the biodegradation of CZ by specific degrading strains.

In this study, we screened the water and soil polluted by CZ production wastewater for a long time, and isolated the special degradation strain TF-1 of CZ for the first time by means of continuous enrichment and purification. The effects of different growth environments (temperature, pH, strain inoculation amount, initial CZ concentration and additional carbon and nitrogen sources) on the degradation effect of the degrading strain were investigated by single factor experiments, and the optimum degradation conditions of the strain were explored. In addition, we tried to propose a way for the strain to degrade CZ by identifying and analyzing the intermediate products of the strain degrading CZ. Finally, in order to investigate the application effect of the strain in the actual sewage treatment, we inoculated the strain into the activated sludge reactor to verify the feasibility of its sewage bioaugmentation treatment.

## 2. Materials and Methods

### *2.1. Enrichment, isolation and identification of CZ*

The CZ used in this study was from Shanghai Aladdin Biochemical Technology Co., Ltd., with a purity of 99%. Other drugs in the experiment were from Sinopharm Chemical Reagent Co., Ltd, Shanghai Aladdin Biochemical Technology Co., Ltd. and other companies, and the purity was analytically pure. The microorganisms in the relevant water and soil were domesticated and screened by inorganic salt medium (MSM) with different CZ concentrations, and the degradation strains were isolated. The composition of the MSM medium is shown in Table 1 below[22]. The soil and water used in this experiment were from the activated sludge of a pesticide company in Nanjing for long-term treatment of CZ production wastewater pollution. After sampling, several slurry samples were dried, crushed and sifted, and then 10 g of samples were inoculated into 100 mL of MSM medium containing 50 mg·L$^{-1}$ CZ, and cultured in an oscillating incubator at 30 ℃ with a rotation speed of 180 rpm. After 3 h of constant temperature culture, 1 mL of the suspension was transferred into 50 mL of inorganic salt medium containing 20 mg·L$^{-1}$ CZ. Under the same conditions, enrichment culture was carried out in an oscillating culture box for 15 days. Then, 1 mL bacterial solution was transferred into a new inorganic salt medium, and the above transfer and culture steps were repeated three times. The concentration of CZ before and after culture in the medium solution was detected and compared. The experimental group with significantly reduced CZ concentration was selected. Continue to carry out new transfer and culture, and gradually increase the concentration of CZ in the inorganic salt medium to 50 mg·L$^{-1}$ for further enrichment and domestication. Then, the multiple dilution method was used, and the dilution multiple was $10^{-3}$-$10^{-8}$. The 100 μL bacterial solution was uniformly coated on the fresh LB solid plate medium at different dilution concentrations. After about one week of culture, a single dispersed colony plate was streaked on the LB solid plate medium for further purification and culture, and repeated many times. Finally, a single strain with obvious morphological characteristics was obtained.

*Table 1 Composition of MSM medium.*

| Medium | Chemical Reagent | Concentration |
|---|---|---|
| MSM | $Na_2HPO_4 \cdot 12H_4O$ | 0.76 g·L$^{-1}$ |
| | $KH_2PO_4$ | 0.20 g·L$^{-1}$ |
| | $MgSO_4 \cdot 7H_2O$ | 0.05 g·L$^{-1}$ |
| | $CaCl_2$ | 10 mL·L$^{-1}$ |
| | Trace Elements Solution SL-4 | 0.76 g·L$^{-1}$ |
| | CZ | 20-100 mg·L$^{-1}$ |

Then, the morphological, physiological and biochemical tests of the strain were carried out according to Bergey 's ' Determination of Bacteriology Manual '. In addition, we further identified the strain by 16S rDNA sequence analysis, and analyzed the nucleic acid sequence of the strain by BLAST (National Center for Biotechnology Information Databases) to determine its genus.

### *2.2. Biodegradation experiment of CZ*

In order to evaluate the biodegradability of TF-1, a 250 mL conical flask was used as an intermittent reactor. Under the basic conditions of 30 ℃, initial pH 9, initial CZ concentration of 50 mg·L$^{-1}$, and strain inoculation amount of 5%, the effects of temperature, initial pH, initial CZ concentration and strain inoculation amount on the degradation of CZ by TF-1 strain were investigated by changing single factor experiments. In addition, the effects of additional carbon or nitrogen sources on the degradation of tricyclazole were also investigated. At least three independent experiments were carried out for each condition, and the standard deviation was calculated by averaging the three independent analysis data. In this study, the logarithmic phase bacteria cultured in LB medium were selected as inoculums, and finally diluted to $OD_{600}$=1.5 by centrifugation, suspension and washing. The initial pH was adjusted by adding HCl and NaOH, respectively.

In this study, HPLC was used to determine the concentration of CZ. The liquid chromatography column was Waters $C_{18}$ column (5 μm, 4.6×150 mm), the detector was a diode array detector, the mobile phase was methanol and ultrapure water, the ratio was 7:3, the flow rate was 1.0 mL·min$^{-1}$, and the injection volume was 5.0 μL. The detection wavelength was 220 nm and the column temperature was 30 ℃.

Shen et al.[23] studied the biodegradation of nitrogen heterocyclic compounds, obtained the Gompertz equation model to reveal its degradation kinetics, and investigated the consumption of pollutants (Sc) per unit culture time during biodegradation. In this study, the model was used to express the degradation kinetics of CZ. The specific mathematical expression of the model is as follows:

$$S_c=\alpha exp(-\beta exp(-kt)$$

In addition, the mathematical expression of the maximum volumetric degradation rate ($V_{max}$, mg·L$^{-1}$·d$^{-1}$) in the biodegradation process is:

$$V_{max}=0.368\alpha k$$

### *2.3. Identification of intermediate products and degradation pathway of CZ*

In the CZ biodegradation experiment, 5 mL of bacterial solution was taken at intervals. The intermediates of CZ biodegradation were detected and analyzed by GC/MS and LC/MS. Before GC/MS analysis, the remaining bacterial solution was extracted with ethyl acetate, and then the extract was concentrated at 80 ℃, and then the concentrated solution was dissolved with methanol solution and determined on the machine. HP-5MS chromatographic column was used for GC/MS analysis, the column temperature was maintained at 280 ℃ for 5 min, and the electron energy was 70 eV. In the LC/MS analysis, the chromatographic column was Waters $C_{18}$ column (3.5 μm, 2.1×150 mm), the mobile phase was methanol and ultrapure water, the ratio was 7:3, the flow rate was 0.2 mL $\cdot$min$^{-1}$, and then the Waters triple quadrupole mass spectrometer was used in the mass spectrometry analysis. Based on the analysis of the intermediate products of CZ degradation, a possible CZ biodegradation pathway was proposed.

### *2.4. CZ biodegradable wastewater small-scale experiment*

In order to explore the feasibility of biodegradation of CZ wastewater, in this study, we inoculated the CZ specific degrading bacteria obtained in the previous study into the activated sludge reactor to treat the production wastewater containing CZ, in order to explore the feasibility of the treatment of CZ wastewater by the specific degrading bacteria. The activated sludge used in this experiment was from the PACT for the treatment of CZ production wastewater from a pesticide production company in Nanjing.

The small-scale test in this study was carried out in an activated sludge reactor with a volume of 35 L. A bubble diffusion device was installed at the bottom of the reactor to ensure that the dissolved oxygen concentration in the reactor was greater than 5.0 mg $\cdot$L$^{-1}$. In addition, we filled polypropylene filter balls in the reactor to solidify microorganisms and maintain microbial activity. The filling ratio was 25%. In addition, when the bioreactor was started, the activated sludge and 0.9% sodium chloride solution were added to control the MLSS of the reactor, and the initial MLSS concentration was about 3.5 g $\cdot$L$^{-1}$.

In the start-up stage of the reactor, the concentration of CZ in the simulated wastewater was 50 mg·L$^{-1}$, and the relevant operating parameters of the reactor are shown in Table 2. After 46 days of operation, the removal efficiency of CZ in the bioreactor was observed to be stable. At this time, in order to improve the removal efficiency of CZ, the specific degradation strains were inoculated in the reactor, and the dosage was 2.5 g (cell dry weight). The reactor was operated for 100 days. The biodegradability of the degrading strains was investigated. In the subsequent stages, the effects of these factors on the operation of the biodegradation reactor were investigated by changing the reactor temperature, pH, HRT and concentration of pollutant. During the operation, the sewage and sludge in the reactor were taken for related detection.

*Table 2 Operating parameters of the reactor at each stage*

| Phase | Purpose | Running time | Operating temperature | pH | Concentration of CZ | HRT |
|---|---|---|---|---|---|---|
| I | Research on system stability | 0-45 d | 30±1 ℃ | 9.5 | 50 mg $\cdot$L$^{-1}$ | 4 d |
| II | Biofortification performance | 46-100 d | 30±1 ℃ | 9.5 | 50 mg $\cdot$L$^{-1}$ | 4 d |
| III | Effect of operating temperature | 101-120 d | 25±1-35±1 ℃ | 9.5 | 50 mg $\cdot$L$^{-1}$ | 4 d |

| | | | | | | |
|---|---|---|---|---|---|---|
| IV | Effect of pH | 121-170 d | 30±1 °C | 9-10.5 | 50 mg L$^{-1}$ | 4 d |
| V | Effect of inoculation concentration | 171-230 d | 30±1 °C | 9.5 | 50-100 mg L$^{-1}$ | 4 d |
| VI | Effect of HRT | 231-260 d | 30±1 °C | 9.5 | 50 mg L$^{-1}$ | 2-5 d |

## 3. Result and discussion

The strain TF-1 obtained in this study was short rod-shaped, light yellow, full and convex in overall shape, and smooth in surface and edge. In the physiological and biochemical experiments of the strain, Gram staining was positive, contact enzyme reaction was positive, V.P test was positive, starch hydrolysis test was positive, nitrate reduction test was positive, and methyl red test was positive.

According to the available data, the TF-1 strain found in this study is the first CZ-specific degrading strain. In order to identify its strain, this study used Illunima novaseq 600 sequencing technology for sequencing, and the original data of sequencing results have been uploaded to the SRA database (BioProject accession number: PRJNA1184199). Subsequently, ABySS software and GapCloser software were used for assembly. The assembled nucleotide sequence has been uploaded to the GenBank database (GenBank accession number: PQ578010). Finally, the nucleotide sequence was compared by BLAST similarity and homology through NCBI database, and the strains with high similarity and homology were selected for cluster analysis and phylogenetic tree drawing (as shown in Figure 1). Based on the comprehensive sequence alignment and phylogenetic tree, it was found that TF-1 strain was closely related to *Bacillus*, and the similarity with *Halalkalibacterium halodurans* strain DSM 497 strain (NR_025446.1) was as high as 99.8%. Therefore, the strain TF-1 was identified as *Halalkalibacterium halodurans* strain TF-1.

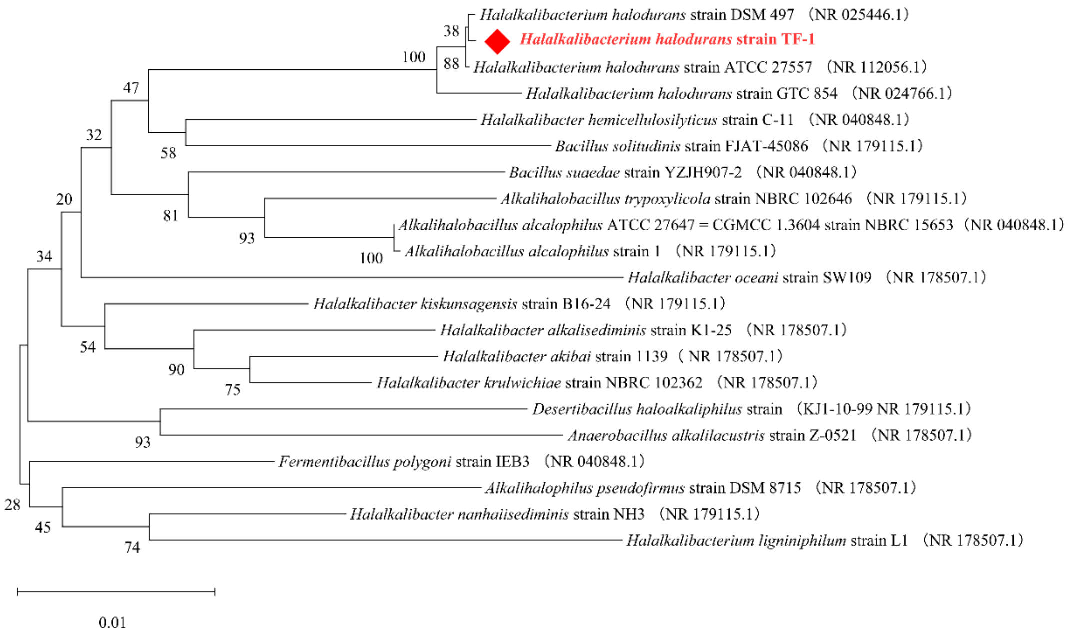

*Figure 1 Phylogenetic tree of TF-1 strain.*

Bacillus plays an important role in the field of microbiology. It is widely distributed, from deserts, Arctic, marine sediments to freshwater, hot springs, etc.[24]. Microbiology has a long history of research on it. At present, Bacillus has been widely used in industry, agriculture and medicine. In recent years, Bacillus has shown outstanding potential in the field of environmental governance, especially in the treatment of complex refractory pollutants such as heavy metals and highly toxic organic compounds[25-26]. Miao et al.[27] used three kinds of corn straw biochar produced at 300 ℃, 500 ℃ and 700 ℃ as carriers to immobilize *Bacillus cereus* MZ-1 when studying the pesticide imidacloprid in the biodegradable water environment. By adjusting the optimal strain addition amount, the removal rate of imidacloprid by the strain was up to 79.85% after 18 h of solidification. Xie et al.[28] isolated a strain of *Bacillus sp.* MY156 from a sewage plant for efficient degradation of dibutyl phthalate (DBP) and diethyl phthalate (DEP). Under the conditions of pH 7.0, temperature 30 ℃, inoculation amount 0.2 ($OD_{600}$), and bacterial load 2%, MY156 strain achieved complete degradation of 300 mg·L$^{-1}$ DBP within 60 h, and the removal rate of 200 mg·L$^{-1}$ DEP exceeded 80% within 5 d. In addition, Bacillus has a significant effect in removing metal ion pollution. A novel Cr (VI) -removing bacterium, *Bacillus paramycoides* Cr6, was isolated from the chromium-contaminated soil of a chromic acid plant in Hebei Province by Gu et al.[29]. Subsequent biodegradation experiments showed that Cr6 strain could completely degrade 100 mg·L$^{-1}$ Cr (VI) within 18 h. The TF-1 strain found in this study once again demonstrated the application potential of Bacillus in the process of pollutant biodegradation and environmental treatment.

### *3.1. Optimization of degradation conditions of strains*

#### *3.1.1. Effect of initial pH on the degradation of CZ by strain*

CZ with a concentration of 50 mg·L$^{-1}$ was added to the inorganic salt medium with an initial pH value of 6,7,8,9,10,11,12, and the degradation of CZ by the strain was investigated at a temperature of 30 ℃ and a strain inoculation amount of 5.0%. The results are shown in Figure 2.

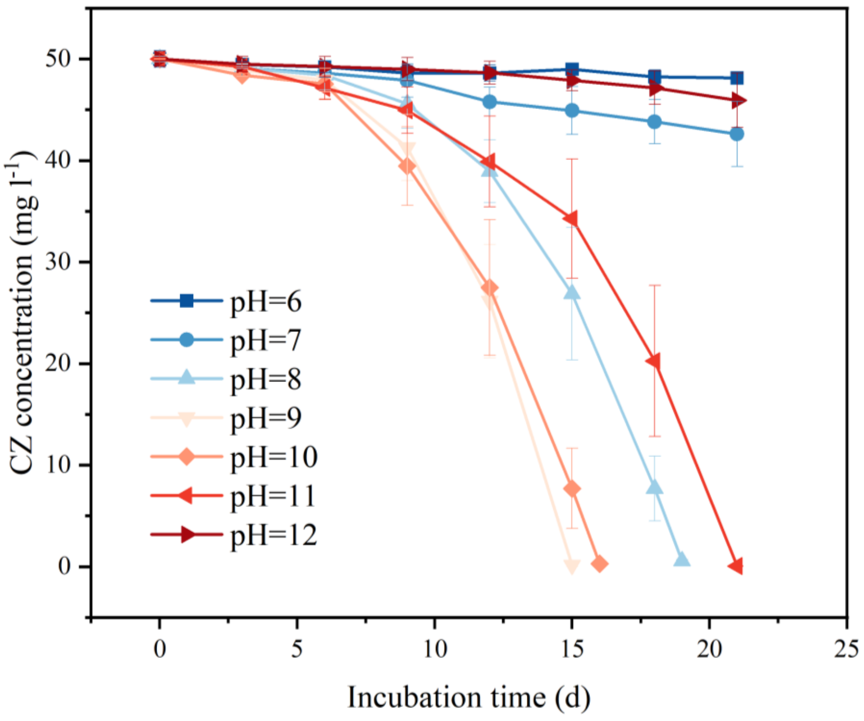

*Figure 2 Effects of different pH on the degradation of CZ by strains.*

It can be seen from the diagram that when the pH was 6 and 7, the residual amount of CZ in the medium within 21 days was 47.8±0.5 mg·L$^{-1}$ and 45.4±1.1 mg·L$^{-1}$, respectively, and the overall removal rate of CZ was only about 5%. When the pH was gradually increased to 9-11, the degradation of CZ in the medium could be basically completed within 15-21 days. When the pH was 9-10, the degradation of CZ could be achieved within 15-16 days. When the pH was further increased to 12, the removal efficiency of CZ was less than 5%. According to the comprehensive research results, the alkaline environment is most conducive to the degradation of CZ by the strain, and the optimal degradation conditions are achieved when the pH is 9, while the acidic or neutral environment cannot guarantee the normal growth and life activities of the halophilic and alkaliphilic strains. Similarly, David et al.[30] studied the degradation of cellulose degradation products in seawater or wastewater by a strain of *Bacillus marmarensis*. They found that the strain grew fastest at pH 9.0-10.5, and the life activities of the strain were almost completely stopped when the pH was lower than 7.0 and higher than 12.5. This is because the alkaliphilic microorganisms generally have a special cell working mechanism, which can acidify the cytoplasm in a certain range of alkaline environment to ensure that the cell biological enzymes are always in the best state.

### *3.1.2. Effect of temperature on the degradation of CZ by strain*

At the temperature of 20 ℃ to 40 ℃, 50 mg L$^{-1}$ CZ was added to the inorganic salt medium, the pH of the medium was controlled at 9, and the inoculation amount of the strain was 5.0%. The degradation of CZ by the strain within 16 days was investigated. The results are shown in the Figure 3. It can be seen from the diagram that the degradation efficiency of CZ by the strain increases first and then decreases with the increase of temperature. When the ambient temperature of the medium was 20 ℃, the CZ residue in the medium was 43.5±1.1 mg L$^{-1}$ after 16 days. When the temperature was increased to 25 ℃, 30 ℃ and 35 ℃, the degradation efficiency of CZ by the strain was significantly improved within 16 days. When the temperature was 30 ℃, the CZ in the medium was basically degraded within 15 days. When the temperature was 25 and 35 ℃, the CZ concentration in the medium was reduced by about 70%-80% after 16 days. Obviously, too high or too low temperature affects the growth of the strain, which in turn affects its degradation efficiency of CZ. Kang et al.[31] also found a similar situation in the study of the biodegradation of nicosulfuron.

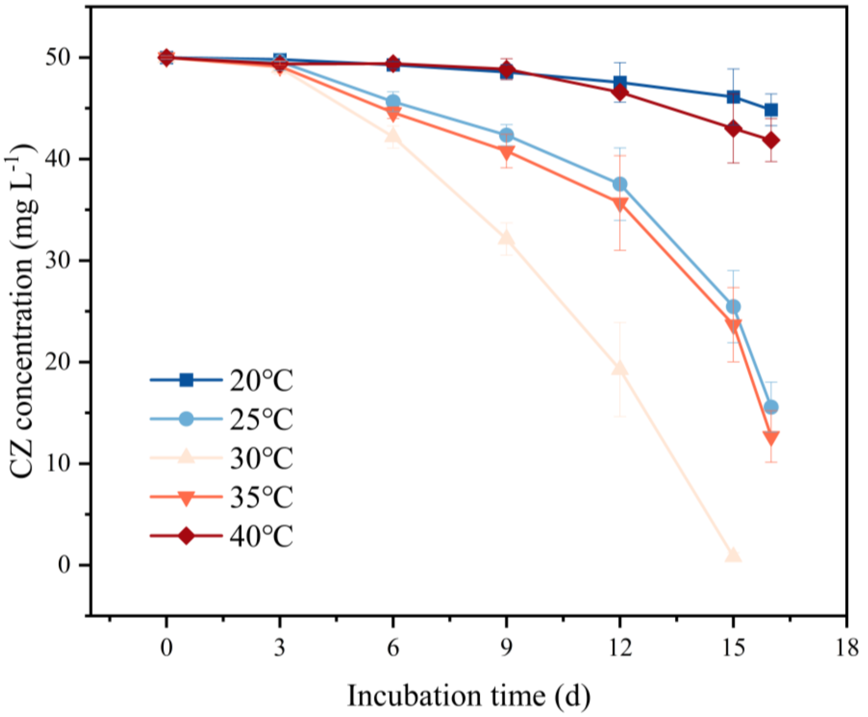

*Figure 3 Effects of different temperatures on the degradation of CZ by strains*

### *3.1.3. Effect of inoculation amount on the degradation of CZ by strain*

Under the conditions of initial pH value of 9, CZ concentration of 50 mg L$^{-1}$ and temperature of 30 ℃, 0.5% to 10% strains were inoculated in several inorganic salt media, respectively. The degradation of CZ under different inoculation amounts was investigated. The results are shown in the Figure 4.

It can be seen from the diagram that CZ in the medium can be degraded after 15-20 days after inoculation with different doses of strains. When the inoculation amount was 0.5%, the degradation efficiency of CZ was significantly lower, which was probably because the insufficient inoculation amount of the strain was greatly affected by the substrate concentration, and the higher substrate concentration inhibited the growth of a small number of strains, thus affecting the degradation efficiency of CZ. When the inoculation amount was increased to 1.0%-5.0%, the degradation efficiency of CZ was significantly improved, and the degradation rate of CZ was higher than 70% after 15 days. When the inoculation amount was 5.0%, the CZ in the medium was completely degraded after 15 days. However, when the inoculation amount was further increased to 10.0%, the degradation efficiency of CZ by the strain decreased, and more than 10% of CZ remained in the medium within 15 days. This is probably because the high inoculation amount leads to the rapid consumption of growth factors such as dissolved oxygen in the medium by a large number of strains, resulting in limited nutrients unable to supply the normal growth and reproduction of the strains, and a large number of strains enter the decline period in advance, affecting the degradation efficiency of CZ. Qian et al.[32] also found that the degradation efficiency of oxytetracycline increased first and then decreased with the increase of strain inoculation amount when studying the degradation of oxytetracycline by a strain of Enterococcus faecalis.

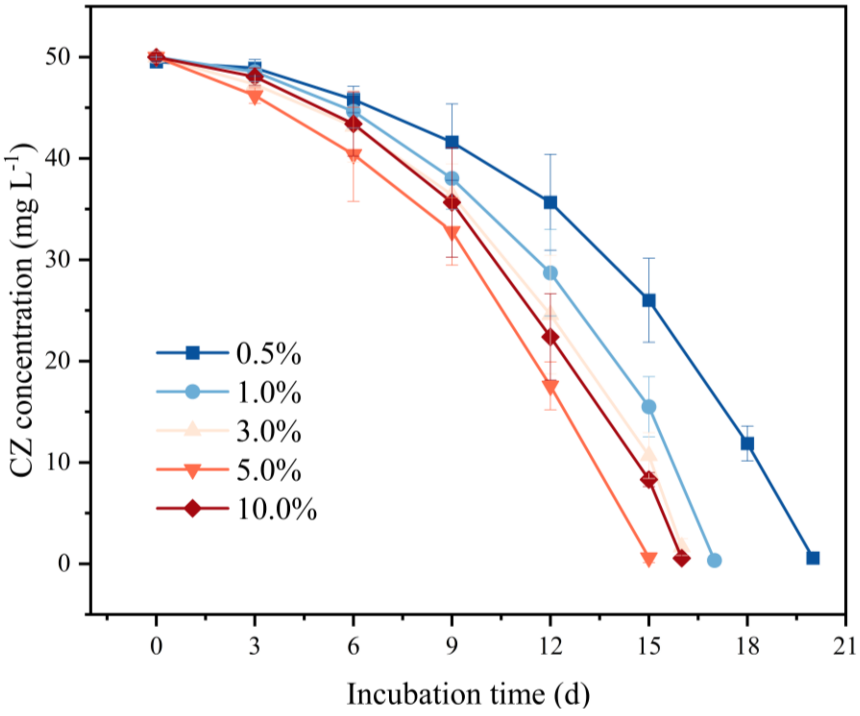

*Figure 4 Effects of different inoculation amount of strains on the degradation of CZ by strains*

### 3.1.4. Effect of initial pollutant concentration on the degradation of CZ by strain

Under the conditions of initial pH value of 9, temperature of 30 ℃ and inoculation amount of 5.0%, CZ with initial concentration form 20 mg Ł$^{-1}$- 80 mg·L$^{-1}$ was speculated in several inorganic salt media. The effect of initial pollutant concentration on the degradation of CZ by the strain was investigated. The specific results are shown in the following Figure 5.

As shown in the figure, in this study, the strain can degrade CZ at a concentration of 20-80 mg Ł$^{-1}$ within 8-18 d. From the change of the degradation curve on the graph, when the initial CZ concentration increased from 20 mg Ł$^{-1}$ to 80 mg Ł$^{-1}$, with the gradual increase of degradation time, the degradation rate at full concentration increased to varying degrees. This may be due to the fact that as the degradation process progresses, the continuous growth of biomass in the medium dilutes the inhibitory effect of initial pollutants and their intermediates on biodegradation. The same phenomenon appeared in the study of pyridine degradation by Rhizobium sp. NJUST18 by Shen et al.[33].

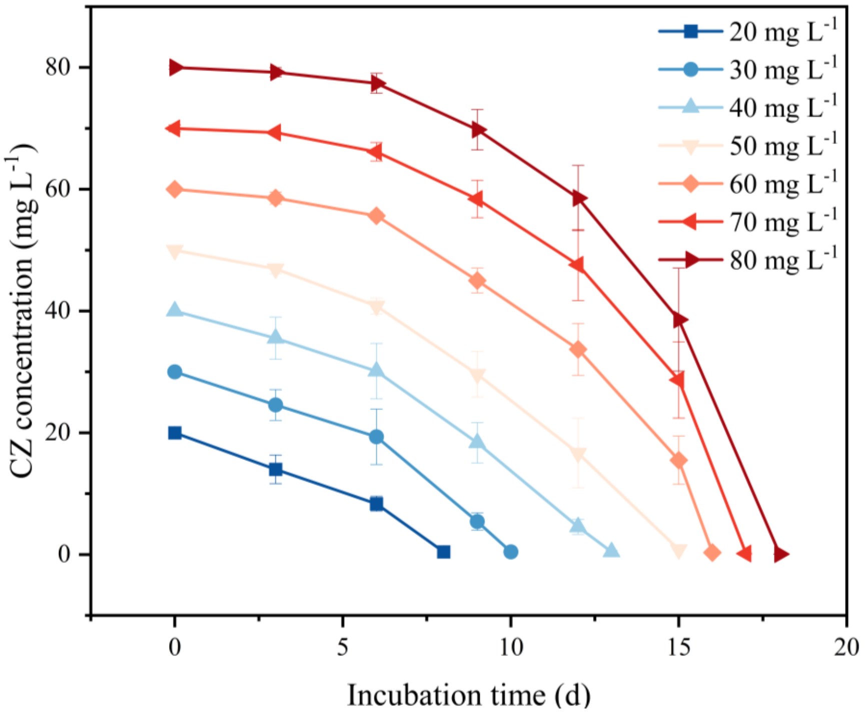

*Figure 5 Effects of different initial CZ concentrations on the degradation of CZ by strains*

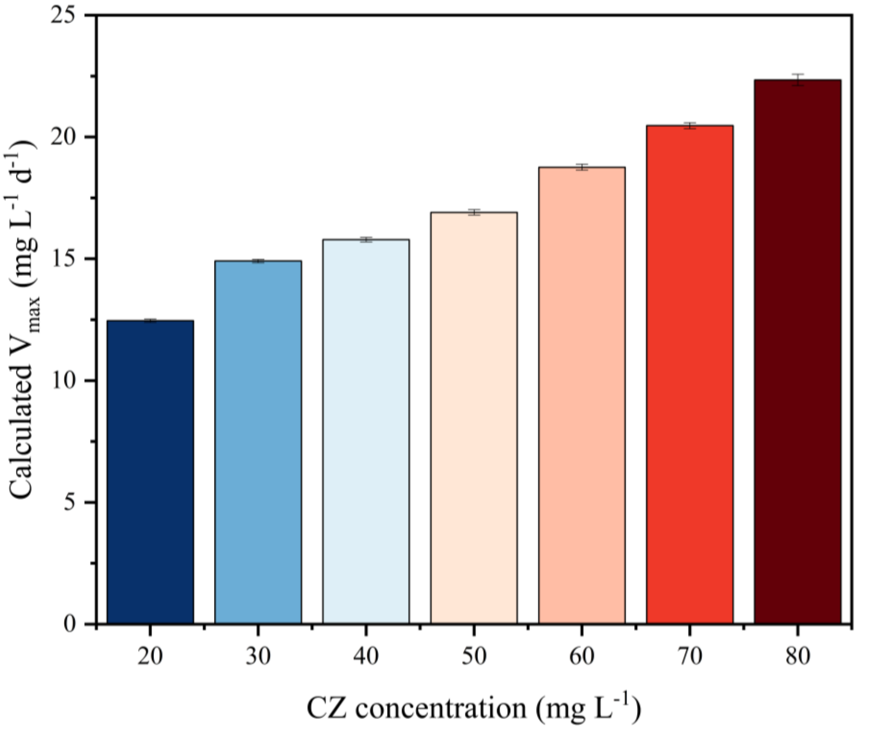

*Figure 6 The maximum volume degradation rate at each initial pollutant concentration*

In addition, through the further study of the degradation curve at each initial concentration, it was found that all the degradation curves conformed to the Gompertz model. According to the mathematical expression of the model, the maximum volume degradation rate at different concentrations was calculated, as shown in Figure 6. It can be seen from the figure that with the increase of the initial pollutant concentration, the maximum volume degradation rate increased from $12.2 \pm 0.06$ mg·L$^{-1}$·d$^{-1}$ to $22 \pm 0.12$ mg·L$^{-1}$·d$^{-1}$. It can be seen that with the increase of the concentration of the contaminated substrate, the degradation of CZ by the strain was not significantly affected, which proved that the strain had outstanding stability and impact resistance, and had the potential to treat complex wastewater.

### *3.1.5. Effect of additional organic carbon source on the degradation of CZ by the strain*

Under the conditions of initial pH value of 9, CZ concentration of 50 mg L$^{-1}$, temperature of 30 ℃, and strain inoculation amount of 5.0%, three organic carbon sources of sucrose, glucose, and yeast extract with concentrations of 500-2000 mg L$^{-1}$ were added to multiple inorganic salt media, and their effects on CZ degradation were investigated. The results are shown in Figure 7.

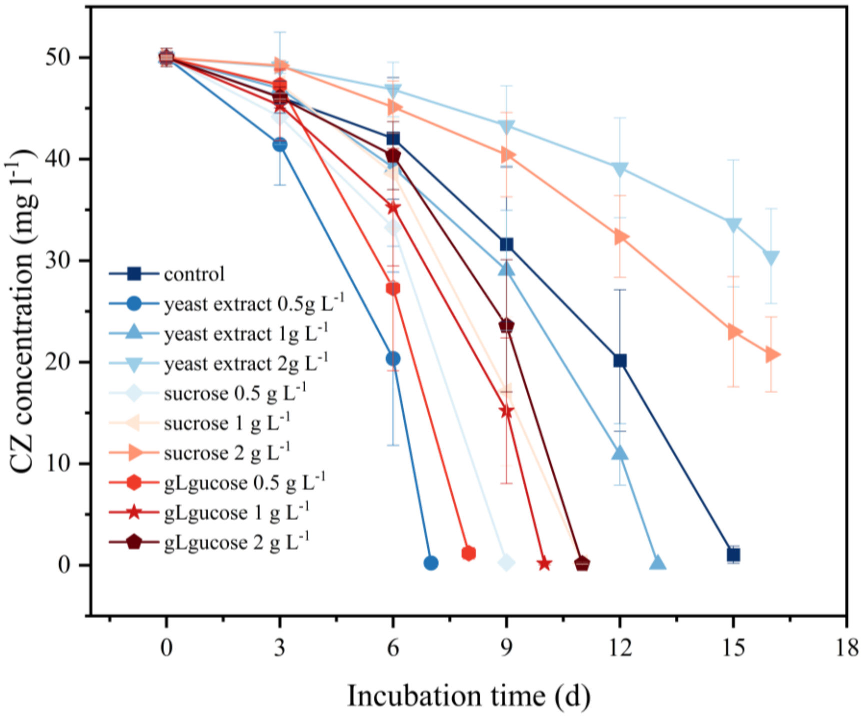

*Figure 7 Degradation of CZ by different concentrations of external organic carbon sources*

It can be clearly seen from the figure that under the addition of three organic carbon sources of 500-1000 mg L$^{-1}$, CZ in the medium can be degraded within 7-13 days. Compared with the control group, the complete degradation of CZ was achieved within 15 days, and the degradation rate was significantly improved. Among them, 500 mg L$^{-1}$ yeast extract had the most significant effect on the degradation rate, which could achieve complete degradation of CZ within 7 days. Vertically, with the increase of the concentration of each organic carbon source, the degradation rate of CZ slowed down, and even the addition of 2000 mg L$^{-1}$ sucrose and yeast extract inhibited the degradation of CZ. Among them, yeast extract with significant effect on CZ degradation at low concentration showed great inhibition effect at high concentration, and the degradation efficiency of CZ was less than 40% within 16 days. Based on the above comparison, the addition of low concentration of organic carbon source has an extremely obvious effect on the degradation of CZ in the medium, and when the concentration of organic carbon source is gradually increased, this gain effect will slow down, and even when the concentration of organic carbon source is added to a certain extent, the addition of organic carbon source will inhibit the biodegradation of CZ. The reason for this situation is likely to be due to the limited growth factors such as dissolved oxygen and microorganisms in the medium. Although there are sufficient carbon sources, microorganisms are unable to digest them effectively. Excessive carbon sources will inhibit the normal growth, metabolism and degradation of microorganisms to a certain extent.

### *3.1.6. Effects of additional inorganic nitrogen sources on the degradation of CZ by strains*

Under the conditions of initial pH value of 9, CZ concentration of 50 mg L$^{-1}$, temperature of 30 ℃, and strain inoculation amount of 5.0%, three different inorganic nitrogen sources of $NH_4^+$, $NO_3^-$ and $NO_2^-$ with a concentration of 2.5-10 mM were added to the medium, and the blank group was used as a control to investigate the effect of external inorganic nitrogen sources on the degradation of CZ by the strain. The results are shown in Figure 8.

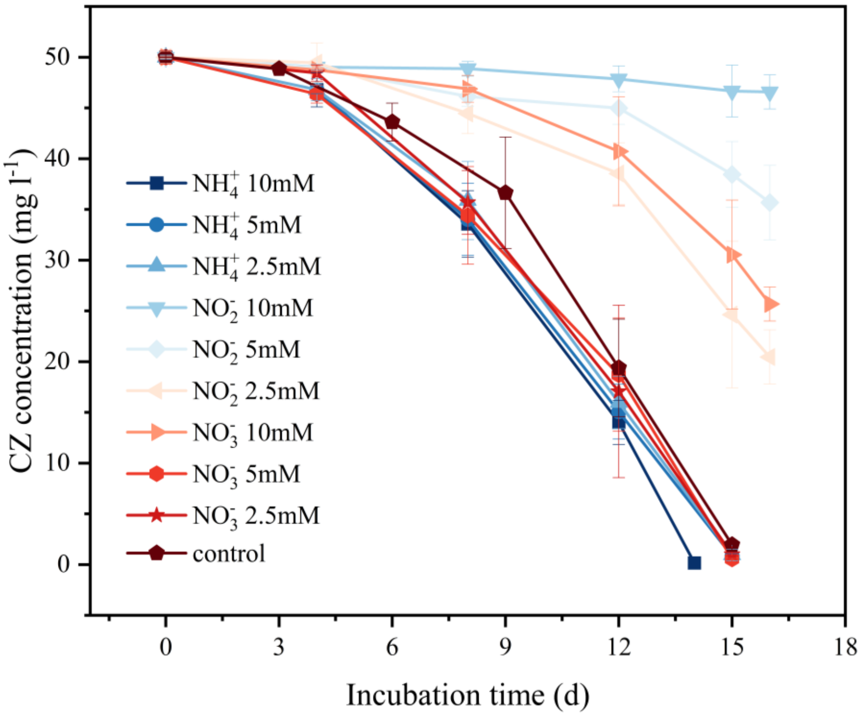

*Figure 8 Degradation of CZ by different concentrations of inorganic nitrogen sources*

From the figure, by comparing with the blank group, the addition of $NH_4^+$ and $NO_3^-$ has little effect on the biodegradation of CZ and even has some gain effect. Basically, all experimental groups can achieve the degradation of CZ in 14-15 d. Only when the concentration of $NO_3^-$ was 10 mM, the degradation of CZ was inhibited, and the degradation rate of CZ was only about 50% after 16 days. This is likely because excessive $NO_3^-$ has an inhibitory effect on the synthesis of growth elements such as microbial nucleotides and proteins, thus affecting the efficacy of biodegradation. Compared with the other two nitrogen sources, $NO_2^-$ at the full concentration of the experiment (2.5 mM, 5 mM, 10 mM) showed an inhibitory effect on the degradation of CZ by the strain. Under the addition of 2.5 mM $NO_2^-$, the removal rate of pollutants was less than 60% before and after 16 days, and the degradation efficiency of CZ continued to decrease with the increase of $NO_2^-$ concentration. Even when 10 mM $NO_2^-$ was added, the degradation of pollutants by the strain was almost completely inhibited, and the degradation efficiency was about 5 % within 16 days. This is likely because $NO_2^-$ is toxic to microorganisms[34]. Using $NO_2^-$ as a nitrogen source will have a great impact on the degradation of microorganisms. In general, the appropriate amount of ammonia nitrogen and nitrate nitrogen had a certain promoting effect on the degradation of CZ by the strain, while nitrite nitrogen had a significant inhibitory effect on the biodegradation of CZ.

### ***3.2. Analysis of intermediate products and degradation mechanism of CZ degradation by strains***

In this study, LC/MS and GC/MS were used to identify the intermediate products of CZ degradation by the strain. A total of 17 intermediate products were found by detection. The 17 intermediate products were named M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15, M16 and M17, respectively. Through the analysis of the structure and properties of various degradation intermediates, we proposed three main CZ degradation pathways A, B, and C, as shown in Figure 9 below.

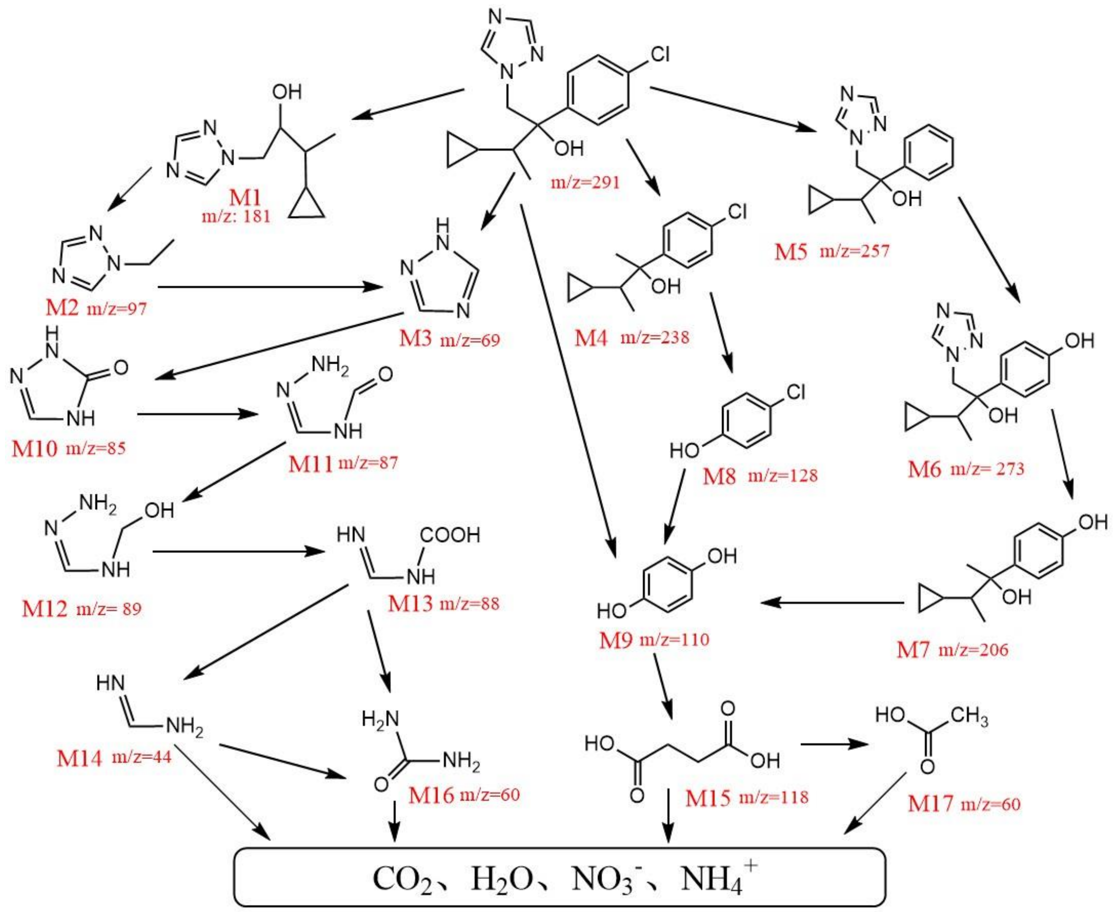

*Figure 9 The main biodegradation pathways of CZ by strains*

Pathway A: In pathway A, CZ first removed the halogen on the benzene ring to generate M5, and then the hydroxyl radical ( ·OH) replaced the position of the halogen on the benzene ring to generate M6; then the C-N single bond in M6 was also attacked and broken by free radicals. M6 removed triazole to form M7, in which triazole was degraded in a certain way (mentioned in pathway B). The substitution reaction of M7 occurred again, and the hydrocarbon chain on the benzene ring was replaced by ·OH to form M9. After M9 is further oxidized, a ring-opening reaction occurs to produce M15 and a subsequent smaller molecular product M17. Both M15 and M17 may be further degraded into small molecular inorganic substances such as $CO_2$, $H_2O$, $NO_3^-$ and $NH_4^+$.

Pathway B: In pathway B, CZ was hydrolyzed to remove the benzene ring and generate M1 and M9, and the subsequent degradation of M9 was basically the same as that of pathway A. In pathway B, CZ was hydrolyzed to remove the benzene ring and generate M1 and M9. Under the action of ·OH, M1 first forms M2, and the C-N in M2 is further attacked by ·OH to form M3. M3 is oxidized to M10 by a single oxygen oxidation process, and then M10 undergoes ring cleavage to form M11. In the degradation process of M11, hydration reaction occurs first to form M12, and then M13 is formed in the subsequent oxidation and deamination reaction of M12, and $NH_4^+$ is released in this process. Then M13 is decarboxylated to form M14, and M14 is oxidized to M15. Both M14 and M15 will be mineralized and decomposed in the subsequent reaction.

Pathway C: In the pathway C, the C-N in the CZ was first attacked and broken to generate M3 and M4, and the subsequent degradation of M3 can be seen in pathway B. In the pathway C, the C-N in the CZ was first attacked and broken to generate M3 and M4. In the subsequent degradation process of M4, the long chain on the benzene ring was first replaced by ·OH attack to form M8, and the chlorine on the subsequent M8 was also replaced by hydroxyl to form M9. The subsequent biodegradation process of M9 was the same as that of M9 in pathway A. Subsequent products were mineralized into small molecules in further reactions.

Through the 17 intermediate products detected by LC/MS and GC/MC, it was speculated that the main metabolic processes involved in the degradation of CZ by the strain included oxidation, hydrolysis ring opening, dehydroxylation, deamination, decarboxylation, dechlorination, hydroxylation, hydration and final mineralization. In addition, the biodegradation of CZ involves these processes catalyzed by a variety of enzymes, including hydroxylase, decarboxylase, dehydrogenase, demethylase, etc. During the whole degradation process, the chemical bonds such as C=N, C-N and C-Cl in CZ were broken under the action of OH to form a series of intermediate products. These intermediate products were further reacted and converted into simple small molecule inorganic substances such as $CO_2$, $H_2O$, $NO_3^-$and $NH_4^+$.

### *3.3. Wastewater small-scale experiment*

Firstly, this study investigated the removal of COD, TOC and CZ in the reactor as shown in the following Figure 10. It can be seen from the diagram that the removal rate of CZ in the reactor was only about 10 %, and the removal rate of COD and TOC was only about 20-30 % without TF-1 bacteria inoculation in the reactor in the first 46 days. After 46 days of operation, TF-1 strain was inoculated into the activated sludge of the reactor. After a period of operation, the removal efficiency of CZ, TOC and COD was significantly improved. Among them, the removal efficiency of CZ is greatly improved to about 98 %, and it can be stably maintained for a long time. The removal rates of COD and TOC in the reactor were steadily increased to about 35 % and 40 %, respectively. It can be seen that TF-1 strain has outstanding potential to be used as a microbial enhancer in actual wastewater treatment.

In addition, the effects of reactor temperature, reactor pH, initial substrate concentration and HRT on the operation of the reactor were also investigated in this study, as shown in Figure 11. Based on the information on the comprehensive map, considering the actual operation cost, this study believes that when the temperature is 30±1 ℃, the pH is 9.5, and the HRT is 4 d, the reactor operation is the best. The research shows that the reactor has good impact resistance and load resistance.

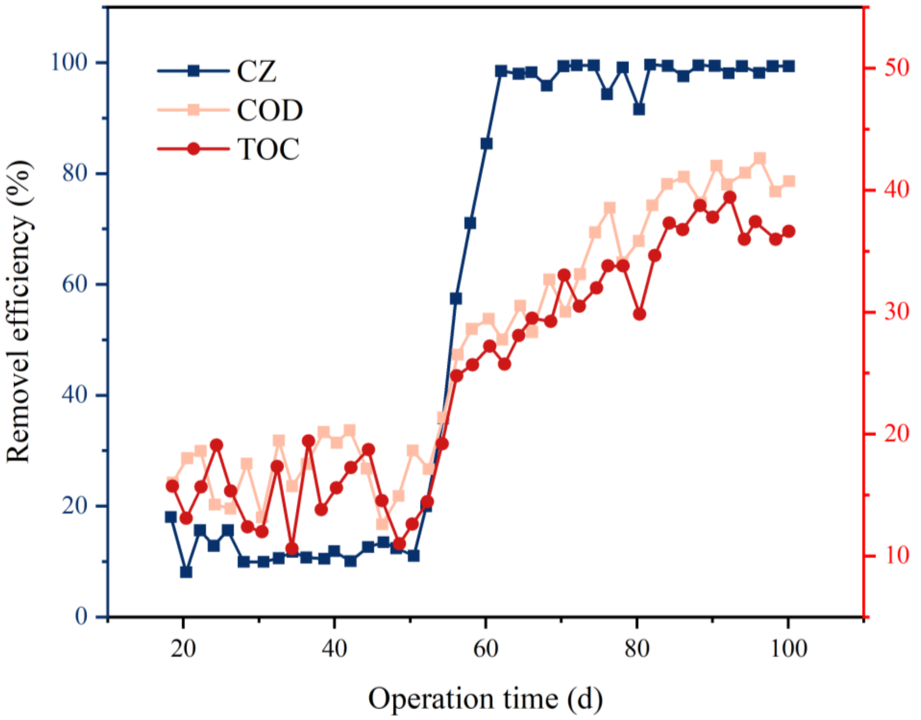

*Figure 10 Degradation of CZ in the reactor*

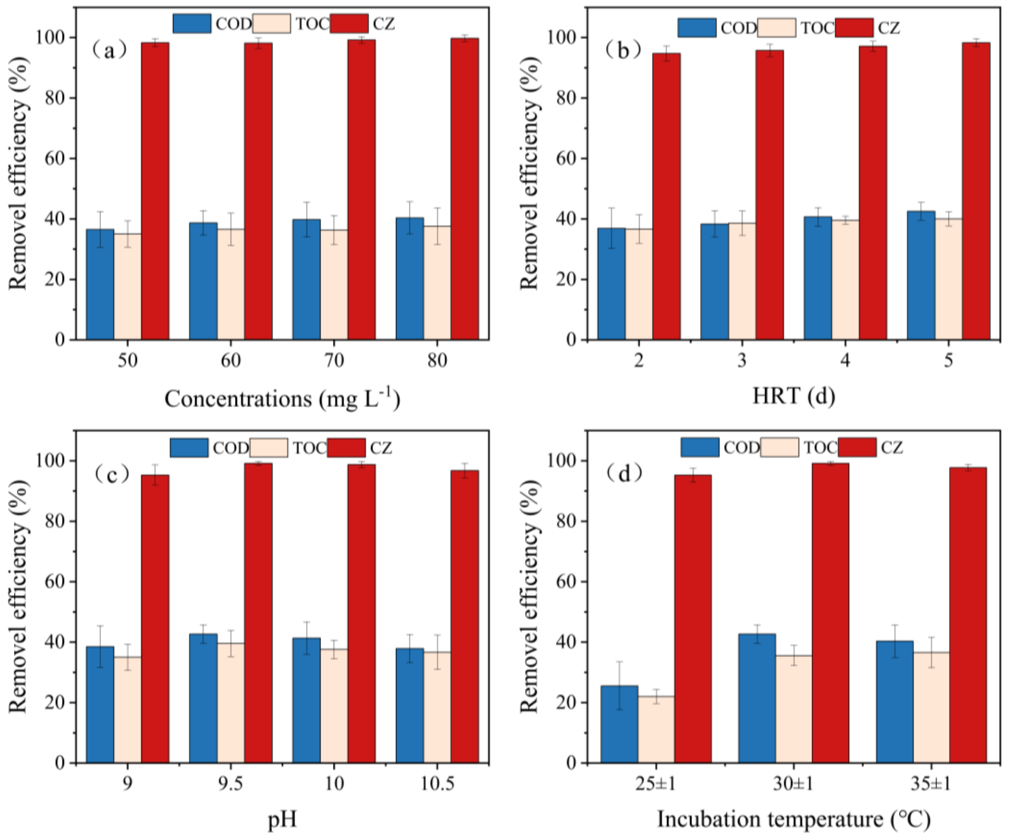

*Figure 11 Degradation of pollutants in the reactor under various factors*

## 4. Conclusion

1) In this study, a highly efficient degradation strain TF-1 capable of using CZ as the sole carbon and nitrogen source was isolated for the first time from the activated sludge of the domesticated sewage treatment plant. The strain was identified as *Halalkalibacterium halodurans* by morphological observation, physiological and biochemical experiments, 16S rRNA gene sequencing and phylogenetic tree analysis. It was named *Halalkalibacterium halodurans* strain TF-1.

2) The suitable degradation conditions of the strain were obtained by single factor experiment as follows: initial pH value was 9, culture temperature was 30 ℃, and inoculation amount was 5.0%. The strain could completely degrade CZ with a concentration of 20-80 mg L$^{-1}$ within 18 days, which proved that it had outstanding impact resistance and degradation performance.

3) Low concentration of organic carbon source speculation is conducive to the degradation of CZ by the strain; however, the addition of high concentrations of organic carbon sources inhibited the degradation of CZ by the strain. The addition of appropriate amount of $NO_3^-$ and $NH_4^+$ was beneficial to the degradation of CZ by the strain, while the addition of $NO_2^-$ showed an inhibitory effect on the whole process of CZ biodegradation.

4) A total of 17 intermediates were obtained by LC/MS and GC/MS. Three degradation pathways were obtained by analyzing the 17 intermediates, including oxidation, hydrolysis ring opening, dehydroxylation, deamination, decarboxylation, dechlorination, hydroxylation, hydration reaction and final mineralization.

5) Further small-scale wastewater experiments proved that microbial inoculation can effectively improve the removal efficiency of CZ by activated sludge and reduce the biological toxicity of wastewater. By changing the operating conditions of the reactor, the optimal conditions for the operation of the activated sludge reactor were explored as follows: temperature of 30±1-35±1 ℃, pH of 9.5, and HRT of 5 d. The study further proved the impact resistance and load resistance of TF-1 strain, and verified the feasibility of bio-enhanced degradation of CZ wastewater by the strain to a certain extent.

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