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Enhancing Microbial Superoxide Generation and Conversion to Hydroxyl Radicals for Enhanced Bioremediation Using Iron-Binding Ligands

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ABSTRACT

Harnessing bacteria for superoxide production in bioremediation holds immense promise, yet its practical application is hindered by slow production rates and the relatively weak redox potential of superoxide. This study delves into a cost-effective approach to amplify superoxide production using an *Arthrobacter* strain, a prevalent soil bacterial genus. Our research reveals that introducing a carbon source along with specific iron-binding ligands, including deferoxamine (DFO), diethylenetriamine pentaacetate (DTPA), citrate, and oxalate, robustly augments microbial superoxide generation. Moreover, our findings suggest that these iron-binding ligands play a pivotal role in converting superoxide into hydroxyl radicals by modulating the electron transfer rate between Fe(III)/Fe(II) and superoxide. Remarkably, among the tested ligands, only DTPA emerges as a potent promoter of this conversion process when complexed with Fe(III). We identify an optimal Fe(III) to DTPA ratio of approximately 1:1 for enhancing hydroxyl radical production within the *Arthrobacter* culture. This research underscores the efficacy of simultaneously introducing carbon sources and

DTPA in facilitating superoxide production and its subsequent conversion to hydroxyl radicals, significantly elevating bioremediation performance. Furthermore, our study reveals that DTPA augments superoxide production in cultures of diverse soils, with various soil microorganisms beyond *Arthrobacter* identified as contributors to superoxide generation. This emphasizes the universal applicability of DTPA across multiple bacterial genera. In conclusion, our study introduces a promising methodology for enhancing microbial superoxide production and its conversion into hydroxyl radicals. These findings hold substantial implications for the deployment of microbial reactive oxygen species in bioremediation, offering innovative solutions for addressing environmental contamination challenges.

Keywords:

Superoxide

Hydroxyl Radical

Iron-Binding Ligand

Bioremediation

DTPA

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1 Introduction

Recent discoveries regarding the production of superoxide by heterotrophic bacteria across a wide range of taxonomic groups (Diaz et al., 2013) have underscored the pivotal role of extracellular reactive oxygen species (ROS) in the degradation of organic contaminants in natural environments, owing to their heightened reactivity. ROS, whether directly or indirectly, have been reported to play a facilitating role in pollutant degradation. For example, in microbial cultures from various environments such as soil (Gu et al., 2016; Huang et al., 2020), marine (Gu et al., 2018), and freshwater (Yin et al., 2016) ecosystems, superoxide and hydrogen peroxide have been observed to degrade pollutants through direct reactions or processes leading to the formation of other oxidizing products (e.g., biogenic manganese oxides) (Learman et al., 2011). This versatility in reactions enables ROS to target a broader range of pollutants.

Among the multitude of ROS encountered in microbial cultures, superoxide stands out as the most frequently detected. However, leveraging microbial extracellular superoxide for environmental remediation comes with its own set of challenges, including the relatively slow production rate and low oxidizing potential of superoxide. These limitations can hinder the achievement of satisfactory degradation rates unless superoxide is efficiently converted into a more potent oxidizer, such as the hydroxyl radical (Dong et al., 2021; Oliveira et al., 2016). Consequently, there is an urgent need to develop cost-effective protocols that can simultaneously enhance the production rate of microbial extracellular ROS and promote the conversion of superoxide into hydroxyl radicals.

In our previous research, we have identified a method to enhance the production rate of microbial superoxide. We demonstrated that fluctuations in carbon source levels, induced by the presence of siderophores (iron-binding ligands secreted by microorganisms to facilitate iron uptake), can stimulate extracellular superoxide production in *Arthrobacter* species cultures (Ning et al., 2022). This stimulation likely occurs through complexation and disruption of iron-bearing molecules within the electron transfer chain (ETC). The addition of carbon sources and deferoxamine (DFO),

a type of siderophore, led to a remarkable maximum superoxide production rate of approximately $2,200 \text{ pmol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$ in *Arthrobacter* cultures cultured in a PYG medium for 8 days, surpassing reported rates in cultures of other heterotrophic microorganism isolates, which typically range from 0 to $25 \text{ pmol} \cdot \text{mL}^{-1} \cdot \text{min}^{-1}$ (Diaz et al., 2013). This discovery underscores the significant role of siderophores in stimulating microbial superoxide production, although their high cost limits their application.

In this study, we observed that iron-binding ligands other than siderophores can also enhance extracellular superoxide production by *Arthrobacter* species, offering a cost-effective approach for accelerating microbial ROS production. Since the reaction rate of iron with superoxide and hydrogen peroxide can be altered after complexation with ligands, it is conceivable that complexation might either promote or inhibit the conversion of superoxide to hydroxyl radicals. Hence, the quest for an appropriate iron-binding ligand capable of simultaneously enhancing superoxide production and facilitating its conversion into hydroxyl radicals becomes imperative.

To address these challenges, our study systematically screened for a suitable iron-binding ligand that promotes superoxide production in an *Arthrobacter* strain. Additionally, we investigated the potential mechanisms involved in Fe(III)-ligand-enhanced hydroxyl radical production within the *Arthrobacter* culture. We also explored the performance of microbial hydroxyl radicals in degrading organic pollutants and evaluated the impact of these iron-binding ligands on superoxide production within the soil microbiome to ascertain the method's universality for other bacterial genera. Our findings open a novel technical avenue based on microbial advanced oxidation for pollutant remediation, contributing to the expanding body of knowledge in the field of environmental bioremediation.

2 Materials and methods

2.1 Soil sample collection and cultivation

Soil samples were collected from a Litchi orchard in Shenzhen, Guangdong, China, at depths of 0 cm, 5 cm, and 10 cm, from three distinct sites within each field. These

samples were subsequently combined in equal proportions to create a single composite sample for each field. To prepare the samples, 45 g of each composite sample was mixed with 100 mL of 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; ACMEC, China) buffer at pH 7.2. The suspensions were then agitated at 170 r/min and 30°C for 1 hour. The resulting soil suspensions were introduced into PYG medium (Peptone Yeast Glucose Broth), which contained glucose (250 mg/L; Aladdin, China), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (500 mg/L; Macklin, China), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (60 mg/L; Macklin, China), peptone (250 mg/L; Meryer, China), yeast extract (250 mg/L; Meryer, China), and 10 mM HEPES at pH 7.2 (Liang et al., 2017), at four different nutrient concentrations, including the original concentration and its diluted solutions (2, 5, and 10 times). The inoculation proportion was 5% (vol/vol), and cultures were maintained at 30°C with agitation at 170 r/min.

2.2 *Arthrobacter* strains and cultivation

The *Arthrobacter* strain *Arthrobacter* sp. QXT-31 (referred to as *A. QXT-31*) used in this study was previously isolated from a manganese mine in Hunan Province, China. Both the *A. QXT-31* strain and the soil microbiome were cultured in PYG medium or diluted PYG medium. For activation, 1 mL of the *A. QXT-31* culture or soil microbiome was inoculated into 30 mL of PYG medium and incubated for 24 hours. Subsequently, cultures were inoculated into fresh PYG medium at a ratio of 3% (vol/vol) and incubated at 30°C with agitation at 170 r/min for subsequent experiments.

2.3 Enhancement of superoxide production by iron-binding ligands

Certain number of *A. QXT-31* cultures (cultured in PYG medium for 2 days) was collected for superoxide detection. Four types of iron-binding ligands (diethylenetriamine pentaacetate - DTPA; Macklin, China, deferoxamine - DFO; J&K Scientific, China, citrate; Aladdin, China, and oxalate; Aladdin, China) were introduced to the *A. QXT-31* culture at varying concentrations (0, 5, 50, 100, 500, and 1000 μM) to observe their influence on superoxide production by *A. QXT-31*. Subsequently, sterile glucose (50 mg/L) was added to the cultures to induce superoxide production.

Superoxide was quantified using MCLA (125 μ M, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo [1,2-a] pyrazin-3(7H)-one; TCI, Japan), with luminescent signals recorded continuously for 2 hours using a microplate reader (Tecan, Switzerland) (Godrant et al., 2009). Further details are provided in Appendix A **Method S1**.

2.4 The role of Fe(III) and iron-binding ligands in hydroxyl radical production

The Fe-ligand solutions were prepared as follows: Four types of iron-binding ligands (DTPA, DFO, citrate, and oxalate) were mixed with a FeCl₃ solution (Aladdin, China) in a molar coordination ratio of 1:1. The mixture was shaken at 170 r/min for 1 hour to allow the complex reaction to proceed completely. To study the effect of Fe(III) and iron-binding ligands on the conversion of superoxide into hydroxyl radicals, the resulting complexed solution (50 μ M, calculated as Fe(III)) was added to a solution containing xanthine (50 μ M; Shanghai Yuanye Bio-Technology Co., Ltd, China) and xanthine oxidase (0.5 U/L; Sigma-Aldrich, USA). The pH of the mixture was maintained at 7.2 by adding 10 mM HEPES buffer (pH 7.2) to ensure a constant rate of superoxide production by the reaction of xanthine and xanthine oxidase (Fridovich, 1970). A solution containing only xanthine (50 μ M) and xanthine oxidase (0.5 U/L) was used as a control. Additionally, a treatment with the addition of Fe(III) (50 μ M) was also included to explore the effect of iron alone on hydroxyl radical production. Tert-butanol was introduced to the treatments mentioned above to quench hydroxyl radicals, serving as a negative control of hydroxyl radicals. Hydroxyl radicals were trapped by 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO; GLPBIO, USA) and detected with electron spin resonance (ESR; Magnettech ESR5000, Bruker, Germany). Further details can be found in Appendix A **Method S2**.

2.5 Influence of Fe(III)/DTPA ratio on hydroxyl radical generation

Fe(III) (FeCl₃) was complexed with DTPA (50 μ M) in ultrapure water in varying ratios (2:5, 4:5, 1:1, 3:2, 2:1) for 15 minutes. The resulting mixture was then introduced to the *A. QXT-31* culture. To initiate superoxide production by the *A. QXT-31* culture, glucose (50 mg/L) was added, and salicylic acid (500 μ M; Aladdin, China) was introduced to

capture the hydroxyl radicals (Martínez-Tarifa et al., 2010). The amount of 2,3-dihydroxybenzoic acid (2,3-DHBA; TRC, Canada) generated in the culture were quantified during a 1-hour reaction time using ultra-performance liquid chromatography (UPLC, ACQUITY, Waters, USA) to quantify hydroxyl radicals. Additional details are provided in Appendix A **Method S3**.

2.6 Bacterial degradation of oxytetracycline via ROS

To investigate bacterial degradation of oxytetracycline through ROS, a solution of Fe(III)-DTPA (at a 1:1 ratio, 50 μ M), glucose (50 mg/L), and oxytetracycline (1 mg/L; Macklin, China) was sequentially added to a 40 mL culture of *A. QXT-31*. The mixture was thoroughly mixed, and samples were collected every 10 minutes to determine the remaining oxytetracycline concentration using UPLC. Further details can be found in Appendix A **Method S4**. As a control, a parallel treatment without Fe(III)-DTPA was conducted to represent oxytetracycline biodegradation solely by superoxide. SOD (120 kU/L; Gansu Yangtaihe Biotechnology Co., Ltd., China) and CAT (50 mg/L; Macklin, China) were added to neutralize the hydroxyl radicals generated in the culture. This enzyme-dependent approach was employed instead of the typical hydroxyl radical quencher tert-butanol to avoid cytotoxicity of tert-butanol.

2.7 Superoxide detection of soil samples

For superoxide detection in soil sample cultures, 1 mL of soil sample culture was collected every 24 hours. Sterile glucose (50 mg/L) was added to the collected culture to induce microbial superoxide production. Two types of iron-binding ligands (DTPA, DFO) were individually introduced into the collected cultures at varying final concentrations (0, 5, 50, 100, 500, and 1000 μ M) to investigate their impact on microbial superoxide production. Superoxide was quantified using MCLA, with luminescent signals recorded continuously for 3 hours using a microplate reader (Godrant et al., 2009). Further details are provided in Appendix A **Method S1**.

2.8 16S rDNA Sequencing and analysis

For soil sample culture (5 mL), cells were harvested by centrifugation (10000 g, 5 min) every 24 hours for DNA extraction. DNA extraction was performed using the Soil Genomic DNA Extraction Kit D2600-50T (Solarbio, China), following the manufacturer's instructions. Extracted DNA was sent to Guangdong Meige Gene Technology Co., LTD for next-generation sequencing of the V3-V4 regions of the 16S rDNA using the 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers (Zeng and An, 2021). Raw data from this study were deposited in the NCBI Sequence Read Archive database under accession number PRJNA944413. Sequencing results were analyzed using Qiime2 (v2020.11) (Bolyen et al., 2019), including denoising with DADA2 (via q2-dada2) (Callahan et al., 2016) and species annotation by comparison with the Silva database (v138.1) (Quast et al., 2012). Linear discriminant analysis effect size (LEfSe) and correlation analysis were employed to identify microbial groups responsible for superoxide production in the soil sample cultures.

2.9 Data analysis method

All experiments in this study were conducted in triplicate, and the figures present the mean value $\pm$ standard deviation. We assessed the significance of differences using one-factor ANOVA with Tukey's HSD test and paired samples t-test, considering a probability value of $P < 0.05$ as significant. Pearson correlation analysis was employed to assess the degree of correlation between variables, with a probability value of $P < 0.05$ considered significant. The strength of the correlation was evaluated using the Pearson correlation coefficient (Pearson's r).

3 Results and Discussion

3.1 Exploring a low-cost iron-binding ligand to enhance microbial production of superoxide

While various siderophores have been shown to boost microbial superoxide production during fluctuations in carbon source levels (Ning et al., 2022), their prohibitive cost makes them unsuitable for practical engineering applications. To address this challenge,

we sought iron-binding ligands that could offer cost-effectiveness while delivering comparable performance. Our selection included DTPA (with a logarithm of the stability constant of coordination compounds with ferric ion, $\log K_{\beta n} = 28.0$, an octadentate ligand), citrate ($\log K_{\beta n} = 11.85$, a quadridentate ligand), and oxalate ($\log K_{\beta n} = 9.4$, a bidentate ligand), and we compared their effectiveness in enhancing superoxide production in *A. QXT-31* cultures with that of DFO ($\log K_{\beta n} = 30.5$, a biosynthetic macrocyclic ligand). These ligands were chosen based on several factors: (1) their broad-range complexation strength; (2) representation of different complexation mechanisms in coordination number; (3) wide industrial use due to affordability (e.g., DTPA); (4) ubiquity as biosynthetic substances in soil (e.g., citric acid and oxalic acid).

As shown in **Fig. 1**, all four iron-binding ligands significantly increased superoxide production in *A. QXT-31* cultures compared to the negative control (one-factor ANOVA, Tukey HSD, $P < 0.05$). Among concentrations of $\leq 500 \mu\text{M}$, DFO generally exhibited the most substantial enhancing effect, followed by DTPA, citric acid, and oxalate (**Fig. 1a**). Given that DTPA is far more cost-effective than DFO, it emerges as a practical alternative for boosting microbial superoxide production. The presence of citric acid and oxalic acid as common secretions from plant roots (Mimmo et al., 2008; Sindhu et al., 2022) suggests that the rhizosphere soil may serve as a natural hotspot for extracellular microbial superoxide. The varying effects of these iron-binding ligands on stimulating microbial superoxide production may be attributed to their differing affinities for iron (reflected by $\log K_{\beta n}$), as indicated by the high correlation coefficient between $\log K_{\beta n}$ and the maximum superoxide signal value (Pearson's $r = 0.875$) (**Fig. 1b**). These findings are consistent with our earlier study, which demonstrated that iron-binding ligands enhance microbial superoxide production by stripping iron from electron transfer chain-bound molecules, facilitating electron leakage, and subsequent extracellular superoxide formation (Ning et al., 2022).

However, it's important to note that while all four iron-binding ligands increased microbial superoxide production, their effectiveness per unit decreased as the

concentration increased (**Fig. 1c**). In the case of DFO, a high concentration of 1000 μM even inhibited microbial superoxide production compared to 500 μM (**Fig. 1a**). These results suggest that simply raising the concentration of iron-binding ligands may not always promote microbial superoxide production. The decrease in efficacy per unit may be attributed to two factors: (1) high concentrations of iron-binding ligands may impair microbial metabolic processes, leading to reduced electron production necessary for superoxide production, and (2) in reaction systems with higher concentrations of iron-binding ligands, such as the generation of superoxide dismutase and peroxidase, an enhanced microbial self-protective response to higher superoxide-induced oxidative stress may scavenge superoxide and hydrogen peroxide.

3.2 Influence of iron-binding ligands on superoxide conversion to hydroxyl radical in the presence of Fe(III)

While all four tested iron-binding ligands demonstrated the ability to enhance superoxide production by *A. QXT-31*, we further investigated their impact on the conversion of superoxide into hydroxyl radicals. Hydroxyl radicals, with their higher redox potential (2.33 eV), hold significant potential for advancing contaminant degradation compared to superoxide, which possesses a lower redox potential (0.65 eV) (Krumova and Cosa, 2016). To mimic the common presence of Fe(III) in aerobic environments, experiments were conducted in the presence of Fe(III). Previous research has established that superoxide effectively reduces Fe(III) to Fe(II) (Rose et al., 2005; Rose and Waite, 2005) (Equation 1 and Equation 4), resulting in the continuous regeneration of Fe(II) through the reduction of Fe(III) by superoxide. Regenerated Fe(II) is anticipated to further enhance hydroxyl radical formation in the presence of superoxide through two established pathways: (1) Fe(II) reacts with hydrogen peroxide generated via superoxide disproportionation (Equation 2), leading to hydroxyl radical formation via the Fenton reaction (Equation 3) (Welch et al., 2002; Xiao et al., 2020), and (2) Fe(II) undergoes a single electron transfer reaction with superoxide in the presence of an appropriate iron-binding ligand (Equation 5), resulting in hydroxyl radical generation (Equation 6) (Wang et al., 2015; Xiao et al., 2020; Xie et al., 2021).

To investigate the impact of Fe(III) complexation with iron-binding ligands on superoxide conversion into hydroxyl radicals, we conducted a study using a chemical superoxide-producing system based on the reaction between xanthine and xanthine oxidase (X/XO), since the X/XO superoxide-producing system can generate superoxide at a much more constant rate compared to the *Arthrobacter* culture. The ligands could affect the reaction involving Fe(III)/Fe(II) in two ways: (1) by altering the redox properties of Fe(III)/Fe(II) (Zhang and Zhou, 2019); (2) by obstructing physical contact between iron and the target through steric effects (Yoshida et al., 1993).

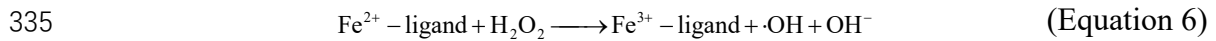
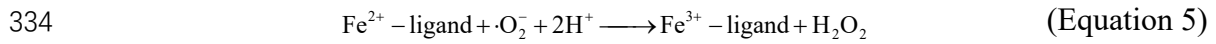
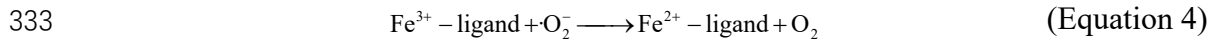
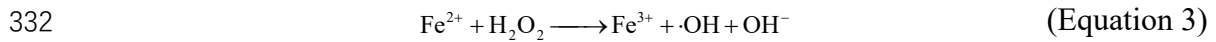
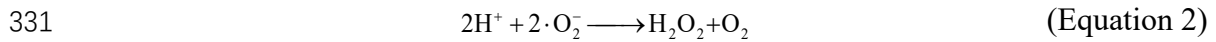
Fig. 2 illustrates the peak density of the hydroxyl radical signal in systems with different types of iron-binding ligands added. Hydroxyl radical signal peaks were exclusively observed in the systems with Fe(III) or Fe(III)-DTPA, underscoring the pivotal role of Fe(III) in hydroxyl radical generation (**Fig. 2a**). The highest peak density of hydroxyl radical signals was observed in the system with DTPA addition (**Fig. 2a**). This can be attributed to the reduction of the redox potential of Fe(III)/Fe(II) through complexation with DTPA (Buettner et al., 1983). This renders the complexed Fe(II) more reactive, facilitating electron loss. Consequently, the efficient production of hydroxyl radicals is likely due to their more reactive generation through single electron transfer reactions (Equations 5-6).

Notably, the complexation of Fe(III) with DFO led to a complete inhibition of hydroxyl radical formation (**Fig. 2a**). To explore the reasons for this inhibition, we attempted to identify the rate-limiting step in the reactions (Equation 4-6) by comparing the consumption rate of Fe(III) and Fe(II) in the X/XO system. Fe(II) in the X/XO system was observed to be oxidized at a higher rate than that in the absence of X/XO (Appendix A **Fig. S1**), indicating the presence of an additional oxidizer in addition to dioxygen. Hence, the result suggested the likely generation of hydrogen peroxide and hydroxyl radicals via the reactions in Equation 5-6. However, after Fe(III) was added to the X/XO system, no significant generation of Fe(II) was observed in up to 45 minutes (Appendix A **Fig. S1**), indicating that the reduction rate of Fe(III) to Fe(II) (Equation 4) is much lower than the oxidation rate of Fe(II) to Fe(III) in the X/XO

system. Therefore, the result suggested that Equation 4 is the rate-limiting step in the series of reactions (Equation 4-6). The inhibition of hydroxyl radical formation in the presence of DFO may be due to the lower rate of reaction in Equation 4, as some studies have shown that the steric effect of ligands with large volume creates a barrier to the reaction between Fe(III) and superoxide (Buettner et al., 1983; Miller et al., 2016; Yoshida et al., 1993). Given that DFO is a siderophore routinely secreted by microorganisms to facilitate iron acquisition, this trait may be an outcome of natural selection. It likely contributes to the inhibition of the conversion of widespread microbial superoxide into hydroxyl radicals, thereby preventing more pronounced oxidative stress in organisms that produce DFO.

Although the characteristic peak signal of hydroxyl radicals (e.g., 1:2:2:1) was not visibly observed in the ESR results of citrate and oxalate (**Fig. 2a**), signal fitting was conducted to distinguish all kinds of radicals. The results revealed the presence of two other types of radicals, $\cdot\text{H}$ and $\cdot\text{R}$ (alkyl radicals) (Appendix A **Fig. S2**). Despite the absence of the characteristic peak signal of hydroxyl radicals, the presence of $\cdot\text{H}$ and $\cdot\text{R}$ may suggest the possibility of hydroxyl radicals' generation during the reaction, since the two former may be reacting products between hydroxyl radicals and citrate/oxalate. To test the hypothesis, the hydroxyl radical quencher tert-butanol was added at the beginning of the reactions; the characteristic peak intensity previously observed after the reaction decreased in the Fe(III)+citrate group but showed no obvious change in the Fe(III)+oxalate group (**Fig. 2a&b**). This result indicates that hydroxyl radicals were only generated in the Fe(III)+citrate group, but not in the Fe(III)+oxalate group. The result also supported the possibility that hydroxyl radical generated in the Fe(III)+citrate group is responsible for the production of fractional $\cdot\text{H}$ and $\cdot\text{R}$ (alkyl radicals), although the detailed mechanisms involved are still unclear in our study. In summary, our results highlight DTPA as the most suitable candidate for facilitating the superoxide conversion into hydroxyl radicals among the four tested iron-binding ligands.





336 **3.3 Optimal Fe(III)/DTPA molar ratio for microbial ROS production**

337 To maximize hydroxyl radical production in the Fe(III)-ligand-mediated microbial
 338 superoxide-producing system, determining the ideal Fe(III)/DTPA molar ratio is crucial.
 339 While Fe(III) has been shown to facilitate the conversion of superoxide into hydroxyl
 340 radicals in the chemical superoxide-producing system based on the xanthine and
 341 xanthine oxidase reaction, an excess of Fe(III) doesn't necessarily result in increased
 342 hydroxyl radical production. This is because an excess of Fe(III) tends to form
 343 complexes with nearly all iron-binding ligands, while uncomplexed iron-binding
 344 ligands are crucial for inducing microbial superoxide production, as demonstrated
 345 previously (Ning et al., 2022). Consequently, we investigated the effects of varying
 346 Fe(III)/DTPA molar ratios on hydroxyl radical production within the *A. QXT-31* culture.

347 **Fig. 3** illustrates that hydroxyl radical production initially increases with the
 348 Fe(III)/DTPA molar ratio, peaking at a ratio of 1:1, before markedly declining at higher
 349 ratios (e.g., 3:2 and 2:1). Notably, at the Fe(III)/DTPA ratio of 1:1, one might expect a
 350 reduction in hydroxyl radical production compared to the 4:5 ratio, as the iron-binding
 351 ligand was presumed to be fully complexed with Fe(III), leaving fewer uncomplexed
 352 ligands to stimulate *A. QXT-31* cells to generate superoxide. However, a higher yield
 353 of hydroxyl radicals was observed at the 1:1 ratio compared to the 4:5 ratio. This
 354 phenomenon could be attributed to the presence of biosynthetic DFO in the reacting
 355 system (e.g., *A. QXT-31* culture), where *A. QXT-31* was observed to persistently
 356 synthesize and secrete DFO in our previous study (Ning et al., 2022). Our previous
 357 study showed that even at concentrations as low as approximately 2 μM , DFO

significantly augmented superoxide production within the *A. QXT-31* culture (Ning et al., 2022). When additional Fe(III) was introduced (e.g., the 3:2 and 2:1 ratios), the DFO secreted by *A. QXT-31* is expected to become fully complexed with Fe(III), resulting in fewer free iron-binding ligands (e.g., DTPA and DFO) available to react with *A. QXT-31* cells to produce superoxide, which is essential for hydroxyl radical generation. Similar results have been observed in our previous study, where the presence of Fe(III) largely weakened the efficiencies of DFO in promoting superoxide production in the *A. QXT-31* culture (Ning et al., 2022). Accordingly, the rapid decrease in hydroxyl radical production observed in treatments with a ratio of 3:2 and 2:1 aligns with our theoretical expectations.

It needs to be emphasized that the optimal Fe(III)/DTPA molar ratio (e.g., 1:1) determined in this study should be used as a rough reference when it comes to application, and the optimal Fe(III)/DTPA molar ratio is susceptible to other factors, such as the content of Fe and ubiquitous iron-binding ligands (such as siderophores) in the environment. Thus, it should be adjusted according to the local conditions. In summary, these findings imply that an approximate Fe(III)/DTPA ratio of 1:1 represents the optimal condition for hydroxyl radical production in the *A. QXT-31* culture, establishing a fundamental reference point for other bacterial species and engineering applications.

3.4 Enhanced pollutant degradation by microbial ROS

The incorporation of DTPA into the *A. QXT-31* culture is expected to amplify microbial degradation performance by facilitating superoxide production and its subsequent conversion into hydroxyl radicals. The significantly higher redox potential of hydroxyl radicals (2.33 eV) in comparison to superoxide (0.65 eV) (Krumova and Cosa, 2016) is anticipated to boost pollutant degradation by microbial ROS. To validate this hypothesis, we selected oxytetracycline, a commonly used antibiotic in aquaculture and livestock breeding, as the target substance.

As depicted in **Fig. 4**, the degradation percentage of oxytetracycline remained

minimal in both the negative control (PYG medium, 0.32%) and the superoxide-producing treatment (*Arthrobacter* culture, 0.66%), signifying low levels of autodegradation and superoxide-mediated degradation of oxytetracycline. In contrast, the degradation percentage (8.6%) in the hydroxyl radical-producing treatment (culture + Fe(III) + DTPA) was significantly higher than that in the negative control (paired samples *t*-test; $P < 0.01$) and the superoxide-producing treatment (paired samples *t*-test; $P < 0.01$), demonstrating that the generation of hydroxyl radicals enhanced oxytetracycline degradation. Hydroxyl radical production was effectively inhibited by a combination of superoxide dismutase (SOD) and catalase (CAT), leading to a decrease in the degradation ratio to 2.4%, representing only 27.9% of the performance in the hydroxyl radical-producing treatment (**Fig. 4a**). Given the low degradation percentage of oxytetracycline in the superoxide-producing treatment, this decrease in the degradation ratio was less likely to be associated with the elimination of superoxide. Furthermore, hydrogen peroxide was not detected in the *A. QXT-31* culture, indicating that the addition of CAT would not impede the degradation performance of oxytetracycline. These results strongly suggest that hydroxyl radicals are the primary drivers behind the indirect degradation of oxytetracycline in the hydroxyl radical-producing treatment (culture + Fe(III) + DTPA).

Fig. 4b demonstrates a positive correlation between oxytetracycline degradation and the signal intensity of accumulated superoxide in the hydroxyl radical-producing treatment (culture + Fe(III) + DTPA), with a Pearson's $r = 0.908$ ($P = 0.092$). This further supports a potential causal relationship between hydroxyl radical production and oxytetracycline degradation. Overall, our findings indicate that the combined addition of DTPA and Fe(III) can significantly augment microbial ROS production in the *A. QXT-31* culture, thereby resulting in the improved degradation of the tested organic pollutants.

3.5 Enhancement of microbial superoxide production in soil samples by DTPA

In our prior study, we showcased the ability of iron-binding ligands to elevate superoxide production in the *A. QXT-31* culture by disrupting iron within the electron

transfer chain (ETC). This process leads to electron leakage and the subsequent generation of extracellular superoxide during carbon source metabolism. Given the prevalence of the ETC in various aerobic bacteria, we hypothesized that DTPA could have a similar superoxide-enhancing effect in other bacterial taxa. To test this hypothesis, we collected soil samples from Shenzhen, Guangdong Province, and subsequently diluted them into PYG medium by factors of 1, 2, 5, and 10 to emulate the varying nutrient levels found in natural environments. We then quantified superoxide production in the soil cultures after introducing a carbon source, with or without the addition of DTPA. Remarkably, in cultures of rhizosphere soil samples, we observed a pronounced increase in superoxide signal intensity upon the addition of 50 μ M DTPA (**Fig. 5a**), confirming that DTPA indeed enhances microbial superoxide production within soil microbiomes.

To further explore the bacterial genera potentially responsible for superoxide production in soil cultures in the presence of a carbon source, we conducted a comparative analysis of the bacterial community within the rhizosphere soil sample. LEfSe analysis was employed to identify the genera most likely to account for differences between superoxide-producing cultures and non-superoxide-producing cultures. The results of superoxide production detection in the rhizosphere soil sample culture under various nutrient concentrations of PYG can be found in Appendix A **Table S1**. *Ferrivobrio*, *Peredibacter*, *Paenibacillus*, *Chryseobacterium*, and *Pedobacter* were identified as significant contributors to the observed differences ($\log_{10}(\text{LDA score}) = 3.356, 3.327, 3.271, 2.993, \text{ and } 2.846$, respectively) (**Fig. 5b**), underscoring their potential role in superoxide production. Additionally, we performed a correlation analysis between the abundance of bacterial genera in each sample and the superoxide production rate. The results showed that *Brevundimonas*, *Caulobacter*, and *Ferrovibrio* exhibited a strong correlation with superoxide generation (Pearson's $r = 0.495, 0.494, 0.524, P < 0.05$). While LEfSe analysis and correlation analysis do not produce identical results, they both highlight *Ferrovibrio* as a prominent candidate associated with superoxide production in these samples, indicating its potential involvement in

444 superoxide generation.

445 However, it's worth noting that, despite our previous findings that *Arthrobacter* can
446 produce superoxide in the presence of DTPA, *Arthrobacter* was not identified as a
447 contributor to superoxide production in these soil cultures. Interestingly, we observed a
448 significant negative correlation between *Arthrobacter* abundance and the corrected
449 superoxide peak signal intensity (maximum superoxide signal intensity normalized to
450 the control) in our Pearson correlation analysis (Pearson's $r = -0.467$, $P = 0.038 < 0.05$)
451 (**Fig. 5c**). These results collectively suggest that microbial genera other than
452 *Arthrobacter* are responsible for superoxide production in the tested soil samples and
453 that DTPA can indeed enhance microbial ROS production within the environment.

454 **3.6 Environmental significance**

455 This study carries substantial implications for the field of environmental science. Firstly,
456 the exploration of low-cost iron-binding ligands, such as DTPA, to simultaneously
457 amplify microbial superoxide production and facilitate the conversion of superoxide
458 into hydroxyl radicals holds significant promise for environmental applications. The
459 prohibitive cost of siderophores has hindered their use in promoting the production of
460 microbial ROS for bioremediation. However, by identifying cost-effective alternatives
461 like DTPA, this study offers a more accessible approach to stimulate microbial ROS,
462 thereby enhancing contaminant remediation. Secondly, the discovery that common root
463 exudates, such as citrate and oxalate, have the capacity to induce microbial superoxide
464 production underscores the rhizosphere as a dynamic hub for microbial superoxide
465 generation. Since the rhizosphere is already known as a hotspot for the degradation of
466 organic pollutants (Azaizeh et al., 2010; Turkovskaya and Muratova, 2019), our
467 findings point to a novel degradative pathway facilitated by microbial ROS,
468 complementing the traditionally recognized enzyme-mediated pathway. Lastly,
469 considering that natural precipitation can transport carbon sources into the soil (Deng
470 et al., 2017), it is plausible that rainfall may amplify the production of microbial ROS
471 in the presence of ubiquitous siderophores in the soil, consequently enhancing the
472 degradation of pollutants by microbial ROS. This insight highlights the dynamic

interplay between environmental factors and microbial processes, paving the way for more comprehensive approaches to environmental management.

4 Conclusions

In summary, our study introduces a cost-effective approach to enhance microbial superoxide production rates and facilitate its conversion into hydroxyl radicals, ultimately improving bioremediation efficiency. A schematic representation was available in **Fig. 6**. We have identified DTPA as an effective enhancer of superoxide production in both the *Arthrobacter* strain and soil microbiomes, as well as a promoter of the conversion of superoxide into hydroxyl radicals in the presence of Fe(III). When combined with Fe(III), DTPA enhances microbial degradation of a tested pollutant within the *Arthrobacter* culture. These findings present a practical method for augmenting microbial superoxide production and its conversion into hydroxyl radicals through the addition of DTPA and carbon sources in environments containing Fe(III). This approach holds significant promise for applications in soil environments and offers valuable insights for guiding the bioremediation of contaminated soil via microbial ROS.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Appendix A Supplementary data

Supplementary data associated with this article can be found in the online version at xxxxxx.

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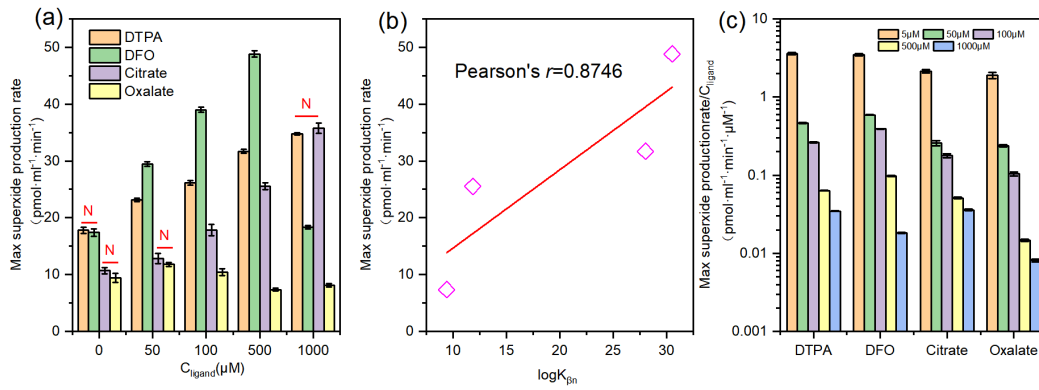


Fig. 1 Superoxide production by the *A. QXT-31* culture in the presence of iron-binding ligands. (a) Maximum superoxide signal intensity in the *A. QXT-31* culture with added iron-binding ligands. Superoxide was immediately determined after adding glucose (50 mg/L) to the *A. QXT-31* culture. A culture without glucose addition served as a negative control, and the maximum rates shown in the figure are the values after subtracting the rate of the negative control. One-factor ANOVA was conducted to test the differences between each treatment, and non-significant differences were marked as "N" ($N: P > 0.05$, Tukey HSD). (b) Pearson correlation analysis between $\log K_{\beta n}$ values of iron-binding ligands and the maximum superoxide signal intensity in the *A. QXT-31* culture with 50 μM of each iron-binding ligand. (c) Maximum superoxide signal per unit iron-binding ligand in the *A. QXT-31* culture after the addition of iron-binding ligands. Superoxide was immediately detected after adding glucose (50 mg/L) to the *A. QXT-31* culture. The differences between any two results were significant at a level of $P < 0.05$ (Tukey HSD).

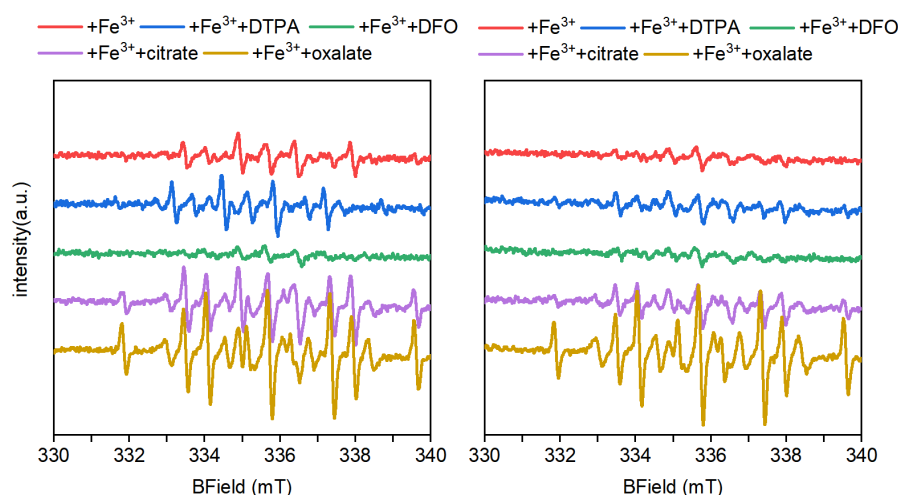


Fig. 2 Influence of Fe(III) on hydroxyl radical production in the presence of iron-binding ligands. (a) ESR spectra of an X/XO solution in the presence of Fe(III) and iron-binding ligands. A chemical superoxide-producing system (X/XO solution, pH = 7.2) was employed to generate superoxide consistently, as opposed to relying on the *Arthrobacter* culture. An X/XO solution (pH = 7.2) without Fe(III) or iron-binding ligand was used as a control, and Fe(III)-complexed ligands (50 μ M) were added to the X/XO solution. (b) ESR spectra of an X/XO solution in the presence of Fe(III) and iron-binding ligands with the addition of 10 mM tert-butanol as a hydroxyl radical quencher. The ESR spectra of a Fenton reaction system are shown as a reference for hydroxyl radical in (a) and (b).

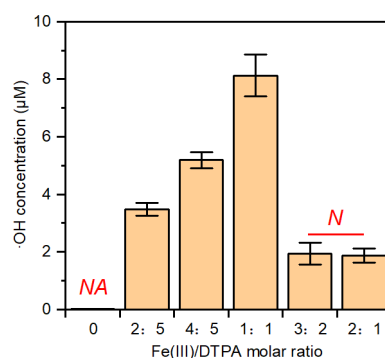


Fig. 3 Influence of Fe(III)/DTPA molar ratio on hydroxyl radical production in the *A. QXT-31* culture. The cultures were harvested 1 h after adding glucose (50 mg/L), and hydroxyl radicals were captured by adding salicylic acid (500 μM) and indirectly quantified as the product of 2,3-DHBA. 'NA' indicates that no hydroxyl radicals were produced. One-factor ANOVA was performed to analyze differences between each group, and non-significant differences ($P > 0.05$, Tukey HSD) were labeled as 'N'.

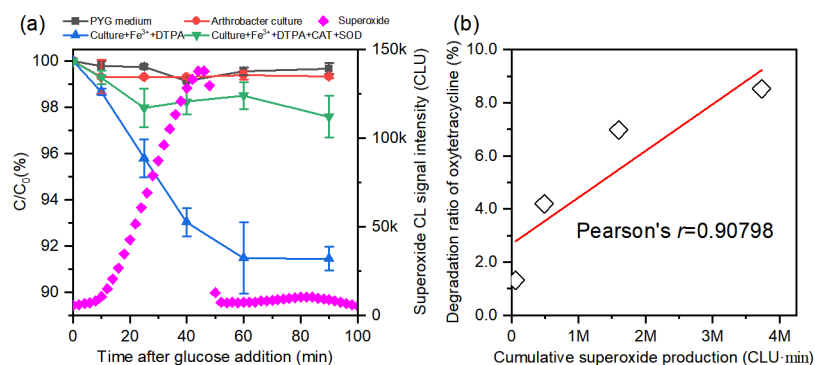


Fig. 4 Pollutant degradation by microbial ROS. (a) Oxytetracycline degradation and superoxide production in the *A. QXT-31* culture. PYG medium: negative control; *A. QXT-31* culture: superoxide-producing treatment; culture + Fe(III) + DTPA: hydroxyl radical-producing treatment; culture + Fe(III) + DTPA + CAT + SOD: suppression treatment. Superoxide intensity was detected in the *A. QXT-31* culture with glucose addition (50 mg/L). The result represents the superoxide production in the hydroxyl radical-producing treatment and the superoxide-producing treatment, both of which used the same *A. QXT-31* culture. (b) Correlation analysis between oxytetracycline degradation percentage and accumulated superoxide. Data from 10 to 60 min of the reaction were selected for this analysis. Accumulated superoxide was calculated as the area between the superoxide signal density and the axes using the trapezoidal approximation method.

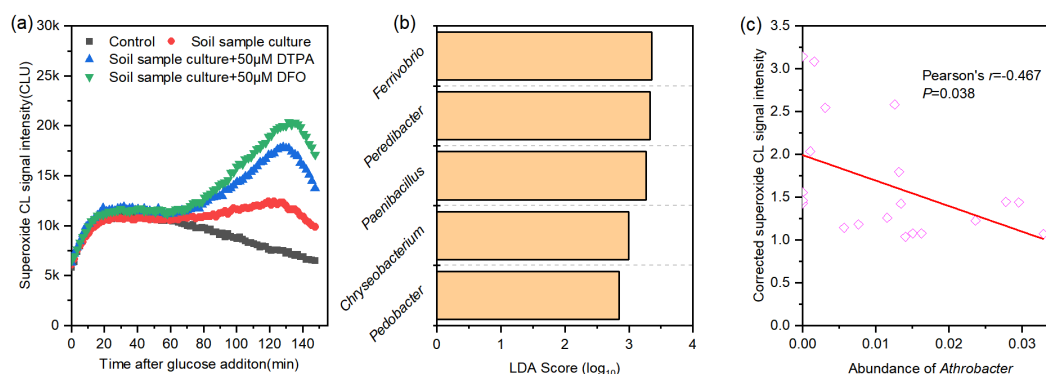


Fig. 5 Microbial superoxide production and identification of potential superoxide-producing bacterial genera in soil cultures. (a) Superoxide production in cultures of a rhizosphere soil sample with 50 μM iron-binding ligand. A cell suspension without the addition of glucose and iron-binding ligand served as a control. Superoxide was measured immediately after adding glucose (50 mg/L). (b) LEfSe analysis of differential bacterial genera in superoxide-producing and non-superoxide-producing cultures to identify potential superoxide-producing bacterial genera. Genera with LDA scores > 2 were considered biomarkers of differences between the two groups. (c) Pearson correlation analysis between the corrected maximum superoxide signal intensity and the *Arthrobacter* abundance in superoxide-producing cultures, showing a significant negative correlation.

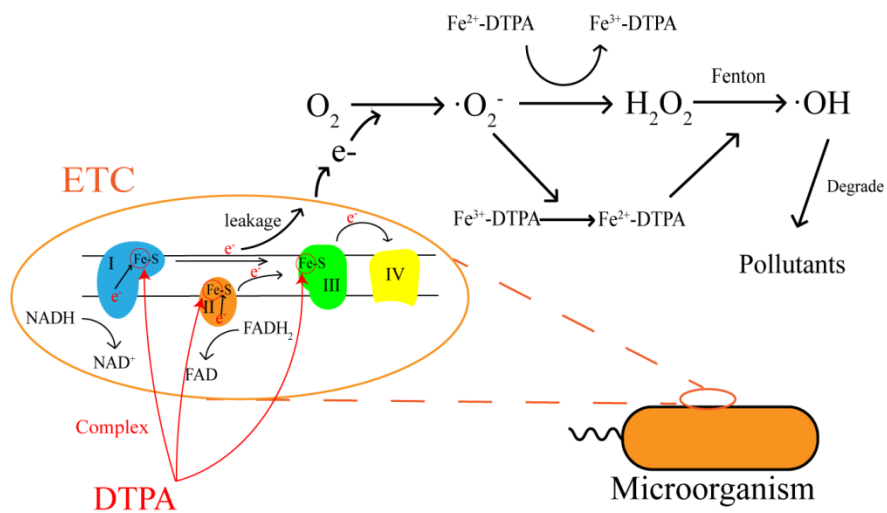


Fig. 6 Mechanism of superoxygen generation and conversion to hydroxyl radicals in the presence of DTPA and Fe(III.)