

Isolation, Characterization and Evaluation of a High-Siderophore-Yielding Bacterium from Heavy Metal Contaminated Soil

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Abstract

Heavy metal resistant siderophore-producing bacteria (SPB) with plant growth promoting traits can assist in phytoremediation of heavy metal contaminated soil. We isolated siderophore producing bacteria from lead and zinc mine soil in Shangyu, Zhejiang, China. The isolate with highest siderophore production, strain SX9, was identified as *Burkholderia* sp. The strain SX9 produced catecholate type siderophore, with highest production at a pH range 6.0 to 8.0, a temperature range 20 to 30 °C and NaCl concentration below 2%. Siderophore production was highest without Fe³⁺ and became gradually lower with increasing Fe³⁺ concentration. Minimal inhibitory concentrations (MIC) of Pb²⁺, Zn²⁺, Cu²⁺ and Cd²⁺ were 4000, 22000, 5000 and 2000 μmol·L⁻¹, respectively. The strain SX9 was sensitive to doxycycline hyclate and rifampicin. The strain had a strong metal solubilization ability: the contents of Cu²⁺, Zn²⁺ and Cd²⁺ in the supernatant were 47.4%, 133.0% and 35.4% higher, respectively, in the strain SX9 inoculated cultures than in the not inoculated controls. The siderophore produced by strain SX9 could combine with Fe³⁺, Zn²⁺ and Cd²⁺ with good effectiveness. The plant growth promoting traits of the strain included indole acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and phosphate solubilization capability. Compared to uninoculated growth medium and SX9 culture supernatant, the germination rate of *Lolium perenne* seeds was higher when inoculated with the strain SX9 culture. In the experiment of seed germination, adding bacterial culture or supernatant could alleviate the toxicity of heavy metals to *Lolium perenne* seed germination. Under Cu²⁺ and Zn²⁺ stress, the strain SX9 promoted the germination rate. Taken together, the strain SX9 had properties beneficial in the microbial enhancement of phytoremediation of soil contaminated with heavy metals.

1 Introduction

The contamination of soil with heavy metals poses major environmental and human health challenges, calling for the remediation of the contaminated soils. Heavy metals are removed from the soil using physical, chemical and biological technologies. Each remediation technique has its own advantages and disadvantages in terms of pollutant removal and cost efficiency (Khalid et al. 2016). Phytoremediation technology has attracted attention due to its economic, environmental friendly and non-secondary pollution characteristics (Ashraf et al. 2019). The discovery of hyperaccumulator plants has enhanced the prospects for phytoremediation. Unfortunately, most of the hyperaccumulators grow and produce biomass slowly, requiring long remediation periods that diminish their applicability in phytoextraction (Ashraf et al. 2019; Kuppusamy et al. 2016).

Therefore, as an alternative strategy, the phytoremediation capacity of non-hyperaccumulating plants that produce biomass faster could be improved. Tolerance to stressful remediation conditions might be achieved by exploiting plant growth-promoting bacteria (PGPB) (Lucian et al. 2014). A large number of bacterial genera include PGPB, e.g. *Pseudomonas*, *Burkholderia* and *Klebsiella* (Pliego et al. 2011). The PGPB promote plant growth through direct and indirect mechanisms. Direct promotion includes the synthesis of substances that have a direct effect on plant growth, e.g. indoleacetic acid (IAA) and

gibberellin, and changes in the availability of nutrients, e.g. nitrogen fixation and phosphate solubilization. Indirect promotion includes the synthesis of substances that inhibit or reduce the effects of adverse factors on plant growth and yield, e.g. siderophores and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The siderophore producing bacteria (SPB) can provide iron to plants and alleviate the toxicity of heavy metals (Sinha and Mukherjee 2008).

Under iron-limiting conditions, microorganisms secrete siderophores that have a high affinity for Fe^{3+} (Saha et al. 2016). Most aerobic and facultative anaerobic microorganisms are capable of synthesizing siderophores, and hundreds of different kinds of siderophores have been characterized (Arton and Hemming 1994). According to the characteristic functional groups, siderophores are classified into four types: hydroxamate, catecholate, hydroxycarboxylate and mixed types, out of which hydroxamate type is the most common (Khan et al. 2017). In addition to binding Fe^{3+} , siderophores can form soluble metal-siderophore chelates with Al^{3+} , Cu^{2+} , Cd^{2+} , Pb^{2+} , Zn^{2+} and other metals (Dimkpa et al. 2009; Rajkumar et al. 2010). The heavy metal chelating ability of siderophores has applications in remediation; inoculation with SPB and adding the supernatant of a SPB culture promoted the growth of plants under heavy metal stress and increased heavy metal uptake by plants (Jiang et al. 2008; Wang et al. 2011).

The purpose of this study was to isolate high-efficient SPB and exploring its characteristics to provide a suitable strain for remediating heavy metal contaminated soils. We isolated bacteria from lead and zinc contaminated soil and assessed their siderophore production. The isolate with highest siderophore production capacity, strain SX9, was tested further: we determined its capacity to produce siderophore under different conditions and tolerance to heavy metals and antibiotics. Furthermore, we investigated the complexation capacity of siderophore produced by strain SX9 for metal ions and the plant growth promoting characteristics of strain SX9, including its ability to promote the growth of *Lolium perenne* under heavy metal stress.

2 Materials And Methods

2.1 Source of soil sample and determination of soil basic properties

Soil sample was collected from a lead and zinc mine in Shangyu, Zhejiang, China (29°59'N, 120°46'E) that has East-Asian monsoon climate with an annual average precipitation of 1400 mm and temperature of 16.4°C. The soil sample was packed in a sterile plastic bag and stored at 4°C before use. The physicochemical properties of soil were determined as described previously (Bao 2000). Heavy metals were extracted with aqua regia- HClO_4 and analyzed with inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 8000, Perkin Elmer Co, USA).

2.2 Growth media and buffers

Liquid media and buffers were prepared by dissolving the below-mentioned components into deionized water to make up a total volume of 1000 mL. For solid media, 1.8% (w/v) agar was added to the liquid media. The media were sterilized at 121°C for 30 min.

LB medium: 10.00 g tryptone, 5.00 g yeast extract, 10.00 g NaCl.

Iron-free modified King B (MKB) medium: 5.00 g acid hydrolyzed casein, 15 mL glycerinum, 2.50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.50 g K_2HPO_4 . Trace iron was removed with 8-hydroxyquinoline.

Chrome azurol sulphonate (CAS) semisolid medium (Wang et al. 2014): 60.50 mg chrome azurol, 72.90 mg hexadecyl trimethyl ammonium bromide (HDTMA), 10 mL of 1 $\text{mmol} \cdot \text{L}^{-1}$ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (dissolved with 10 $\text{mmol} \cdot \text{L}^{-1}$ HCl), 50 mL of 0.1 $\text{mol} \cdot \text{L}^{-1}$ phosphate buffer, 0.9% (w/v) agar.

Dworkin and Foster (DF) medium: 2.00 g $(\text{NH}_4)_2\text{SO}_4$, 4.00 g KH_2PO_4 , 6.00 g Na_2HPO_4 , 0.20 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.00 g glucose, 2.00 g gluconic acid, 2.00 g citric acid, 1.00 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10.00 μg H_3BO_3 , 11.19 μg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 124.60 μg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 78.22 μg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10.00 μg MoO_3 , pH 7.2.

ADF medium: as DF medium, except that the N source is 3.0 mmol ACC instead of $(\text{NH}_4)_2\text{SO}_4$.

National Botanical Research Institute's Phosphate Growth (NBRIP) medium: 10.00 g glucose, 0.50 g $(\text{NH}_4)_2\text{SO}_4$, 0.30 g NaCl, 0.30 g KCl, 0.30 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.00 g $\text{Ca}_3(\text{PO}_4)_2$.

1 $\text{mol} \cdot \text{L}^{-1}$ phosphate buffer: 5.91 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 24.27 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 2.50 g NH_4Cl , 0.75 g KH_2PO_4 , 1.25 g NaCl, pH 6.8.

2.3 Isolation of bacteria

10.0 g soil sample was suspended into 80 mL of sterile water, and the suspension was shaken at 150 rpm for 30 min at 28°C. Ten-fold serial dilutions of the suspension were inoculated onto Luria-Bertani (LB) agar plates as described previously (Liu et al. 2019). Plates were incubated at 28°C for 48 h. Well-separated colonies were picked and inoculated onto LB agar plates until a pure culture was obtained.

2.3.1 Qualitative analysis of SPB

The isolates were inoculated onto iron-free modified King B (MKB) medium agar plates and cultured at 28°C for 48 h. Chrome azurol sulphonate (CAS) semisolid medium was cooled to approximately 60°C and poured onto the MKB agar. After 2–4 h, a color change from blue to orange around a colony indicated siderophore production. Forty-four isolates with distinct orange circles around the colonies were chosen for further analyses. The analyses here and further on were done in triplicate.

2.3.2 Quantitative analysis of SPB

The isolates were cultured in iron-free MKB medium at 28°C for 48 h at 150 rpm. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected and 1 mL supernatant was mixed with 1 mL of CAS

reagent (Arora and Verma 2017; Schywn and Nielands 1987). A negative control was made by mixing 1 mL sterile water and 1 mL of CAS reagent. After incubation for 30 min at room temperature, the absorbance value of the mixture (A_s) was measured at the wavelength of 630 nm. The absorbance of uninoculated iron-free MKB medium mixed with CAS reagent was determined as the reference ratio (A_r). The experiment was carried out in triplicate. The percent siderophore unit (SU) was calculated using the following formula:

$$\% \text{ SU} = (A_r - A_s) / A_r \times 100$$

The larger the SU value, the stronger the ability of an isolate to produce siderophore. The isolate with highest % SU value, referred as strain SX9 from hereon, was chosen for further analyses.

2.4 Characterization of strain SX9

The morphological and chemotaxonomic characterization of strain SX9 was done as described in *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). Genomic DNA of strain SX9 was extracted using Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech Co., Ltd, Shanghai, China). The 16S rRNA gene of the strain was amplified with polymerase chain reaction (PCR) using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al. 1991). The PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The PCR products were checked in 1.0% agarose gel and purified using DiaSpin DNA Gel Extraction Kit (Sangon Biotech Co., Ltd, Shanghai, China). The amplified product was sequenced at Sangon Biotech Co., Ltd (Shanghai, China). The 16S rRNA gene sequence of the strain SX9 was compared to closely related sequences in the National Centre of Biotechnology Information (NCBI) GenBank database using BLAST online (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was constructed using neighbor-joining method in MEGA 6.0 with a bootstrap value of 1,000 (Koichiro et al. 2013). The sequence was submitted to GenBank with the accession number MN658668.

2.5 Siderophore type determination

Arnow's test (Arnow 1937), Csaky's test (Csáky et al. 1948) and Shenker's test (Shenker et al. 1992) were used to determine catechol type, hydroxamate type and hydroxycarboxylate type siderophores, respectively.

Arnow's test. 1 mL of culture supernatant was mixed with 0.1 mL of $5 \text{ mol}\cdot\text{L}^{-1}$ HCl. Subsequently, 1 mL nitrite-molybdate reagent (10.00 g NaNO_2 and 10.00 g Na_2MoO_4 dissolved in 100 mL of deionized water) was added. In the presence of catechol, the nitrite in the solution is decomposed to form a yellow NO ligand. Next, 0.5 mL of $2 \text{ mol}\cdot\text{L}^{-1}$ NaOH was added. In the presence of catechol type siderophore, the solution becomes red and remains unchanged for at least 1 h.

Csaky's test. 1 mL of culture supernatant was mixed with 1.0 mL of 1 N H₂SO₄, boiled for 6 h, 3 mL of 35% sodium acetate solution was added and mixed well, and 1 mL of the solution was diluted with deionized water in a 1:5 ratio. Next, 0.5 mL of sulfanilic acid solution (1.00 g sulfanilic acid dissolved in 100 mL of 30% acetic acid) and 0.2 mL of iodine solution (1.30 g iodine dissolved in 100 mL acetic acid) were added and the solution was incubated at room temperature for 5 min. After this, 0.2 mL of sodium thiosulphate (2.00 g sodium thiosulphate dissolved in 100 mL deionized water) and 0.1 mL of α -naphthylamine solution (3.00 g α -naphthylamine in 1,000 mL of 30% acetic acid) were added and the solution was incubated at room temperature for 20–30 min. In the presence of hydroxamate siderophore, the solution becomes pink.

Shenker's test. 1 mL supernatant was mixed with 1 mL 250 $\mu\text{mol}\cdot\text{L}^{-1}$ CuSO₄ and 2 mL acetic acid buffer (pH 4), and incubated at room temperature for 30 min. The presence of hydroxycarboxylate siderophore–copper compound was determined using full wavelength scanning. A maximum absorption peak at 190–280 nm indicated that the solution contains hydroxycarboxylate siderophore.

2.6 Siderophore production by strain SX9

To study siderophore production over time, strain SX9 was grown for 24 h and 2.5 mL of the culture, adjusted to A₆₀₀ of 1.0, was inoculated into 250 mL iron-free MKB, followed by incubation at 28°C for 48 h at 150 rpm. Cell density as OD₆₀₀ and siderophore production as % SU were recorded at 4 h intervals.

The effects of pH and NaCl and Fe³⁺ concentrations on the growth and siderophore production of strain SX9 were studied using MKB medium, inoculation volume of 1% (v/v) and incubation at 28°C and 150 rpm. The effect of temperature was studied similarly, except that the cultures were incubated at temperatures from 15 to 35°C. The effect of pH was examined in medium with the initial pH adjusted to 5.0, 6.0, 6.8, 7.4, 8.0 and 9.0. Salt tolerance was assessed at 0, 0.5, 1, 2, 3 and 5% (w/v) NaCl concentrations. The effect of Fe³⁺ concentration was assessed at 0, 0.5, 1, 2, 3, 5, 10 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$ Fe³⁺ concentrations.

2.7 Minimal inhibitory concentrations (MICs)

To determine the MICs of twelve metal salts and eight antibiotics, strain SX9 was grown in LB medium with predetermined concentrations of metal salts and antibiotics (Table 1) at 28°C for 48 h at 150 rpm (Yu et al. 2013).

Table 1 Physiological and biochemical characteristics of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China.

Item	Result	Item	Result
Methylene red	+	Gram-staining	-
Voges-Proskauer	-	Fluorochrome assay	+
Oxidase	+	Catalase	+
Amylohydrolysis assay	-	Nitrate reductase	-
Indol reaction	-	Urease activity	-
H ₂ S production	-	Gelatin hydrolysis	-
Fructose utilization	+	Mannose utilization	+
Xylose utilization	+	Maltose utilization	+
Lactose utilization	+	Galactose utilization	+

2.8 Solubilization of heavy metals

50 mL of liquid MKB with 50 mg of Cu²⁺ as CuO, 50 mg of Zn²⁺ as ZnO or 50 mg of Cd²⁺ as CdCO₃ was inoculated with 1 mL of logarithmic phase strain SX9 inoculum and incubated at 28°C and 150 rpm. After 72 h, 5 mL of the culture was centrifuged at 8,000 rpm for 10 min and filtered through a 0.22 μm needle cartridge membrane filter (Jin Teng, China). The Cu²⁺, Zn²⁺ and Cd²⁺ concentrations in the filtrate were determined using inductively coupled plasma optical emission spectrometry (ICP-OES).

2.9 Complexation capacity assays

To assess the ability of siderophore to chelate metal ions under alkaline conditions, a standard curve was made using demertamine mesylate, and the content of siderophores was determined using the CAS method (Ferreira et al. 2019). FeCl₃·6H₂O, CuSO₄·5H₂O, ZnSO₄·7H₂O and Cd(NO₃)₂·4H₂O were added to a fixed volume of the culture filtrate (Ferreira et al. 2019). The pH of the solution was set to 9.0 ± 0.1, the solution was incubated in room temperature for 30 min, the pH of solution was adjusted to 9.0 again, and the solution was incubated for 3 h. The solution was centrifuged at 3,000 g for 10 min and filtered through a 0.45 μm pore size membrane. The concentrations of metal ions in the solution was determined using ICP-OES. The iron complexation capacity of the siderophore in solution was calculated by plotting the ratio [metal ion]_{soluble}/[S] versus [metal ion]_{added}/[S], where [metal ion]_{soluble} is the concentration of metal ion in the filtrate, [metal ion]_{added} is the total concentration of metal ion added and [S] is the concentration of siderophore determined using the CAS method (Ferreira et al. 2019).

2.10 Plant growth promoting characteristics

IAA synthesis assay. The strain SX9 was inoculated into LB liquid medium containing 200 mg·L⁻¹ L-tryptophan and incubated at 150 rpm for 48 h at 30°C. 5 mL of the culture was centrifuged at 8,000 rpm

for 10 min. 2 mL of the supernatant was mixed with 4 mL Salkowski's reagent, incubated for 30 min at room temperature in the dark, and the absorbance of the solution was measured at 530 nm (Bahadur et al. 2020). A standard curve was made by measuring the absorbance of IAA standard solution diluted to concentrations of 0, 3, 5, 8, 10 and 15 mg·L⁻¹.

ACC deaminase activity assay. The strain SX9 was inoculated into 20 mL Dworkin and Foster (DF) medium. After incubation at 150 rpm for 24 h at 30°C, the cells were collected by centrifugation at 8,000 rpm for 10 min at 4°C. The cells were washed twice with DF liquid medium without (NH₄)₂SO₄, suspended in 10 mL of ADF medium and incubated at 150 rpm for 24 h at 30°C. Protein content was determined using colorimetry with bovine serum albumin as the standard (Penrose and Glick 2010). The ACC deaminase activity was calculated using the standard curves of α-ketobutyric acid (α-KA) and protein as the amount of α-KA generated per mg of bacterial protein per hour (Penrose and Glick 2010). The ACC deaminase activity unit is μmol α-KA·(h·mg)⁻¹.

Phosphate solubilization. The strain was inoculated into National Botanical Research Institute's Phosphate Growth (NBRIP) medium and incubated at 30°C for 7 days at 150 rpm (Kong and Hong 2020). After centrifugation at 6,000 rpm for 10 min, phosphorus content of 1 mL supernatant was determined using the Mo-Sb colorimetric method. A not inoculated culture was used as a negative control.

2.11 Seed germination tests

To assess the effect of strain SX9 inoculation on *Lolium perenne* seed germination in the presence of heavy metal contaminants, *Lolium perenne* seeds were soaked in 75% alcohol for 5 min, washed 4 times with sterile deionized water, 50 seeds per plate were spread on filter paper in glass petri dishes, and inoculated with 5 mL of bacterial culture, bacterial culture supernatant or uninoculated growth medium. Metal salt solutions with increasing concentrations of Cu²⁺ (0, 100, 200, and 400 mg·L⁻¹), Zn²⁺ (0, 200, 500, and 1,000 mg·L⁻¹) and Cd²⁺ (0, 5, 10, and 20 mg·L⁻¹) were added to the plates. The plates were kept at a constant temperature of 28°C. Seeds were considered germinated when the root protruded from the seed coat by at least 2 mm. The final germination rate was calculated after 7 days. Five seedlings per plate were randomly chosen to determine the shoot and root lengths (Aka and Babalola 2016).

2.12 Statistical analysis

Statistical analyses and visualization were done using Microsoft Excel (version 2016) and Origin Pro 2018C (OriginLab Corporation, Northampton, USA), respectively. Statistical significance of the differences was tested using one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. Differences were considered statistically significant at $p < 0.05$.

3 Results And Discussion

3.1 Characteristics of soil sample

The pH of the soil sample from a lead and zinc mine in Shangyu, Zhejiang, China, was 5.15, typical to acidic soils in south of China. The contents of organic matter, available nitrogen, available phosphorus and available potassium were $21.17 \text{ g}\cdot\text{kg}^{-1}$, $81.43 \text{ mg}\cdot\text{kg}^{-1}$, $1.56 \text{ mg}\cdot\text{kg}^{-1}$ and $8.21 \text{ mg}\cdot\text{kg}^{-1}$, respectively. The concentrations of zinc, lead, copper and cadmium were $18521.51 \text{ mg}\cdot\text{kg}^{-1}$, $2506.86 \text{ mg}\cdot\text{kg}^{-1}$, $432.27 \text{ mg}\cdot\text{kg}^{-1}$ and $1.47 \text{ mg}\cdot\text{kg}^{-1}$, i.e. above the limits of heavy contamination (15618 – 2018 2018).

3.2 Isolation of SPB and characterization of strain SX9

A total of 44 bacterial strains were isolated from the soil sample. The isolate with the highest SU value, strain SX9 (93.7%) was selected for further investigation.

The colonies of strain SX9 were round, yellow and non-transparent with smooth surface on LB plates (Fig. 1). Transmission electron microscopy (TEM) showed that SX9 cells were rod-shaped with multiple polar flagella (Fig. 2). The strain SX9 was Gram-negative, methylene red, fluorochrome, oxidase and catalase positive, and utilized fructose, mannose, xylose, maltose, lactose and galactose. In the 16S rRNA gene sequence analysis, the strain SX9 was assigned to *Burkholderia* sp. (Fig. 3). Based on a positive Arnow's test (Fig. 4) and negative Casky's and Shenker's tests, the strain SX9 produced catechol type siderophore. In iron-free MKB medium, the growth of strain SX9 reached stationary phase after 20 h of incubation. Similar to previous studies (Patel et al. 2018; Sayyed et al. 2019), siderophore production reached maximum in the stationary phase (Fig. 5).

3.3 Effects of pH, temperature and NaCl and Fe^{3+} concentrations on siderophore production by strain SX9

The solubility of substances, the permeability of cell membrane and the rates of enzymatic reactions are affected by pH, temperature and NaCl. Both the OD_{600} and SU values of strain SX9 were highest at pH 7.4 ($P < 0.05$) (Fig. 6). Similarly, the production of siderophores by *Achromobacter* sp. RZS2 was highest at pH 7.5 (Sayyed et al. 2019). The OD_{600} and SU values were lowest at pH 5.0 and second lowest at pH 9.0 ($P < 0.05$), yet relatively stable between pH 6.0 and 8.0. The optimum temperature range for cell growth and siderophore production was 25 to 30°C ($P < 0.05$) (Fig. 7). At 20°C and at 35°C, growth was slightly lower than at the optimum range ($P < 0.05$). Siderophore production was slightly lower at 20°C than at the optimum range ($P < 0.05$) and second lowest at 35°C ($P < 0.05$). Both the OD_{600} and SU values of strain SX9 were lowest at 15°C ($P < 0.05$). The growth and siderophore production of strain SX9 was gradually inhibited at NaCl concentrations higher than 2% and 1%, and no growth was observed at 5% NaCl ($P < 0.05$) (Fig. 8). Thus, when applying strain SX9 to bioremediation in field, maintaining the pH from 6.0 to 8.0 would be advisable. Based on the effects of environmental factors on siderophore production, the bioremediation efficiency of strain SX9 is at its highest at temperatures from 20 to 30°C and NaCl concentration below 2%.

Iron is an essential element of living organisms and plays an important role in metabolism, and free Fe^{3+} concentration in the environment is the primary factor affecting the synthesis of siderophore. The OD_{600}

value of strain SX9 was highest $3 \mu\text{mol}\cdot\text{L}^{-1} \text{Fe}^{3+}$ ($P < 0.05$), yet the tested Fe^{3+} concentrations had only a minor effect on the growth of strain SX9 (Fig. 9). The siderophore production by strain SX9 was highest without Fe^{3+} , and the SU values became gradually lower with increasing Fe^{3+} concentration ($P < 0.05$) (Fig. 9). The siderophore production by *P. fluorescens* and *P. putida* were at the maximum at $1 \mu\text{mol}\cdot\text{L}^{-1} \text{Fe}^{3+}$ (Sayyed et al. 2005). Increasing Fe^{3+} concentrations repressed siderophore synthesis by fluorescent pseudomonads, and at Fe^{3+} concentrations from 20 to $27 \mu\text{mol}\cdot\text{L}^{-1}$ siderophore production was stopped (Dave and Dube 2000; Sayyed et al. 2005). Similarly, the SU value of strain SX9 was only 2.7% at $20 \mu\text{mol}\cdot\text{L}^{-1} \text{Fe}^{3+}$.

3.4 Heavy metal tolerance and solubilization

Knowledge on the ability of bacterial strains to grow in the presence of contaminants is essential to estimate the applicability of the strains in bioremediating the contaminated soils. The strain SX9 was resistant to multiple metals, yet the strain was sensitive to Hg^2 (Table 2). The MIC values of Pb^{2+} , Cu^{2+} and Cd^{2+} were 4000, 5000 and 2000 μM , respectively. The high tolerance to Zn^{2+} , with a MIC of 22000 μM , may have been due to adaptation to the high level of zinc pollution in the sampled soil.

Table 2 Minimal inhibitory concentrations (MIC) of metal ions to the growth of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China.

Compound	MIC ($\mu\text{mol}\cdot\text{L}^{-1}$ / $\mu\text{g}\cdot\text{mL}^{-1}$)*
Ba ²⁺	40,000
Cd ²⁺	2,000
Co ⁴⁺	1,200
Cu ⁺	4,500
Cu ²⁺	5,000
Fe ²⁺	16,000
Fe ³⁺	16,000
Hg ²⁺	15
Mn ²⁺	70,000
Ni ²⁺	3,600
Pb ²⁺	4,000
Zn ²⁺	22,000
Ampicillin	3,500
Cephameycin	5,000
Doxycycline hyclate	10
Erythromycin	200
Gentamicin	80
Kanamycin	125
Rifampicin	15
Streptomycin	300
* Metal salts as $\mu\text{mol}\cdot\text{L}^{-1}$, antibiotics as $\mu\text{g}\cdot\text{mL}^{-1}$	

In bioremediating heavy metal contaminated soil, the availability of the metals directly affects the efficiency of remediation. The SPB may increase the solubility of heavy metals (Guo et al. 2011; Jiang et al. 2008). Guo *et al.* found that maximum values of water-soluble Cd, Zn and Pb in the solution inoculated with strain D54 were 28.3, 36.9 and 8.2 $\text{mg}\cdot\text{L}^{-1}$ and it was concluded that inoculation with strain D54 could help *S. alfredii* remove more metals from heavy metal contaminated soils (Guo et al.

2011). In agreement, the strain SX9 had a strong metal solubilization ability. In the SX9 inoculated cultures containing CuO, ZnO and CdCO₃, the concentrations of Cu²⁺, Zn²⁺ and Cd²⁺ in the supernatant were 25.94 ± 0.54 mg·L⁻¹, 2.21 ± 0.31 mg·L⁻¹ and 17.35 ± 0.37 mg·L⁻¹, respectively. The contents of Cu²⁺, Zn²⁺ and Cd²⁺ in the supernatant were 47.4%, 133.0% and 35.4% higher, respectively, in the strain SX9 inoculated cultures than in the not inoculated controls (*P* < 0.05).

3.5 Metal complexation capacity

Even though siderophores help the SPB to acquire iron, the complexation of siderophores with heavy metals decreases their free concentration and thus their toxicity (Schalk et al. 2011). In general, one μmol·L⁻¹ of siderophore was expected to complex one μmol·L⁻¹ of metal ion; the catechol-type siderophore (bacillibactin) produced by *Bacillus subtilis* can bind Fe³⁺ at 1:1 ratio (Dertz et al. 2006). However, the complexation capacity of strain SX9 siderophore was relatively low for Fe³⁺; the increase in complexed Fe³⁺ was only a fraction of the added (Fig. 10a). The complexation capacity of different siderophore types varies, and variation exists even within the same type (Ferreira et al. 2019). Possibly the complex formed by the strain SX9 catechol-type siderophore was not stable under alkaline conditions, or the added Fe³⁺ had precipitated.

According to the results of complexation of Cu²⁺ (data not shown), it is impossible to judge the Cu²⁺ complexation capacity of strain SX9. For both Zn²⁺ and Cd²⁺, the ratio of ions in complex versus ions added increased linearly till [ion]_{added} / [S] ratio of approximately 2, after which the adding of ions did not result in increases in complexed ions (Fig. 10b, 10c), indicating that the ions were complexed with fairly good effectiveness and were stable up to that amount of ions added.

3.6 Antibiotic tolerance

Environmental and clinical isolates are often both heavy metal and antibiotic resistant, either due to co-location of the resistance genes, e.g. on a plasmid, or due to a shared resistance mechanism, e.g. an efflux pump, that provides resistance against both metals and antibiotics (Pal et al. 2017). To avoid spreading highly antibiotic resistant strains, MICs of antibiotics to strain SX9 were examined. Results showed that strain SX9 will not evolve into superbugs while it is put into the environment. The strain SX9 was resistant to multiple antibiotics and sensitive to doxycycline hyclate and rifampicin with MIC values of 10 and 15 μg·mL⁻¹, respectively (Table 2). Doxycycline hyclate is an inhibitor of protein synthesis. However, similar sensitivity to other protein synthesis inhibitors such as gentamicin, streptomycin and kanamycin was not detected. The high resistance to cell wall synthesis inhibiting ampicillin and cephamycin implied that the strain SX9 produced β-lactamase.

3.7 Plant growth promoting characteristics of strain SX9

The plant growth promoting (PGP) characteristics of bacteria include the production of plant growth hormone IAA, the control of ethylene levels through ACC deaminase activity and the secretion of organic acids to dissolve nutrients, especially phosphorus. The PGP bacteria (PGPB) protect plants from

biological and abiotic stresses (Tiwari et al. 2016). In this study, the strain SX9 produced 9.50 ± 0.40 $\text{mg}\cdot\text{L}^{-1}$ IAA in L-tryptophan containing LB medium, and the ACC deaminase activity of strain SX9 was 19.13 ± 1.50 $\mu\text{mol } \alpha\text{-KA (mg}\cdot\text{h)}^{-1}$. Thus, the IAA synthesis and ACC deaminase capabilities of strain SX9 were superior to those of *Burkholderia* sp. J62 and D54 that promoted the growth and heavy metal accumulation capacity of *Zea mays* L., *Lycopersicon esculentum* Mill. and *Sedum alfredii* Hance (Guo et al. 2011; Jiang et al. 2008). The phosphate solubilization capacity of the strain SX9 was 106.60 ± 6.65 $\text{mg}\cdot\text{L}^{-1}$. The phosphate solubilization activity of the 8 strains isolated by Liu *et al.* ranged from 12.80 to 140.48 $\text{mg}\cdot\text{L}^{-1}$ (Liu et al. 2019), this indicated that the phosphate solubilization capacity of strain SX9 reached a medium level. The PGP characteristics of the strain SX9 suggest that the strain might be applicable in the plant assisted bioremediation of contaminated sites.

Lolium perenne can be applied in bioremediation due to its tolerance to heavy metals, fast growth rate and heavy metal accumulation capacity (Arienzo et al. 2004). SPB may contribute to the tolerance; for example, the siderophores of *B. tequilensis* CD36 combined with Ni^{2+} and Pb^{2+} and alleviated the metal stress of *Miscanthus* seeds (Li 2018). Therefore, we determined the effect of strain SX9 on the germination and growth of *L. perenne* under Cu, Zn and Cd stress. Without the heavy metals, the germination rate of seeds was the highest and roots were shortest when inoculated with the strain SX9 culture ($P < 0.05$), and inoculation did not result in differences in shoot length (Table 3). Similar to a previous study (Aris et al. 2011), the ability to promote seed germination may have been due to the IAA produced by strain SX9.

Table 3

Growth characteristics of *Lolium perenne*, inoculated with bacterial growth medium, strain SX9 culture or strain SX9 culture supernatant, under Cu²⁺ stress.

	Cu ²⁺ (mg·L ⁻¹)	Medium	Culture	Supernatant
Germination rate (%)	0	64.67 ± 3.06 Ab	73.33 ± 3.06 Aa	62.00 ± 2.00 Ab
	100	45.33 ± 1.15 Bb	42.67 ± 3.06 Bb	64.00 ± 3.46 Aa
	200	20.00 ± 2.00 Cc	35.33 ± 2.31 Cb	52.67 ± 3.06 Ba
	400	0.00 ± 0.00 Dc	7.33 ± 1.15 Db	41.33 ± 3.06 Ca
Shoot length (cm)	0	4.97 ± 1.24 Aa	3.80 ± 0.44 Aa	3.40 ± 0.56 Ba
	100	3.83 ± 0.45 Ab	3.33 ± 0.57 Ab	5.00 ± 0.44 Aa
	200	2.23 ± 0.31 Bb	1.97 ± 0.21 Bb	3.40 ± 0.30 Ba
	400	0.00 ± 0.00 Cb	1.90 ± 0.36 Ba	2.17 ± 0.55 Ca
Root length (cm)	0	4.17 ± 0.58 Aa	1.60 ± 0.10 Ab	3.67 ± 0.06 Aa
	100	0.17 ± 0.12 Bb	0.17 ± 0.06 Bb	0.60 ± 0.20 ABab
	200	0.13 ± 0.06 Bb	0.17 ± 0.12 Bb	0.47 ± 0.06 Ba
	400	0.00 ± 0.00 Cc	0.10 ± 0.00 Bb	0.40 ± 0.10 Ba
The values are mean ± standard deviation (<i>n</i> = 3). Different capital and lowercase letters indicate statistically significant differences between Cu ²⁺ concentrations and inoculants, respectively (<i>P</i> < 0.05).				

Some metal ions mainly inhibits the abscisic acid (ABA) catabolism of plants and causes oxidative stress (Ye et al. 2014). In the Cu²⁺ stress negative control, inoculated with the growth medium, the seed germination rate was lower the higher the Cu²⁺ concentration (*P* < 0.05), and at the highest concentration of 400 mg·L⁻¹ the seeds did nor germinate (Table 3). Similarly, for the seeds inoculated with strain SX9 culture and culture supernatant, the germination rate decreased with increasing Cu²⁺ concentration (*P* < 0.05), yet the seeds germinated even at the highest Cu²⁺ concentration. SPB can provide sufficient bioavailable Fe³⁺ to plants, which can promote plant growth and reduce the toxicity of heavy metals to plants (Marcus and Mohamed 2007). Interestingly, even though without the heavy metals the germination rate was promoted more by the strain SX9 culture than by the supernatant, the supernatant promoted the germination rate more than the culture at all Cu²⁺ concentrations tested (*P* < 0.05). At the 200 mg·L⁻¹ and 400 mg·L⁻¹ Cu²⁺ concentrations, the roots of *L. perenne* inoculated with the strain SX9 culture were shorter than the roots of *L. perenne* inoculated with the supernatant (*P* < 0.05). Thus, the growth promotion under Cu²⁺ stress was likely not due to the siderophore that was expected to remain in the supernatant. Characterizing the PGP property effective under Cu²⁺ stress requires further analyses.

In the Zn²⁺ stress negative control, inoculated with the growth medium, the seed germination rate was lower above 200 mg·L⁻¹ Zn²⁺ concentration ($P < 0.05$) (Table 4). At the 500 mg·L⁻¹ and 1000 mg·L⁻¹ Zn²⁺ concentrations, the germination rates of seeds inoculated with the strain SX9 culture and supernatant were higher than that in the negative control ($P < 0.05$). Possibly the siderophore effectively bound Zn²⁺, making it less toxic. At the highest Zn²⁺ concentration of 1000 mg·L⁻¹, inoculation did not result in differences in shoot and root lengths.

Table 4

Growth characteristics of *Lolium perenne*, inoculated with bacterial growth medium, strain SX9 culture or strain SX9 culture supernatant, under Zn²⁺ stress

	Zn ²⁺ (mg·L ⁻¹)	Medium	Culture	Supernatant
Germination rate (%)	0	64.67 ± 3.06 Ab	73.33 ± 3.06 Aa	62.00 ± 2.00 Bb
	200	64.00 ± 2.00 Ab	70.00 ± 2.00 Ba	69.33 ± 3.06 Aa
	500	49.33 ± 3.06 Bb	56.67 ± 1.15 Ca	60.67 ± 2.31 Ba
	1,000	44.67 ± 2.31 Bb	49.33 ± 1.15 Da	49.33 ± 3.06 Ca
Shoot length (cm)	0	4.97 ± 1.24 Aa	3.80 ± 0.44 Ba	3.40 ± 0.56 ABA
	200	2.13 ± 0.21 Bb	4.77 ± 0.21 Aa	4.63 ± 0.81 Aa
	500	4.30 ± 0.30 Aa	2.53 ± 0.61 BCb	3.70 ± 0.56 Aa
	1,000	2.77 ± 0.32 Ba	3.13 ± 0.45 Ba	2.40 ± 0.66 Ba
Root length (cm)	0	4.17 ± 0.58 Aa	1.60 ± 0.10 Ab	3.67 ± 0.06 Aa
	200	2.77 ± 0.70 Bb	1.70 ± 0.53 Ab	3.13 ± 0.81 ABA
	500	1.90 ± 0.53 Bb	1.40 ± 0.17 Ab	2.97 ± 0.38 Ba
	1,000	1.67 ± 1.25 Ba	1.40 ± 0.62 Aa	0.90 ± 0.20 Ba
The values are mean ± standard deviation ($n = 3$). Different capital and lowercase letters indicate statistically significant differences between Zn ²⁺ concentrations and inoculants, respectively ($P < 0.05$).				

Low concentration of Cd²⁺ have been found to promote the germination of seeds (Chen et al. 2013). Similarly, in the Cd²⁺ stress experiment, the seed germination rate was higher with than without Cd²⁺ ($P < 0.05$) (Table 5). At the highest Cd²⁺ concentration of 20 mg·L⁻¹, inoculation did not result in differences in germination rates, yet shoots were shortest in the negative control and roots were longest when inoculated with the culture supernatant ($P < 0.05$). In addition to the siderophore, the growth promotion may have been due to the production of IAA, ACC deaminase and other substances (Li 2018). Bacterial IAA can help break seed dormancy by stimulating the division of plant cells to promote the development of root and shoot (Aka and Babalola 2016). Similar enhancement of seed germination by inoculation with *Bacillus* sp. RM-2 and bacilli formulations has been observed in food crops such as cowpea, maize

and pearl millet (Minaxi et al. 2012). Taken together, the results indicated that strain SX9 may be applicable to support the growth of *L. perenne* in remediating heavy metal contaminated soil.

Table 5

Growth characteristics of *Lolium perenne*, inoculated with bacterial growth medium, strain SX9 culture or strain SX9 culture supernatant, under Cd²⁺ stress

	Cd ²⁺ (mg·L ⁻¹)	Medium	Culture	Supernatant
Germination rate (%)	0	64.00 ± 2.00 Cb	79.33 ± 1.15 Ba	56.67 ± 3.06 Bc
	5	83.33 ± 3.06 Bb	90.67 ± 3.06 Aa	87.33 ± 1.15 Aab
	10	94.67 ± 1.15 Aa	94.00 ± 2.00 Aa	82.67 ± 1.15 Ab
	20	90.67 ± 3.06 Aa	90.67 ± 1.15 Aa	89.33 ± 6.11 Aa
Shoot length (cm)	0	4.17 ± 0.65 Ba	2.97 ± 0.67 Bab	2.13 ± 0.35 Cb
	5	6.50 ± 0.70 Ab	3.63 ± 0.42 ABc	7.77 ± 0.25 Aa
	10	3.87 ± 0.51 Bab	3.17 ± 0.47 ABb	4.47 ± 0.31 Ba
	20	2.43 ± 0.45 Cb	4.27 ± 0.55 Aa	3.90 ± 0.61 Ba
Root length (cm)	0	1.50 ± 0.30 Bb	2.00 ± 0.30 Cb	3.13 ± 0.90 Ba
	5	1.67 ± 0.15 Bb	3.70 ± 0.46 Aa	4.63 ± 0.81 ABa
	10	1.73 ± 0.55 ABb	2.00 ± 0.70 BCb	5.33 ± 1.10 Aa
	20	2.53 ± 0.50 Ab	2.93 ± 0.21 BCb	5.60 ± 0.92 Aa
The values are mean ± standard deviation (n = 3). Different capital and lowercase letters indicate statistically significant differences between Cd ²⁺ concentrations and inoculants, respectively (P < 0.05).				

4 Conclusion

Forty-four bacterial strains were isolated from heavy metal contaminated soil. An efficiently siderophore producing strain, SX9, was identified as *Burkholderia* sp. The strain SX9 tolerated and solubilized heavy metals and promoted plant growth with and without heavy metal stress. Therefore, *Burkholderia* sp. SX9 has potential to assist in phytoremediation of heavy metal contaminated soils. Assessing the applicability of the strain requires further study, especially on the performance of the strain at in situ phytoremediation.

5 Declarations

Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

All authors read and approved the final manuscript. The work described has not been published. It is not under consideration for publication elsewhere. Its publication has been approved by all coauthors. If the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Authors' contributions

Writing - Original Draft: Yajun Wang. Visualization: Yajun Wang and Yaqian Li. Formal analysis: Yajun Wang, Wei Huang and Yaqian Li. Resources, Writing - Review & Editing, Supervision: Fang-Bo Yu and Petri Penttinen.

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Competing interests

The authors declare that they have no conflict of interest.

Availability of data and materials

The 16S rRNA gene sequence of strain SX9 was submitted to the National Center for Biotechnology Information (NCBI) GenBank with the accession number MN658668. The dataset generated during and/or analysed within the current study are available from the corresponding author on reasonable request.

6 References

1. 15618-2018 GB (2018) Soil environmental quality risk control standard for soil contamination of agricultural land. Ministry of Ecology and Environment of China, and State Administration for Market Regulation of China p 4
2. Aka RJN, Babalola OO (2016) Effect of bacterial inoculation of strains of *Pseudomonas aeruginosa*, *Alcaligenes faecalis* and *Bacillus subtilis* on germination, growth and heavy metal (Cd, Cr, and Ni) uptake of *Brassica juncea*. Int J Phytoremediat 18(2):200-209. <https://doi.org/10.1080/15226514.2015.1073671>
3. Arienzo M, Adamo P, Cozzolino V (2004) The potential of *Lolium perenne* for revegetation of contaminated soil from a metallurgical site. Sci Total Environ 319(1-3):13-25. [https://doi.org/10.1016/S0048-9697\(03\)00435-2](https://doi.org/10.1016/S0048-9697(03)00435-2)
4. Arnow LE (1937) Colorimetric determination of the components of 3, 4-dihydroxyphenylalanine tyrosine mixtures. J Biol Chem 118(2):531-537. [https://doi.org/10.1016/S0021-9258\(18\)74509-2](https://doi.org/10.1016/S0021-9258(18)74509-2)
5. Arora NK, Verma M (2017) Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. 3 Biotech 7(6):381. <https://doi.org/10.1007/s13205-017-1008-y>
6. Arton LL, Hemming BC (1994) Iron chelation in plants and soil microorganisms. Soil Sci 157(5):331-332. <https://doi.org/10.1097/00010694-199405000-00010>
7. Ashraf S, Ali Q, Zahir ZA, Ashraf S, Asghar HN (2019) Phytoremediation: environmentally sustainable way for reclamation of heavy metal polluted soils. Ecotox Environ Safe 174:714-727. <https://doi.org/10.1016/j.ecoenv.2019.02.068>
8. Bahadur ST, Vikram S, Deepika G, Mrinalini P, Arti Y, Preksha S, Akbar A, Prem KD (2020) Identification, characterization and evaluation of multifaceted traits of plant growth promoting rhizobacteria from soil for sustainable approach to agriculture. Curr Microbiol 77(11):3633-3642. <https://doi.org/10.1007/s00284-020-02165-2>
9. Bao SD (2000) Soil and agricultural chemistry analysis, 3th ed. China Agriculture Press
10. Chen W, Zhang MM, Song YY, Zhang DG (2013) Inhibitory effect of heavy metal stress on the seed germination of four turfgrass types. Acta Agrestia Sinica 21(3):556-563. <https://doi.org/10.11733/j.issn.1007-0435.2013.03.022>
11. Csáky TZ, Hassel O, Rosenberg T, Turunen E, Tuhkanen A (1948) On the estimation of bound hydroxylamine in biological materials. Acta Chem Scand 2:450-454. <https://doi.org/10.3891/acta.chem.scand.02-0450>
12. Dave BP, Dube HC (2000) Regulation of siderophore production by iron Fe(III) in certain fungi and fluorescent pseudomonads. Indian J Exp Biol 38(3):297. <https://doi.org/10.1039/AN9578200498>
13. Dertz EA, Stintzi A, Stintzi A, Raymond KN (2006) Siderophore-mediated iron transport in *Bacillus subtilis* and *Corynebacterium glutamicum*. J Biol Inorg Chem 11:1087-1097. <https://doi.org/10.1007/s00775-006-0151-4>
14. Dimkpa CO, Svatos A, Dabrowska P, Schmidt A, Boland W, Kothe E (2009) Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp. Chemosphere 74(1):19-25. <https://doi.org/10.1016/j.chemosphere.2008.09.079>

15. Ferreira CM, Boas AV, Sousa CA, Soares HM, Soares EV (2019) Comparison of five bacterial strains producing siderophores with ability to chelate iron under alkaline conditions. *AMB Express* 9(1):78. <https://doi.org/10.1186/s13568-019-0796-3>
16. Guo JK, Tang SR, Ju XH, Ding YZ, Liao SQ, Song NN (2011) Effects of inoculation of a plant growth promoting rhizobacterium *Burkholderia* sp. D54 on plant growth and metal uptake by a hyperaccumulator *Sedum alfredii* Hance grown on multiple metal contaminated soil. *World J Microb Biot* 27(12):2835-2844. <https://doi.org/10.1007/s11274-011-0762-y>
17. Holt JH, Krieg NR, Sneath P, Staley JT, Williams ST (1994). *Bergey's manual of determinative bacteriology* ninth edition. Baltimore: Williams & Wilkins.
18. Jiang CY, Sheng XF, Qian M, Wang QY (2008) Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. *Chemosphere* 72(2):157-164. <https://doi.org/10.1016/j.chemosphere.2008.02.006>
19. Khalid S, Shahid M, Niazi NK, Murtaza B, Bibi I, Dumat C (2016) A comparison of technologies for remediation of heavy metal contaminated soils. *J Geochem Explo* 182:247-268. <https://doi.org/10.1016/j.gexplo.2016.11.021>
20. Khan A, Singh P, Srivastava A (2017) Synthesis, nature and utility of universal iron chelator-siderophore: a review. *Microbiol Res* 212-213:103-111. <https://doi.org/10.1016/j.micres.2017.10.012>
21. Koichiro T, Glen S, Daniel P, Alan F, Sudhir K (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30(12):2725-2729. <https://doi.org/10.1093/molbev/mst197>
22. Kong P, Hong CX (2020) Endophytic *Burkholderia* sp. SSG as a potential biofertilizer promoting boxwood growth. *PeerJ* 8(2):e9547. <https://doi.org/10.7717/peerj.9547>
23. Kuppusamy S, Palanisami T, Megharaj M, Venkateswarlu K, Naidu R (2016) In-situ remediation approaches for the management of contaminated sites: a comprehensive overview. *Rev Environ Contam T* 236(18):1-115. https://doi.org/10.1007/978-3-319-20013-2_1
24. Li YY (2018) Screening of siderophore-high-yield plant growth promoting rhizobacteria and its potential application in soil remediation master. Dissertation, Jiangnan University
25. Liu CJ, Lin H, Dong YB, Li B, Wang L (2019) Identification and characterization of plant growth-promoting endophyte RE02 from *Trifolium repens* L. in mining smelter. *Environ Sci Pollut R* 26(17):17236-17247. <https://doi.org/10.1007/s11356-019-04904-w>
26. Lucian PV, Gabriela B, Florian S, Nicolae M, Maria G, Cristian A (2014) Study on bioremediation of heavy metal-contaminated soils using the bacteria-plants synergy. *Agron Ser Sci Res* 57(2):125-128.
27. Marcus M, Mohamed AM (2007) Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol R* 71(3):413-451. <https://doi.org/10.1128/MMBR.00012-07>
28. Minaxi LN, Yadav RC, Saxena J (2012) Characterization of multifaceted *Bacillus* sp RM-2 for its use as plant growth promoting bioinoculant for crops grown in semi arid deserts. *Appl Soil Ecol* 59:124-135. <https://doi.org/10.1016/j.apsoil.2011.08.001>

29. Pal C, Asiani K, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson JD, Hobman JL (2017) Metal resistance and its association with antibiotic resistance. *Adv Microb Physiol* 70:261-313. <https://doi.org/10.1016/bs.ampbs.2017.02.001>
30. Patel PR, Shaikh SS, Sayyed RZ (2018) Modified chrome azurol S method for detection and estimation of siderophores having affinity for metal ions other than iron. *Env Sust* 1(1):81-87. <https://doi.org/10.1007/s42398-018-0005-3>
31. Penrose DM, Glick BR (2010) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plantarum* 118(1):10-15. <https://doi.org/10.1034/j.1399-3054.2003.00086.x>
32. Pliego C, Kamilova F, Lugtenberg B (2011) Plant growth-promoting bacteria: fundamentals and exploitation. *Bacteria in agrobiolgy: Crop ecosystems* (pp.295-343). Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-18357-7_11
33. Rajkumar M, Ae N, Prasad MNV, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol* 28(3):142-149. <https://doi.org/10.1016/j.tibtech.2009.12.002>
34. Saha M, Sarkar S, Sarkar B, Sharma BK, Bhattacharjee S, Tribedi P (2016) Microbial siderophores and their potential applications: a review. *Environ Sci Pollut R* 23(5):3984-3999. <https://doi.org/10.1007/s11356-015-4294-0>
35. Sayyed RZ, Badgajar M, Sonawane HM, Mhaske MM, Chincholkar SB (2005) Production of microbial iron chelators (siderophores) by fluorescent pseudomonads. *Indian J Biotechnol* 4(4):484-490.
36. Sayyed RZ, Seifi S, Patel PR, Shaikh SS, Enshasy HE (2019) Siderophore production in groundnut rhizosphere isolate, *Achromobacter* sp. RZS2 influenced by physicochemical factors and metal ions. *Env Sust* 2(2):117-124. <https://doi.org/10.1007/s42398-019-00070-4>
37. Schalk IJ, Hannauer M, Braud A (2011) New roles for bacterial siderophores in metal transport and tolerance. *Environ Microbiol* 13(11):2844-2854. <https://doi.org/10.1111/j.1462-2920.2011.02556.x>
38. Schywn B, Niellands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47-56. [https://doi.org/10.1016/0003-2697\(87\)90612-9](https://doi.org/10.1016/0003-2697(87)90612-9)
39. Shenker M, Oliver I, Helmann M, Hadar Y, Chen Y (1992) Utilization by tomatoes of iron mediated by a siderophore produced by *Rhizopus arrhizus*. *J Plant Nutr* 15(10):2173-2182. <https://doi.org/10.1080/01904169209364466>
40. Sinha S, Mukherjee SK (2008) Cadmium-induced siderophore production by a high Cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. *Curr Microbiol* 56(1):55-60. <https://doi.org/10.1007/s00284-007-9038-z>
41. Tiwari S, Sarangi KB, Thul ST (2016) Identification of arsenic resistant endophytic bacteria from *Pteris vittata* roots and characterization for arsenic remediation application. *J Environ Manage* 180:359-365. <https://doi.org/10.1016/j.jenvman.2016.05.029>
42. Wahyudi AT, Astuti RI, Giyanto (2011) Screening of *Pseudomonas* sp. isolated from rhizosphere of soybean plant as plant growth promoter and biocontrol agent. *Am J Agr Biol Sci* 6(1):134-141.

<https://doi.org/10.3844/ajabssp.2011.134.141>

43. Wang Q, Xiong D, Zhao P, Yu B, Tu B, Wang G (2011) Effect of applying an arsenic-resistant and plant growth-promoting rhizobacterium to enhance soil arsenic phytoremediation by *Populus deltoides* LH05-17. *J Appl Microbiol* 111(5):1065-1074. <https://doi.org/10.1111/j.1365-2672.2011.05142.x>
44. Wang WX, Lun ZX, Ling LZ, Peng WM, Wei WW (2014) Detection of siderophore production from hydrogen-oxidizing bacteria with CAS overlay plate method. *Microb China* 41(008):1692-1697. <https://doi.org/https://doi.org/10.13344/j.microbiol.china.130462>
45. Weisburg GW, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173(2):697-703. <https://doi.org/10.1128/jb.173.2.697-703.1991>
46. Ye NH, Li HX, Zhu GH, Liu YG, Liu R, Xu WF, Jing Y, Peng XX, Zhang JH (2014) Copper suppresses abscisic acid catabolism and catalase activity, and inhibits seed germination of rice. *Plant Cell Physiol* 55(11):2008-2016. <https://doi.org/10.1093/pcp/pcu136>.
47. Yu FB, Shan SD, Luo LP, Guan LB, Qin H (2013) Isolation and characterization of a *Sphingomonas* sp. strain F-7 degrading fenvalerate and its use in bioremediation of contaminated soil. *J Environ Sci Heal B* 48(3):198-207. <https://doi.org/10.1080/03601234.2013.730299>

Figures



Figure 1

Colony morphology of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China, on LB agar.

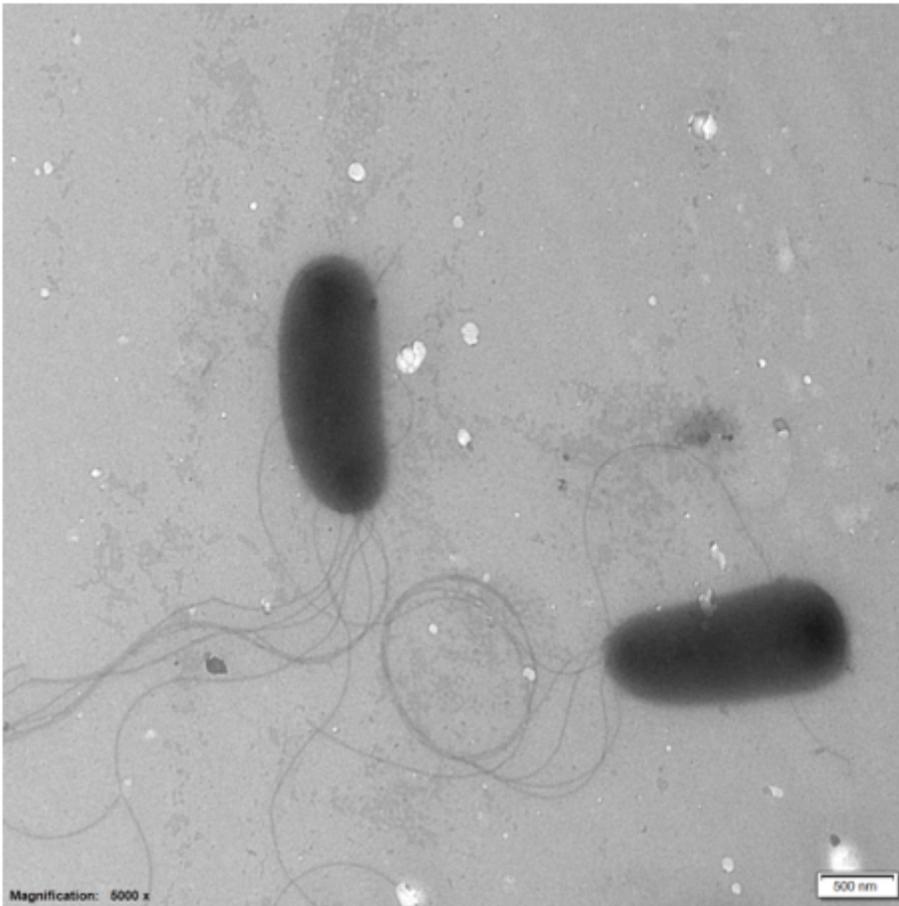


Figure 2

TEM micrograph of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China.

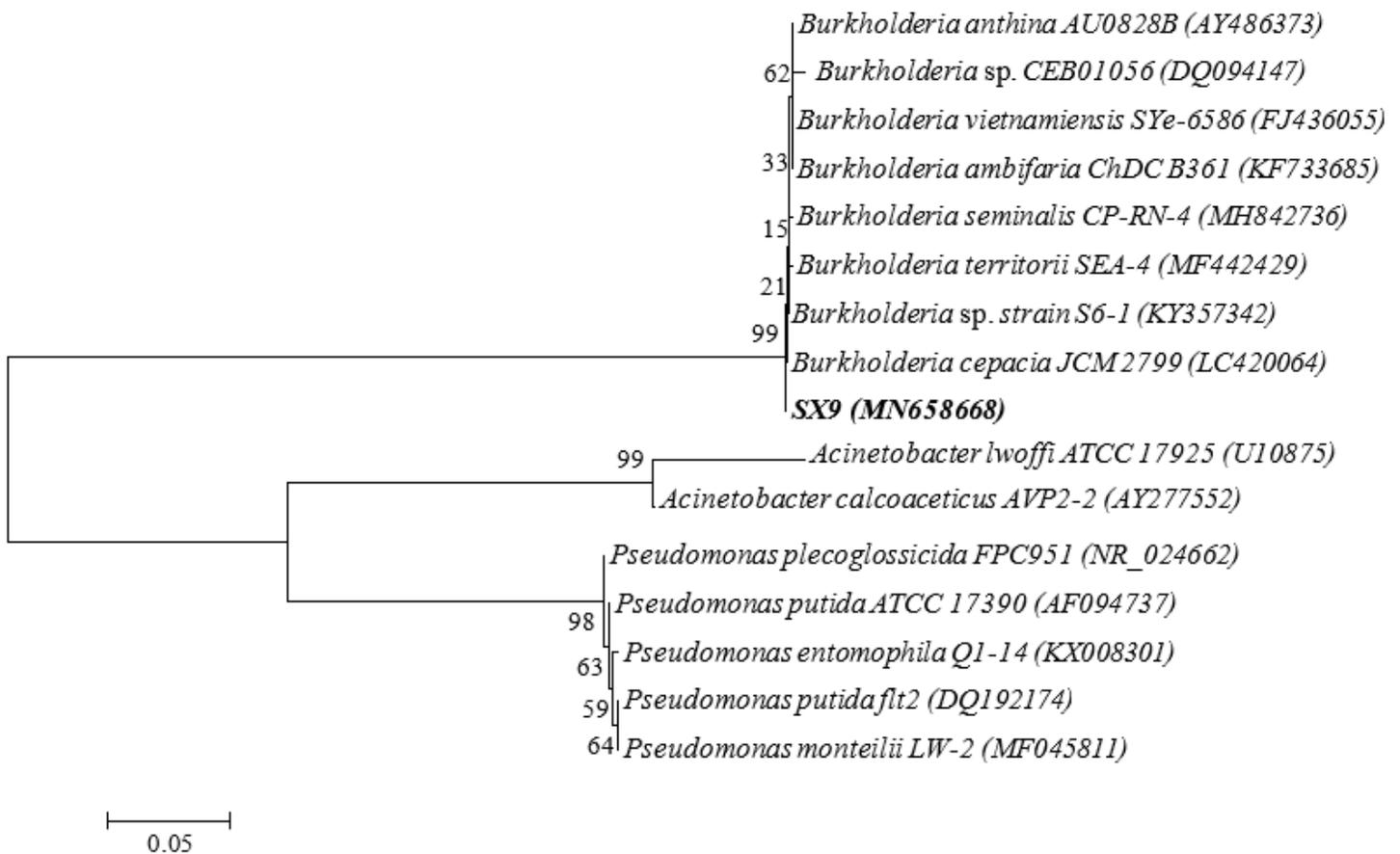


Figure 3

Neighbor-joining tree based on 16S rDNA sequences showing the phylogenetic relatedness between strain SX9 (in bold) isolated from lead and zinc mine soil in Shangyu, Zhejiang, China, and reference strains. Bootstrap values $\geq 50\%$ are shown on the branches. Genbank accession numbers are in parentheses. Scale bar = 5% substitutions per site.

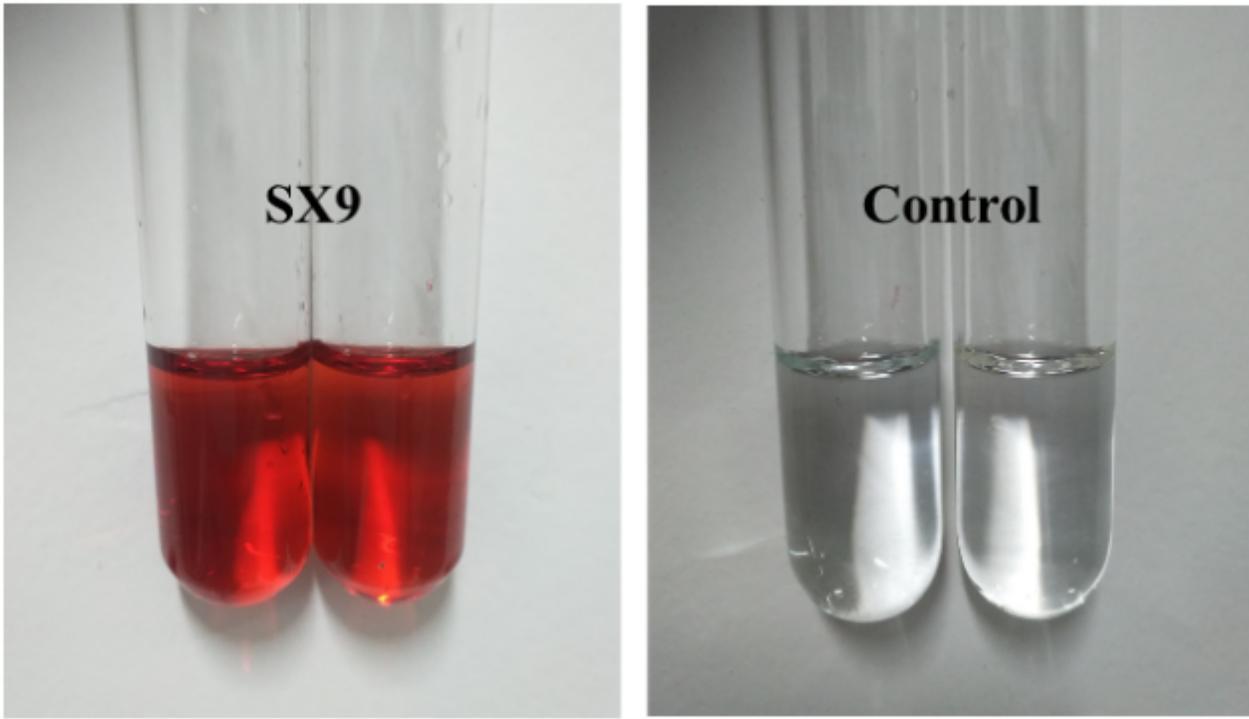


Figure 4

Arnow's test of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China.

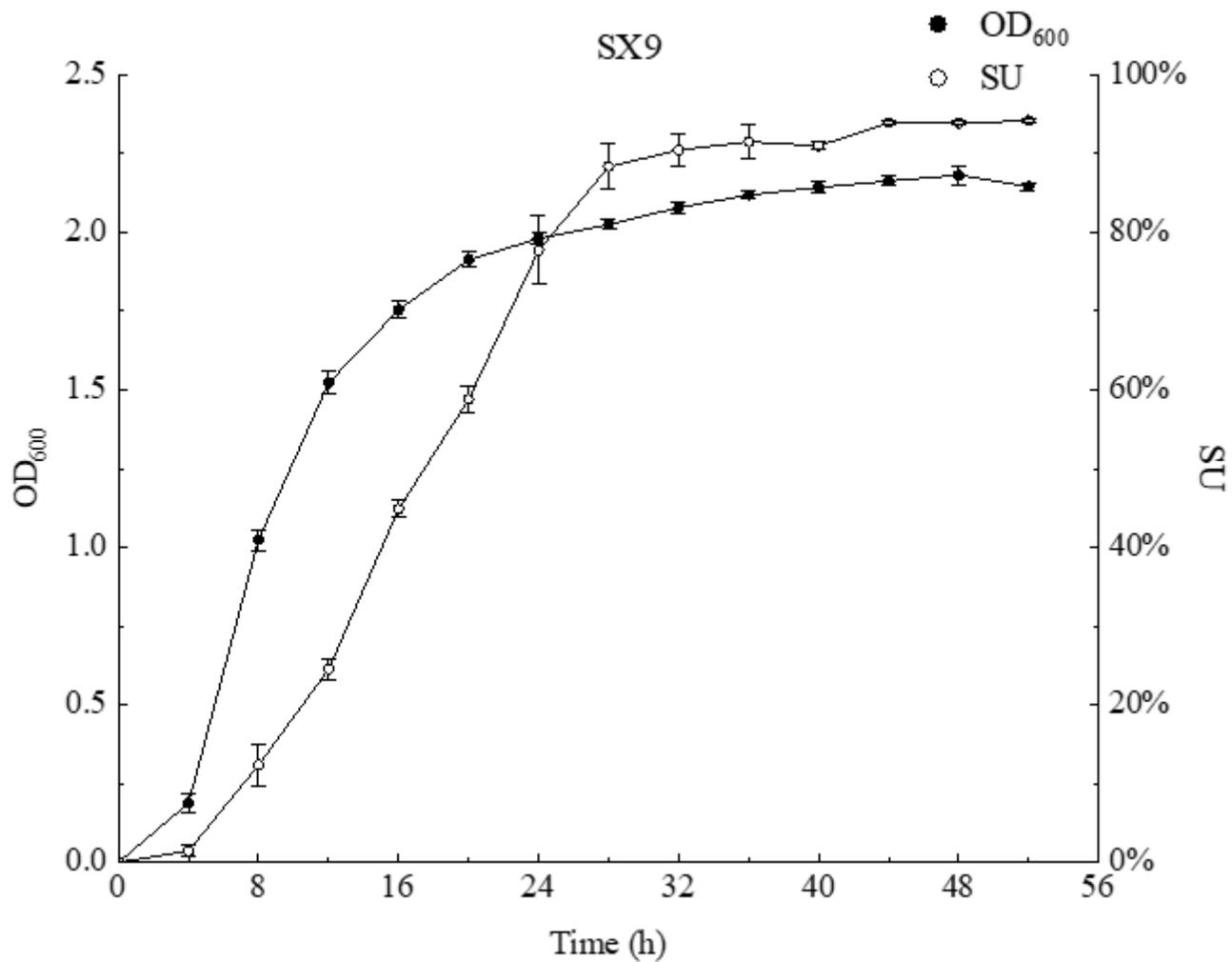


Figure 5

Growth and siderophore production by strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China. SU = percent siderophore unit.

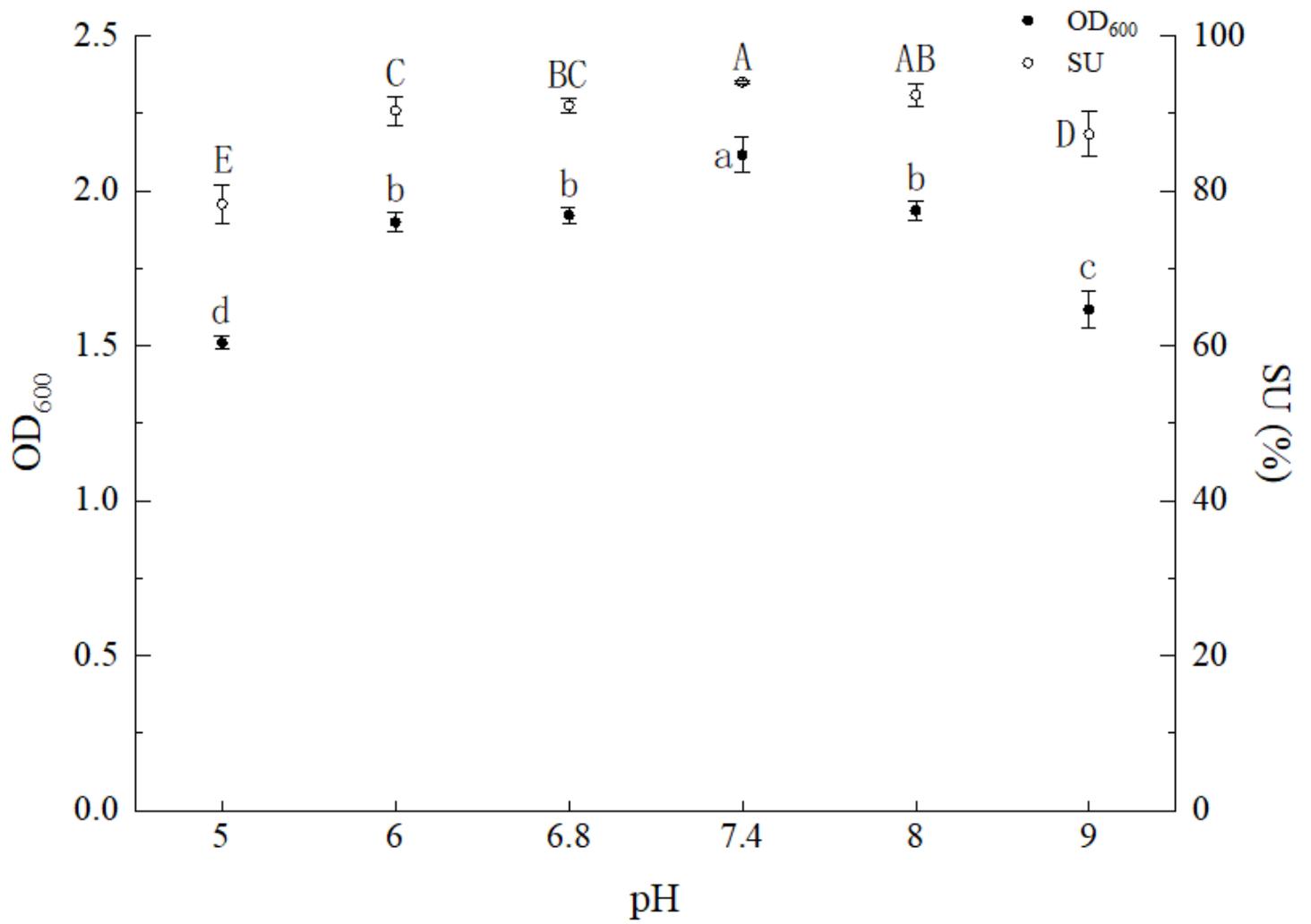


Figure 6

Effect of pH on the growth and siderophore production of strain SX9. SU = percent siderophore unit. Different lowercase and capital letters above points indicate statistically significant differences between growth and SU values, respectively.

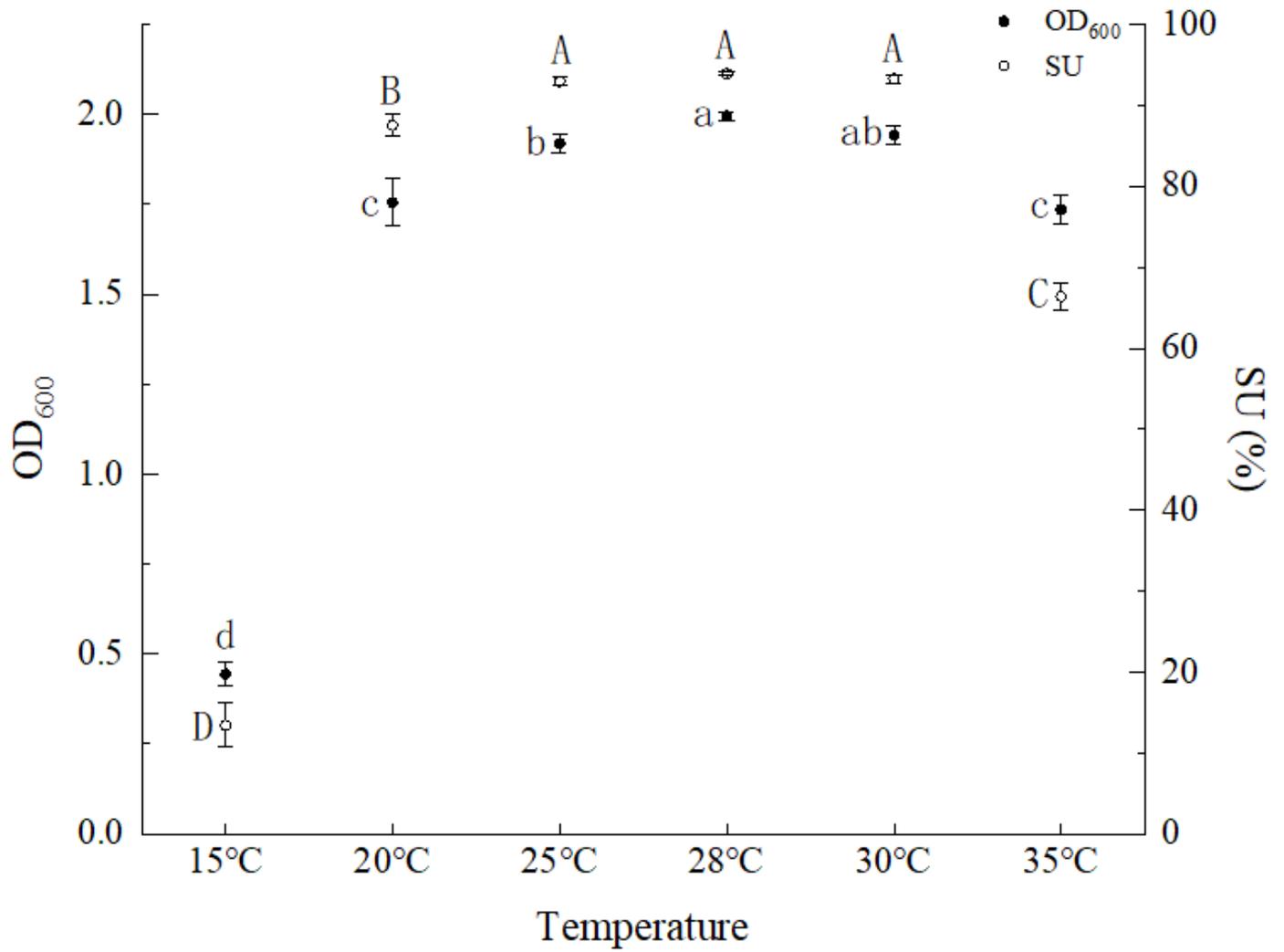


Figure 7

Effect of temperature on the growth and siderophore production of strain SX9. SU = percent siderophore unit. Different lowercase and capital letters above points indicate statistically significant differences between growth and SU values, respectively.

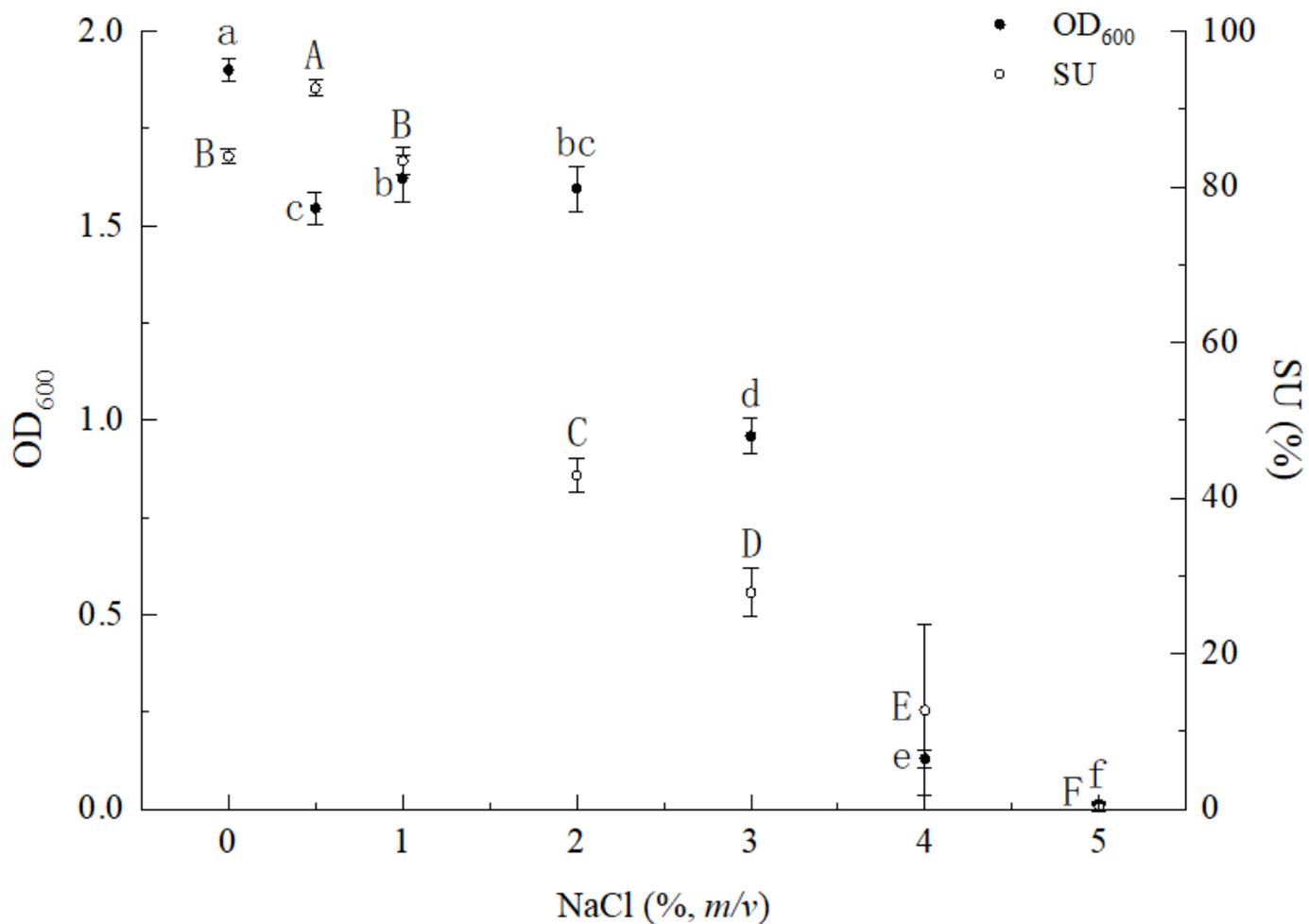


Figure 8

Effect of NaCl concentration on the growth and siderophore production of strain SX9. SU = percent siderophore unit. Different lowercase and capital letters above points indicate statistically significant differences between growth and SU values, respectively.

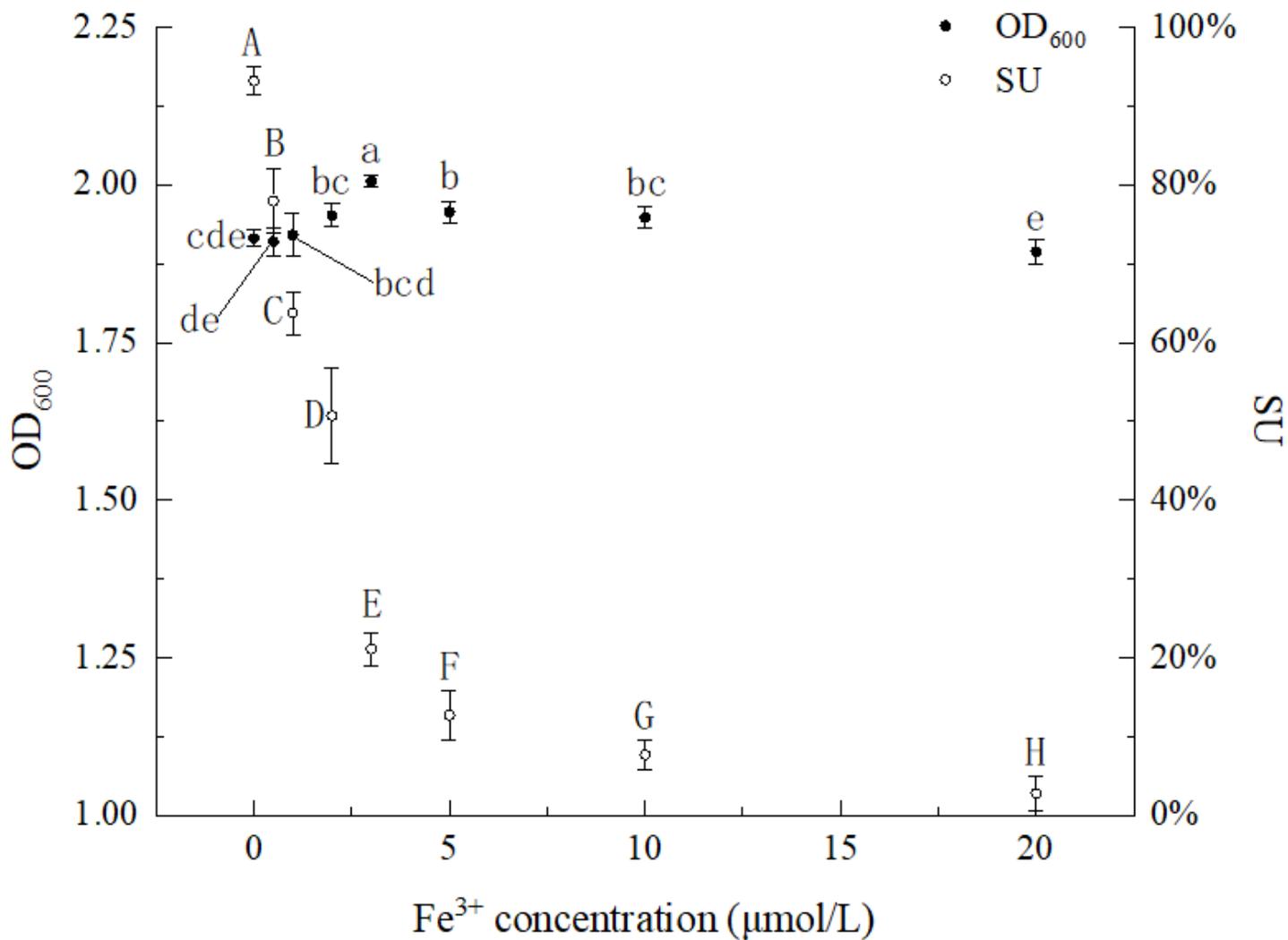


Figure 9

Effect of Fe³⁺ concentration on the growth and siderophore production of strain SX9. SU = percent siderophore unit. Different lowercase and capital letters above points indicate statistically significant differences between growth and SU values, respectively.

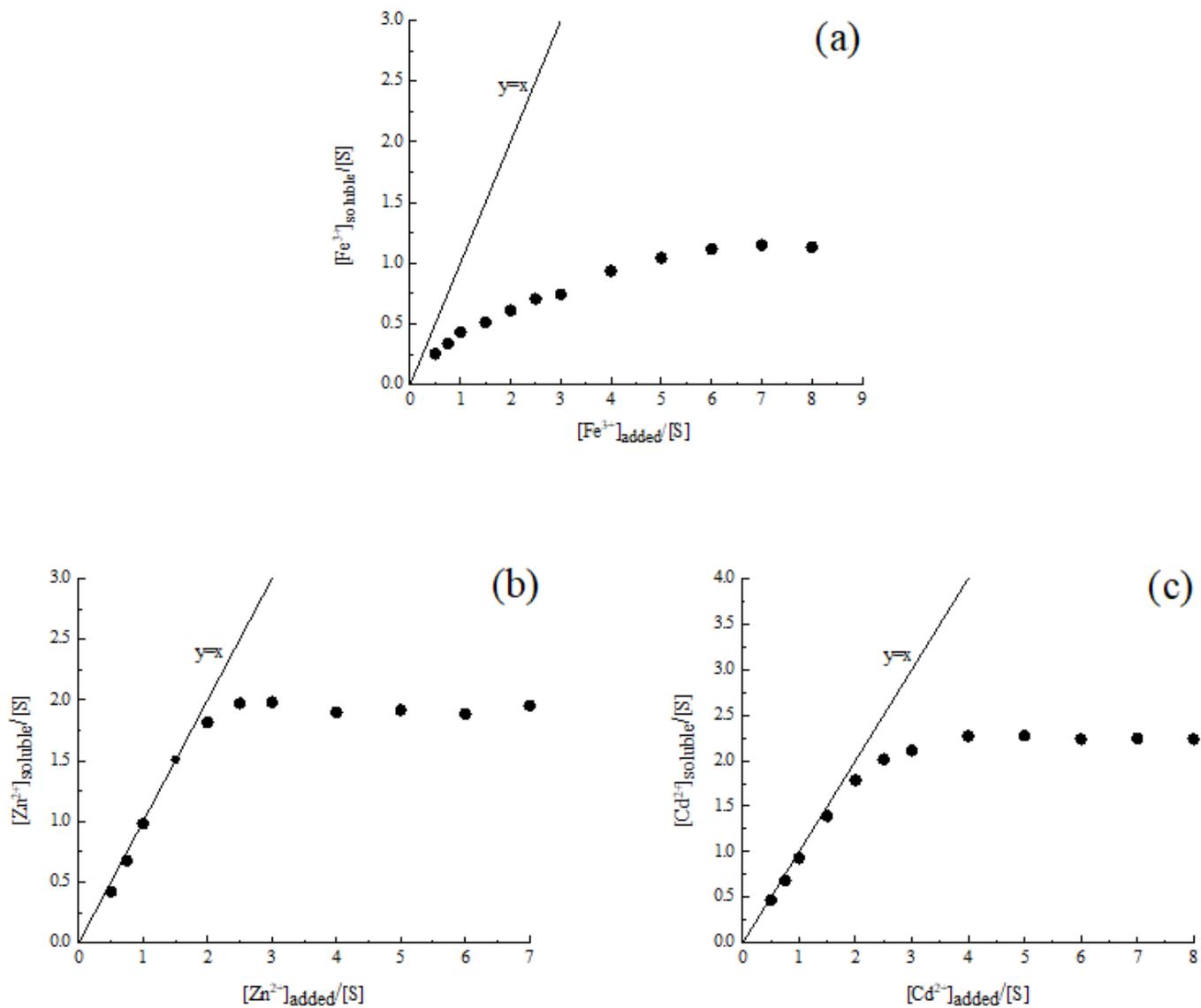


Figure 10

The metal ion complexing ability of the siderophores of strain SX9 isolated from lead and zinc mine soil in Shangyu, Zhejiang, China.