Screening and Culture Condition Optimization of a Siderophore-producing Endophytic Bacterium from *Saposhnikova divaricata* (Trucz.) Schischk. and Its Growth-Promoting Evaluation on Mulberry Seedlings

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Abstract [**Objectives**] This study was conducted to explore the culture conditions for siderophore production and preliminarily investigate the growth-promoting effects of strains. [**Methods**] Endophytic bacteria secreting siderophores were isolated and screened from the roots of the plant *Saposhnikovia divaricata* (Trucz.) Schischk. The siderophore-producing capability was confirmed through qualitative detection and gene cluster analysis. [**Results**] The screened endophytic bacterium PB-2 belonged to the genus *Paenibacillus* sp. and could produce catechol-type or carboxylate-type siderophores. When cultured in MKB medium with 15 g/L glycerol as the carbon source and 6 g/L glycine as the nitrogen source for 3 d, the strain exhibited efficient siderophore secretion. Appropriate concentrations of Fe^{3+}, Co^{2+}, Cu^{2+} and Al^{3+} promoted siderophore production, with 15 μmol/L Fe^{3+} being the most effective. Under the combined influences of siderophores, volatile compounds and other factors, strain PB-2 altered the root morphology of mulberry seedlings and increased their biomass. The primary root length decreased by 9.15%, while the number and length of root hairs increased by 37.93% and 16.37%, respectively, and the total biomass increased by 10.26%, all showing significant differences ($P<0.05$). Additionally, strain PB-2 enhanced the activities of defense-related enzymes in mulberry seedling leaves. The activities of superoxide dismutase (SOD) and peroxidase (POD) increased significantly ($P<0.05$) by 247.27% and 189.47%, respectively, compared with the control group. The activities of phenylalanine ammonialyase (PAL) and polyphenol oxidase (PPO) also showed varying degrees of increase. [**Conclusions**] This study provided a theoretical basis for rational utilization of endophytic growth-promoting strains secreting siderophores in the sustainable development of agriculture.

Key words Endophytic bacterium; *Paenibacillus* sp.; Siderophore; Promoting growth

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Iron (Fe) is an essential element for the growth and development of living organisms, participating in numerous metabolic processes[1-3]. Although Fe is abundant in the Earth's crust, it predominantly exists in a stable insoluble Fe^{3+} form, resulting in low bioavailability[3-4]. To overcome this challenge, various plants and microorganisms have evolved distinct strategies to acquire Fe from their environment[5-6]. Among these, the use of siderophores is one of the key approaches employed by microbes and plants to transport external iron ions[7-8].

Siderophores are small-molecule compounds that can specifically chelate Fe^{3+}[4] to form "siderophore-Fe^{3+}" complexes, which supply Fe for the growth and development of microorganisms and plants[7-8]. Bacteria, fungi, plants and algae can produce siderophores under iron-deficient conditions, with microbial siderophores being the most extensively studied. There have also been numerous reports on microbial siderophores controlling plant diseases and promoting plant growth. For example, Liu *et al.*[9] found that siderophores exhibited inhibitory effects against *Xanthomonas oryzae* pv. Oryzae. Siderophores extracted from the fermentation broth of *Pseudomonas aeruginosa* F2 and *Pseudomonas fluorescens* JY3 effectively controlled wheat seedling damping-off caused by *Fusarium oxysporum* and *Rhizoctonia solani*[10]. Additionally, siderophores produced by *Bacillus subtilis* DSM10 promoted plant growth and alleviated chlorosis in plants caused by iron deficiency in alkaline soils[11].

Endophytes are microbial communities that reside within plant tissues and constitute the essential component of the plant micro-ecosystem[12]. Endophytes isolated from various plants can directly or indirectly regulate plant growth through nitrogen fixation, phosphate solubilization, and phytohormone production[13-15], as well as by synthesizing secondary metabolites and secreting siderophores[16-17]. Due to their diversity, rapid reproduction, and abundant metabolic products, endophytic bacteria have emerged as a resource library for achieving sustainable agricultural development.

Saposhnikovia divaricata (Turcz.) Schischk. is a perennial herb with medicinal effects such as dispelling wind, relieving exterior syndrome, eliminating dampness, alleviating pain, and stopping spasm. In this study, endophytic bacteria were isolated from *S. divaricata* roots by conventional methods, and the chrome azurol S (CAS) assay were applied to screen siderophore-producing strains, which were analyzed and predicted for their siderophore biosynthesis pathways. Furthermore, the culture conditions of the screened strains for siderophore production were optimized, and their plant growth-promoting effects were evaluated.

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This study laid a foundation for developing and applying siderophore-producing microorganisms while providing theoretical support for the exploitation and utilization of plant endophytes.

Materials and Methods

Experimental materials

Plant materials: *S. divaricata* was collected from Laozhuangke, Linghei Village, Gaoliang Township, Shizong County, Qujing City, Yunnan Province. The plants were collected with surrounding soil to maintain root integrity.

Mulberry seeds: Fengchi mulberry (*Morus* spp.) was provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang).

Main culture media

PDA medium, LB medium and NA medium were used for routine culture of strains. MSA medium[18], MKB medium[19], SM medium[20] and AM medium[21] were adopted for siderophore production. RCV medium[22] was used for plate culture of mulberry seeds. CAS assay medium[23] was used in qualitative analysis of siderophore production by the strains.

Isolation of plant endophytes

Mature roots were washed with running water to remove surface attachments, and then cut into small segments (1 – 2 cm). The segments were sequentially soaked in 1.5% sodium hypochlorite for 5 min and 75% ethanol for 5 min, followed by multiple times of rinsing with sterile water. The root segments were air-dried on the clean bench to remove surface moisture, and then longitudinally bisected. The cut surfaces were pressed onto PDA medium and incubated at 28 ℃ for 3 – 4 d, during which bacterial colonies growing around the roots were isolated and repeatedly transferred to fresh PDA medium for purification. The selected strains were stored in the refrigerator (4 ℃) for subsequent experiments.

Identification of plant endophytes

The bacterial strains to be identified were cultured in LB liquid medium for 24 h, and centrifugation were performed at 5 000 r/min and 4 ℃ for 10 min to collect bacterial cells. Genomic DNA was extracted using the centrifugal column-type Bacterial Genomic DNA Extraction Kit from Sangon Biotech (Shanghai) Co., Ltd. PCR amplification was performed using universal primers (forward primer: 5′-AGAGTTTGATCCTGGCTCAG-3′, reverse primer: 5′-GGTTACCTTGTTACGACTT-3′). Sequencing was conducted by Sangon Biotech (Shanghai) Co., Ltd., and the sequence was analyzed by BLAST comparison in the NCBI database.

Qualitative analysis of siderophore production by plant endophytes

The endophytic bacterial strains were spot-inoculated at the center of CAS assay medium. If the strains produced siderophores, the siderophores would form chelates with Fe^{3+}, resulting in the formation of yellow circles. The types of siderophores produced by the strains were analyzed using the Arnow test[24], ferric perchlorate test[24], and Shenker test[25].

Analysis of siderophore biosynthesis gene clusters in the strain

The strain was inoculated onto LB solid medium and cultured at 37 ℃ for 24 h. Phosphate buffer (pH 7.0) was added to the culture plates, and the bacterial cells were gently scraped off, followed by centrifugation at 8 000 r/min for 10 min for cell collection. Whole-genome sequencing was performed by PANOMIX Biomedical Tech. Co., Ltd. (Suzhou). The genomic sequences of the strain were analyzed online using antiSMASH software (version 6.0.0), and siderophore production was predicted.

Screening of basal culture medium for siderophore production

Using LB medium as the control, the effects of MKB medium, MSA medium, SM medium, and AM medium on siderophore production by the strains were analyzed. The strain was inoculated into liquid media (50 ml/150 ml) at 5% (v/v) inoculum size and cultured at 37 ℃ with shaking at 120 r/min for 48 h. The supernatants were then obtained by centrifugation at 10 000 r/min for 10 min. Siderophore activity unit SU (Siderophore Unit) was determined according to the method of Ge *et al.*[23] to represent the siderophore production of the strain: $SU = [(Ar - As)/Ar] \times 100\%$, where Ar is the absorbance value of uninoculated medium mixed with an equal volume of CAS solution, and As is the absorbance value of the supernatant from the inoculated strain mixed with an equal volume of CAS solution. The As/Ar ratio was used to analyze siderophore production capacity of the strain[26]. The As/Ar ratio of bacteria with high siderophore production capacity was lower than 0.5.

Determination of bacterial growth curve and optimization of culture time

The strain was inoculated into the determined culture medium at 5% (*v/v*) inoculum size and cultured at 37 ℃ with shaking at 120 r/min. Samples were collected every 2 h to measure the absorbance at 600 nm. The growth curve was plotted with time as the x-axis and absorbance as the y-axis. Under the same conditions, samples were collected every 24 h. The siderophore secretion curve was plotted with time as the x-axis and SU (Siderophore Unit) as the y-axis.

Analysis of culture conditions for siderophore production

Effects of different metal ions on siderophore production The medium was supplemented with Fe^{3+} at final concentrations of 5, 10, 15, 20, and 25 μmol/L, as well as Al^{3+}, Mn^{2+}, Co^{2+}, and Cu^{2+} at final concentrations of 0, 10, 20, 40, and 80 μmol/L, respectively. The strain was cultured at 37 ℃ with shaking at 120 r/min, and siderophore production was measured during the stable phase of siderophore synthesis.

Effects of different carbon and nitrogen sources on siderophore production Eight carbon sources (glucose, sucrose, soluble starch, maltose, mannitol, glycerol, sodium carbonate, and sodium bicarbonate) and seven nitrogen sources (peptone, yeast extract, urea, glycine, ammonium chloride, acid-hydrolyzed casein, and ammonium oxalate) were separately added to the medium. The strain was cultured at 37 ℃ with shaking at 120 r/min.

Siderophore production was measured during the stable phase to determine the optimal carbon and nitrogen sources. Based on these results, the optimal concentrations of the selected carbon and nitrogen sources were further determined.

Effects of different pH values on siderophore production Based on the determined optimal carbon and nitrogen sources, the medium pH was adjusted to 5, 6, 7, 8, and 9. The strain was cultured at 37 ℃ with shaking at 120 r/min, and samples were collected during the stable phase of siderophore production. After neutralizing the pH of the test liquid, CAS blue detection solution was added to measure siderophore yield.

Evaluation of growth-promoting effects of the strain on mulberry seedlings

Mulberry seedling treatment Plump and uniform mulberry seeds were selected. They were surface-sterilized by soaking in 5% sodium hypochlorite solution for 15 min, and then rinsed for multiple times with sterile water to remove residual sodium hypochlorite.

Treatment 1: Mulberry seeds were sown on RCV solid medium plates, with 15 μl of bacterial suspension (2×10^7 cfu/ml) added 4 cm below each seed. An equal volume of sterile water was used as the control. After sealed with breathable sealing film, the plates were vertically placed in a growth chamber at 28 ℃ and 60% humidity for culture with a 12 h/12 h (light/dark) cycle. After 15 d, mulberry seedling development was assessed to analyze the comprehensive growth-promoting effects of the strain.

Treatment 2: The strain was shake-cultured (37 ℃, 120 r/min) in liquid LB medium (50 ml/150 ml) for 72 h, followed by centrifugation at 10 000 r/min for 10 min. The supernatant pH was adjusted to 2, added with an equal volume of ethyl acetate by two separate times with continuous shaking for 30 min, and then left to stand for 24 h. The ethyl acetate phase was concentrated and dried using a rotary evaporator at 0.1 MPa and 40 ℃, and the concentrate was then dissolved in a small amount of methanol and diluted with sterile water to 0.08 mg/ml to obtain the crude siderophore extract[27]. Next, 15 μl of the crude siderophore extract was used to replace the bacterial suspension in treatment 1 to analyze the growth-promoting effects of siderophores on plants.

Treatment 3: In the bipartite Petri dish, mulberry seeds were sown on one side containing RCV solid medium, while the bacterial strain was inoculated on the other side containing LB solid medium. Culture was performed under the aforementioned conditions to analyze the growth-promoting effects of volatile compounds produced by the strain on plants.

Index determination The biomass of mulberry seedlings was determined by weighing. Primary root length was measured using a standard ruler. Root tip images were captured using the Nikon E100 microscope under 40 × 10 times, and the number of root hairs in the 3 mm apical segment of the primary root was counted. Root hair length was measured using Image J software[28]. Superoxide dismutase (SOD) activity was determined by nitroblue tetrazolium (NBT) photoreduction assay[29], with one enzyme activity unit (U) defined as 50% inhibition of NBT photoreduction reaction. The guaiacol oxidation method was applied to determine peroxidase (POD) activity[30], with one enzyme activity unit (U) defined as a 0.01 change in OD_{470} per minute. The pyrocatechol oxidation method was adopted to measure polyphenol oxidase (PPO) activity[31], with one enzyme activity unit (U) defined as a 0.01 change in OD_{398} per minute. Phenylalanine ammonialyase (PAL) activity was determined by the phenylalanine deamination method[32], with one enzyme activity unit (U) defined as a 0.01 change in OD_{290} per minute.

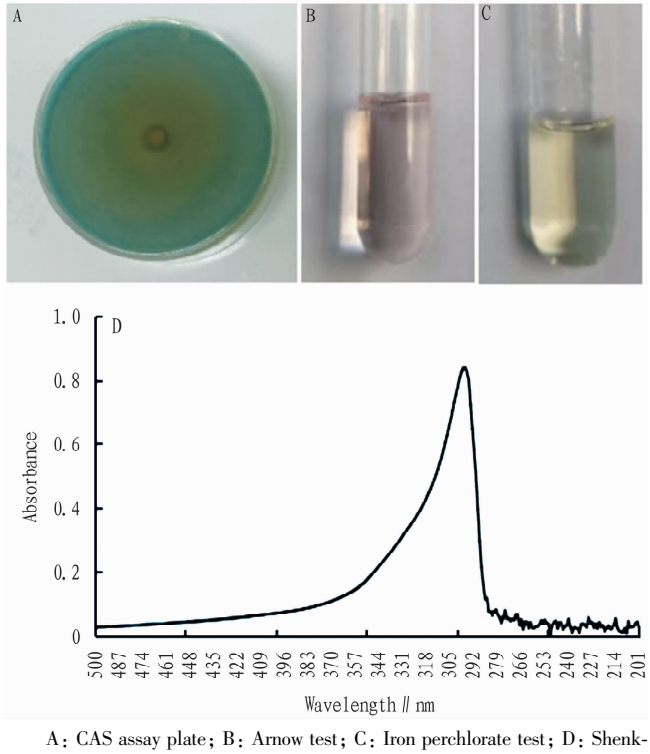
Data analysis

Data processing and graphing were performed using Microsoft Excel 2019. Statistical significance ($P < 0.05$) was analyzed using IBM SPSS Statistics 25 software.

Results and Analysis

Isolation of plant endophytes and screening of siderophore-producing strains

A total of five endophytic bacterial strains were isolated from *S. divaricata* roots, named as PB-1, PB-2, PB-3, PB-4, and PB-5, respectively. When strain PB-2 was spot-inoculated on blue CAS assay plate and statically cultured at 37 ℃ for 6 d, distinct yellow siderophore circle appeared around the colony (Fig. 1A).



A: CAS assay plate; B: Arnow test; C: Iron perchlorate test; D: Shenker test.

Fig. 1 Qualitative analysis of siderophore production by strain PB-2

When the LB fermentation supernatant of strain PB-2 was subjected to the Arnow test and ferric perchlorate test, the solution turned red in the Arnow test (Fig. 1B) but remained unchanged

in the ferric perchlorate test (Fig. 1C), suggesting that strain PB-2 may produce catechol-type siderophores. During the Shenker test, a maximum absorption peak was observed at 190 – 280 nm (Fig. 1D), indicating that strain PB-2 might also produce carboxylate-type siderophores.

Identification of endophytic strain PB-2

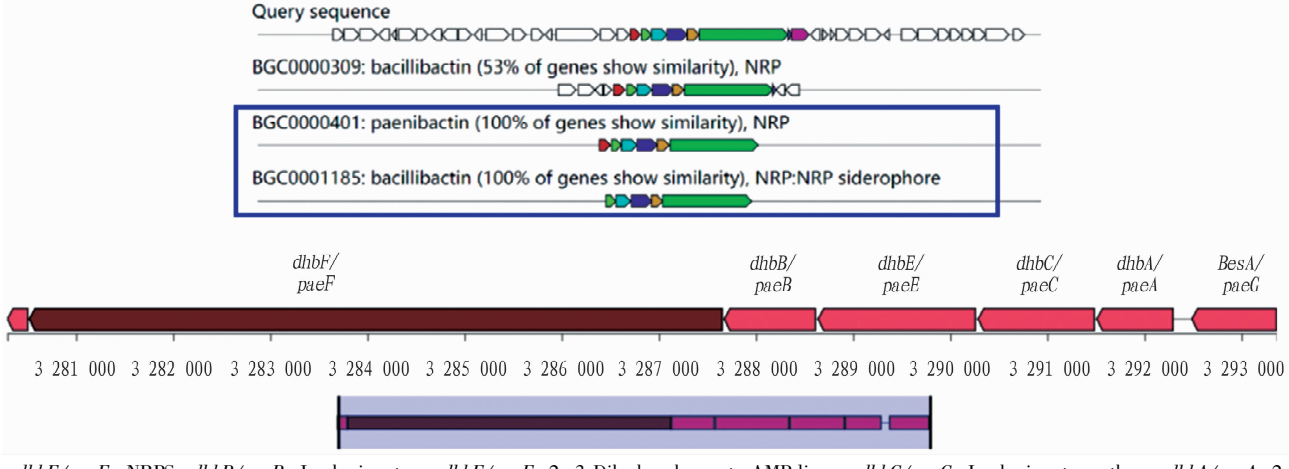
The PCR product of the genomic DNA from strain PB-2 was sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing, and the obtained 16S rRNA gene sequence had a total length of 1 403 bp. Homology search using NCBI-BLAST software (Table 1) revealed that strain PB-2 belonged to the genus *Paenibacillus* sp.

Prediction of siderophore production pathways in endophytic strain PB-2

Whole-genome sequencing was performed for strain PB-2, and secondary metabolite gene clusters were analyzed using the antiSMASH software to predict its siderophore biosynthesis pathways. The genome of strain PB-2 contained one nonribosomal peptide synthetase (NRPS) gene cluster responsible for synthesizing the siderophore bacillibactin (*bestA*, *dhbA*, *dhbC*, *dhbE*, *dhbB*, *dhbF*) or the siderophore paenibactin (*paeG*, *paeA*, *paeC*, *paeE*, *paeB*, *paeF*), with 100% homology (Fig. 2). Both bacillibactin and paenibactin were catechol-type siderophores, belonging to cyclic NRPS products.

Table 1 Molecular identification results of strain PB-2

Sequence ID	Strain name	Similarity//%
NR_137255.1	*Paenibacillus tyrfis* MSt1	100
MN428206.1	*Paenibacillus tyrfis* PK1-25	99.93
MN428207.1	*Paenibacillus tyrfis* PK2-1.1	99.86
CP121215.1	*Paenibacillus elgii* L146	99.86



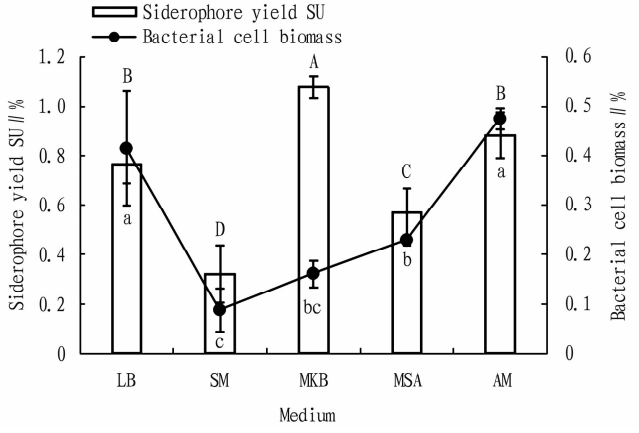
dhbF/paeF: NRPS; *dhbB/paeB*: Isochorismatase; *dhbE/paeE*: 2, 3-Dihydroxybenzoate-AMP ligase; *dhbC/paeC*: Isochorismate synthase; *dhbA/paeA*: 2, 3-dihydro-2, 3-Dihydroxybenzoate dehydrogenase; *besA/paeG*: Esterase.

Fig. 2 Siderophore synthesis gene clusters in the genome of strain PB-2

Screening of basal medium for siderophore production by endophytic strain PB-2

Under the same inoculation conditions, the siderophore yield (SU) and bacterial biomass varied significantly among different media (MKB, MSA, SM, and AM) after 48 h of culture (Fig. 3). The highest siderophore production was observed in MKB medium, followed by LB and AM media. In contrast, the highest bacterial cell biomass was achieved in LB and AM media, followed by MKB and MSA. These results indicated that siderophore production was not proportional to bacterial cell growth. Since MKB medium was the most suitable for siderophore synthesis, it was selected as the basal medium for subsequent experiments.

In the MKB medium, samples were taken every 24 h to measure the Ar and As of siderophores, and the As/Ar ratio was calculated to analyze the siderophore production capacity of the strain. The As/Ar ratio ranged from 1 to 0, with intervals of 0.2, and each decrease of 0.2 added a " + ". As shown in Table 2, the siderophore production capacity was relatively weak on the first and second days, with As/Ar above 0.5. After the third day, the siderophore production capacity increased, and the As/Ar ratio gradually fell below 0.5. The strain PB-2 was classified as a high-yield siderophore-producing strain[26].



Different letters indicate significant differences between treatments ($P < 0.05$), the same below. In the figure, uppercase letters represent differences in siderophore production, while lowercase letters represent differences in bacterial cell biomass.

Fig. 3 Siderophore production and bacterial cell biomass of strain PB-2 in different media

Table 2 Analysis on the siderophore production capacity of strain PB-2

Time//d	As/Ar	Siderophore production capacity
0	0.986 7	+
1	0.969 8	+
2	0.705 9	+ +
3	0.505 4	+ + +
4	0.501 0	+ + +
5	0.456 3	+ + +

Growth curve and siderophore secretion curve of endophytic strain PB-2

Bacterial growth can be divided into lag phase, logarithmic phase, stationary phase, and death phase. In MKB liquid medium, strain PB-2 exhibited a lag phase from 0 to 10 h, entered the logarithmic phase from 12 to14 h, reached maximum biomass at 16 h (stationary phase), and began to decline after 22 h (death phase) (Fig. 4A). With prolonged culture time, siderophore yield (SU) continuously increased, and was stabilized on the third day with an SU value of 49.33% (Fig. 4B). The combined results of the two curves indicated that their growth rates and stabilization time were inconsistent, suggesting that the growth of strain PB-2 and its siderophore secretion were asynchronous.

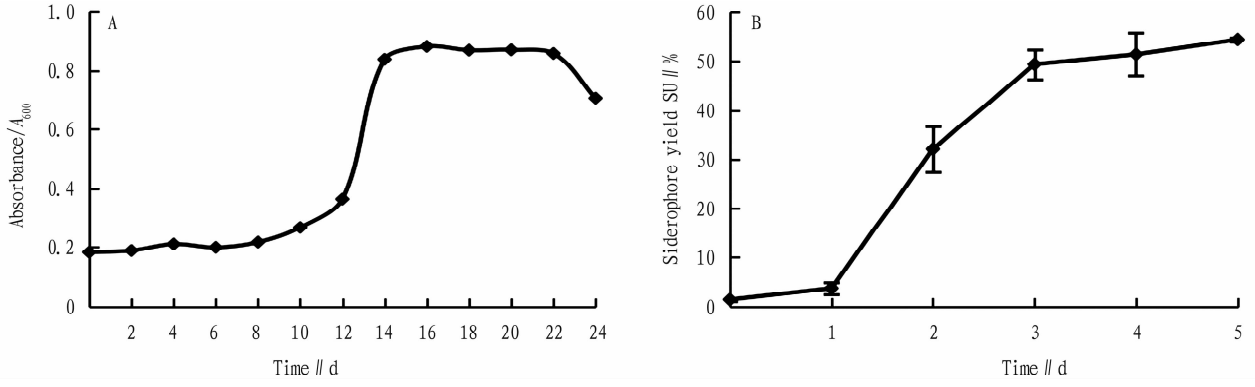


Fig. 4 Growth curve and siderophore secretion curve of strain PB-2

Induction conditions of siderophore production by endophytic strain PB-2

Effects of different metal ions on siderophore production After shaking culture for 3 d in the MKB liquid medium with final Fe^{3+} concentrations of 5, 10, 15, 20, and 25 μmol/L, the siderophore production of strain PB-2 reached its maximum (19%) at the Fe^{3+} concentration of 15 μmol/L (Fig. 5A). An appropriate amount of Al^{3+} promoted siderophore production of strain PB-2, and the highest siderophore yield (SU) was achieved at the Al^{3+} concentration of 20 μmol/L (Fig. 5B). The siderophore yield (SU) did not show significant changes with varying Mn^{2+} concentrations (Fig. 5C). An appropriate concentration of Cu^{2+} also promoted siderophore production in the strain, and the highest SU value was observed at 40 μmol/L (Fig. 5D). Co^{2+} initially slightly enhanced but later significantly inhibited siderophore production, with the SU value reaching 23.62% at the Co^{2+} concentration of 10 μmol/L (Fig. 5E). To compare the promoting effects of different metal ions at their optimal concentrations, MKB medium was supplemented with 15 μmol/L Fe^{3+}, 20 μmol/L Al^{3+}, 40 μmol/L Cu^{2+}, and 10 μmol/L Co^{2+}, respectively. The corresponding SU values were 25.17%, 9.72%, 13.13%, and 19.42%, respectively (Fig. 5F). Among them, 15 μmol/L Fe^{3+} resulted in the highest siderophore yield, demonstrating the most significant promoting effect.

Effects of different carbon and nitrogen sources on siderophore production In MKB liquid medium containing 15 μmol/L Fe^{3+}, the siderophore production of strain PB-2 showed certain differences under different carbon sources (15 g/L) (Fig. 6A). The highest siderophore yields were observed with starch, mannitol, and glycerol as carbon sources, reaching 50.62%, 47.64%, and 49.52%, respectively, with no significant differences among them. Since 15 g/L glycerol was the original carbon source in MKB medium, it was selected as the carbon source for subsequent experiments.

Strain PB-2 was inoculated into MKB liquid medium containing 15 g/L glycerol as the carbon source and 15 μmol/L Fe^{3+}. Different nitrogen sources (5 g/L) exhibited varying effects on siderophore production (Fig. 6B). The highest siderophore yield (SU = 72.23%) was achieved when glycine as the nitrogen source, whereas the original nitrogen source (acid-hydrolyzed casein) in MKB medium only yielded an SU value of 14.76%. Therefore, glycine was selected to replace acid-hydrolyzed casein as the nitrogen source. Glycine were tested at concentrations of 3, 4, 5, 6, and 7 g/L. The highest siderophore production was observed at 6 g/L glycine (Fig. 6C), which was consequently selected as the nitrogen source for the medium.

Effects of different pH values on siderophore production The siderophore production of the strain showed no much differences at initial medium pH values of 5, 6, 7, and 8. However, a significant decrease in siderophore yield was observed in the fermentation broth at pH 9 (Fig. 7). Since the original pH of MKB medium was approximately 7, no pH adjustment was required for the prepared medium.

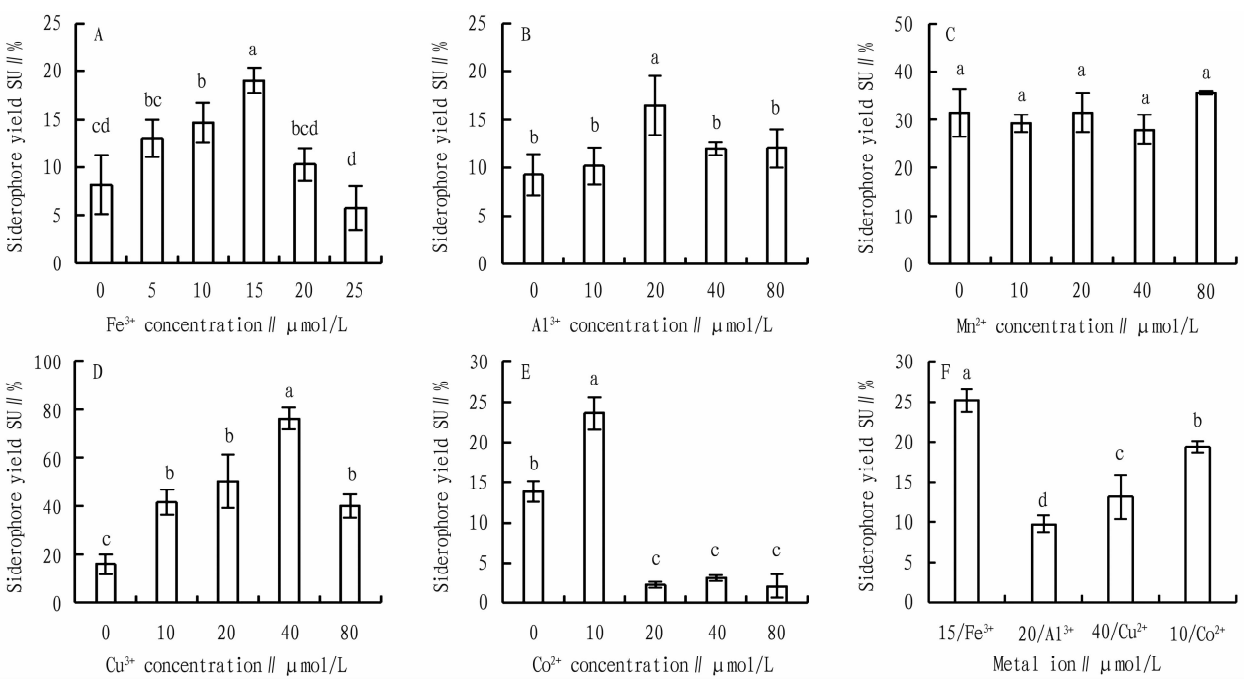


Fig. 5 Effects of different metal ions on the siderophore production of strain PB-2

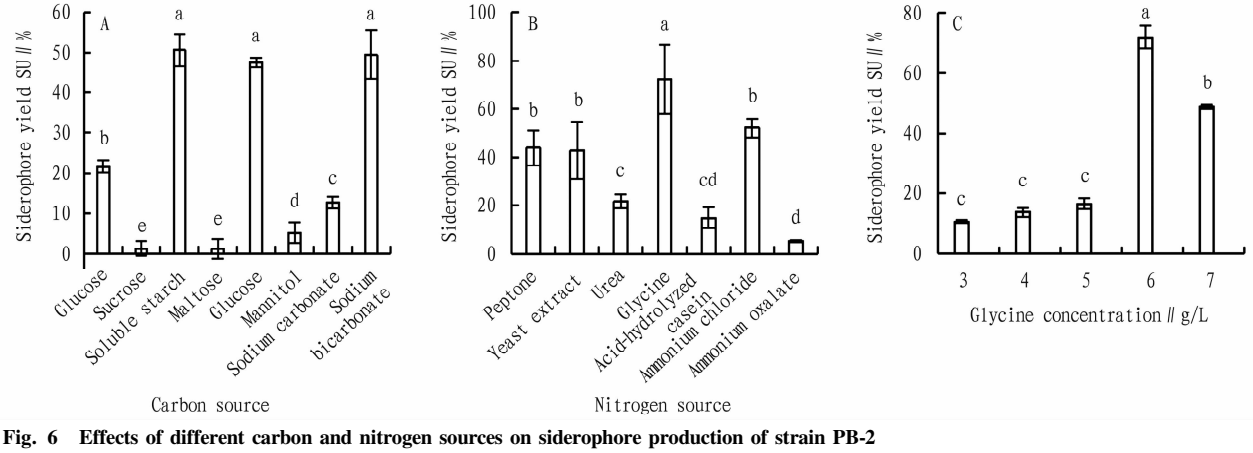


Fig. 6 Effects of different carbon and nitrogen sources on siderophore production of strain PB-2

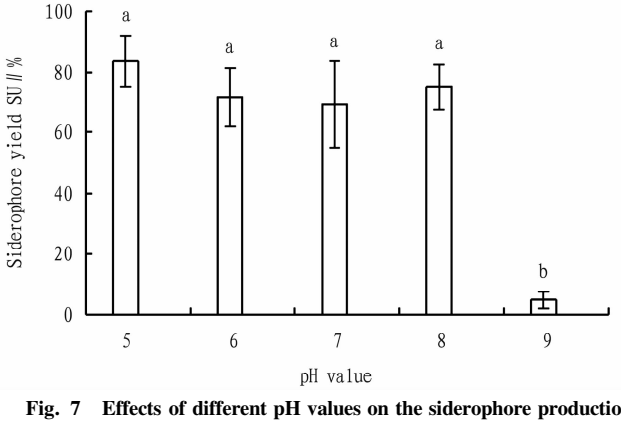


Fig. 7 Effects of different pH values on the siderophore production of strain PB-2

Growth-promoting effects of endophytic strain PB-2 on mulberry seedlings

Compared with the control group, the mulberry seedlings treated with PB-2 showed a 9.15% reduction in primary root length ($P<0.05$). However, the number and length of root hairs increased significantly by 37.93% and 16.37%, respectively. The underground biomass increased by 15.59% ($P<0.05$), while the aboveground biomass showed no significant change. Nevertheless, the total biomass increased significantly (Table 3, treatment 1). These results indicated that strain PB-2 had a notable growth-promoting effect on mulberry seedlings.

After treatment with crude siderophore extract, the primary root length decreased significantly by 21.72% compared with the control. The aboveground biomass and total biomass increased significantly by 30.42% and 42.76% compared with the control, respectively, while no significant changes were observed in other indexes (Table 3, treatment 2). The volatile compounds showed no significant effects on the primary root length of mulberry seedlings, though a 10.57% reduction was observed. However, both root hair number and length increased significantly by 57.42% and 18.73%, respectively. The underground biomass also increased

significantly by 16.34% compared with the control, while no significant effects were observed on aboveground and total biomass (Table 3, treatment 3). These results demonstrated that both the crude siderophore extract and volatile compounds from strain PB-2 promoted mulberry seedling growth through distinct mechanisms.

Table 3 Growth-promoting effects of strain PB-2 on mulberry seedlings

Index	Treatment 1		Treatment 2		Treatment 3	
	Control	Strain PB-2	Control	Siderophores	Control	Volatile compounds
Primary root length//cm	4.37 ±0.13 a	3.97 ±0.10 b	2.44 ±0.26 a	1.91 ±0.19 b	2.65 ±0.55 a	2.37 ±0.45 a
Root hair number//3 mm	121.80 ±16.20 b	168.00 ±20.23 a	173.75 ±0.25 a	222.50 ±33.50 a	113.50 ±12.79 b	178.67 ±17.46 a
Root hair length//μm	212.31 ±16.44 b	247.06 ±13.89a	198.83 ±40.66a	186.48 ±53.28a	179.52 ±31.34b	213.15 ±35.89a
Aboveground biomass//g/10 plants	0.057 3 ±0.003 4 a	0.061 0 ±0.001 1 a	0.081 2 ±0.000 2 b	0.105 9 ±0.011 1 a	0.084 1 ±0.008 6 a	0.083 8 ±0.008 0 a
Underground biomass//g/10 plants	0.044 9 ±0.004 3 b	0.051 9 ±0.000 2 a	0.086 7 ±0.030 5 a	0.133 8 ±0.012 0 a	0.040 4 ±0.002 7 b	0.047 0 ±0.004 4 a
Total biomass//g/10 plants	0.102 3 ±0.003 3 b	0.112 8 ±0.001 2 a	0.167 9 ±0.030 3 b	0.239 7 ±0.023 1 a	0.124 5 ±0.009 6 a	0.130 8 ±0.007 8 a

Different lowercase letters following data in the same column indicate significant differences between treatments ($P<0.05$).

Compared with the control group, the mulberry seedlings treated with PB-2 showed significantly enhanced SOD and POD activities in leaves, with increases of 247.27% and 189.47% respectively. Although PAL and PPO activities did not show statistically significant increases compared with the control, their values rose by 44.46% and 9.30%, respectively (Table 4), indicating that the strain could improve defensive enzyme activities in mulberry seedling leaves.

Table 4 Effects of strain PB-2 on defense enzyme activities of mulberry seedlings

Treatment	SOD activity//U/g FW	POD activity//U/(min · g FW)	PAL activity//U/(min · g FW)	PPO activity//U/(min · g FW)
Control	12.63 ±2.05 b	22.80 ±5.50 b	24.00 ±8.00 a	25.80 ±2.08 a
PB-2	43.86 ±9.43 a	66.00 ±16.23 a	34.67 ±12.86 a	28.20 ±4.53 a

Different lowercase letters following data in the same column indicate significant differences between treatments ($P<0.05$).

Discussion and Conclusion

This study isolated a high-yielding siderophore-producing strain PB-2 from the roots of *S. divaricata*. Based on 16S rRNA homology analysis, it was identified as a strain of the genus *Paenibacillus* sp. Genome sequencing analysis using antiSMASH software revealed that strain PB-2 possessed the siderophore synthesis gene cluster for bacillibactin or paenibactin. Both bacillibactin and paenibactin are catechol-type siderophores, differing in that glycine replaces alanine[33], which is consistent with the Arnow test confirming the presence of catechol-type siderophores. *Paenibacillus* strains can produce various substances such as polypeptide antibiotics, antagonistic proteins, and phytohormones, demonstrating potential as biopesticides and biofertilizers, and they have been widely applied in agriculture[34-36]. The strain PB-2 screened in this study exhibited high siderophore production, along with the ability to synthesize hormones such as auxin and cytokinin, and displayed broad-spectrum antimicrobial activity (unpublished data). It is expected to provide a new strain for plant growth-promoting bacterial resources.

The carbon source in media provides raw materials and energy for the formation of cellular carbon skeletons and metabolic products, while the nitrogen source serves as the origin for synthesizing nitrogen-containing substances such as bacterial proteins, nucleic acids and siderophores[37]. Deng *et al.* [38] determined that *Serratia plymuthica* GR-39 exhibited higher siderophore production with 6 g/L sucrose and 5 g/L peptone. Similarly, Li *et al.* [39] found that the optimal carbon and nitrogen sources for *P. aeruginosa* Gxun-2 were 10 g/L glycerol and 1 g/L ammonium sulfate, respectively. In contrast, strain PB-2 in this study showed higher siderophore yield in MKB medium supplemented with 15 g/L glycerol and 6 g/L glycine. The pH value affects microbial growth and reproduction, and the synthesis of metabolites, as well as the activity of microbial enzymes and the absorption and utilization of nutrients by microorganisms[40]. *P. aeruginosa* achieved the highest siderophore yield at pH 6[41], while *Serratia plymuthica* GR-39[38] produced siderophores optimally at pH 8.0. The results of this study demonstrated that the pH range of 5.0 – 8.0 was suitable for siderophore production by strain PB-2. These findings indicated that, in addition to external factors such as medium composition and pH, siderophore yield was also closely related to the genera and species of strains.

Metal ions and their concentrations influence siderophore secretion. The study found that 15 μmol/L Fe^{3+}, 20 μmol/L Al^{3+}, 40 μmol/L Cu^{2+} and 10 μmol/L Co^{2+} promoted siderophore production in strain PB-2, though their effects varied in the following order: $Fe^{3+} > Co^{2+} > Cu^{2+} > Al^{3+}$. In contrast, Mn^{2+} showed no significant impact on siderophore production within the tested range of less than 80 μmol/L. Dimkpa *et al.* [42] found that strains *Streptomyces acidiscabies* E13 and *Streptomyces tendae* F4 produced almost no siderophores at Fe^{3+} concentrations of 35 and 100 μmol/L, respectively. In *P. aeruginosa*, siderophore production was strongly induced by Zn^{2+}, while Ni^{2+} and Mn^{2+} had little effects, and Cu^{2+} exhibited significant inhibition[43]. These findings indicated that different metal ions varied considerably in their effects on siderophore production, and the same metal ion may also exert distinct effects on different bacterial strains.

The plant growth-promoting effects of bacterial strains are directly reflected in changes of plant biomass. Qu *et al.* [44] found that *Paenibacillus abekawaensis* ZLT11, isolated from the rhizosphere of *Mikania micrantha*, increased rice biomass. Similarly,

Guo et al.[45] reported that tomato seedlings treated with the fermentation broth of *Paenibacillus polymyxa* CF05 showed a 272% increase in fresh weight and a 266.7% increase in dry weight. After treatment with crude siderophore extract from strain PB-2, the aboveground biomass and total biomass of mulberry seedlings significantly increased ($P < 0.05$). Meanwhile, volatile organic compounds (VOCs) significantly enhanced the underground biomass by 16.34%, and under the combined influence of multiple substances, the total biomass of mulberry seedlings treated with PB-2 increased by 10.26%, reaching a significant level ($P < 0.05$), which was aligned with the aforementioned research findings. Similarly, under multifactorial interactions, strain PB-2 significantly shortened the primary root length of mulberry seedlings, while markedly increased root hair number and length ($P < 0.05$). The reduction in root length might weaken the absorption of water and nutrients by the roots[46], but the increase in root hair number and length was sufficient to compensate for or even surpass the absorption and supply of water and nutrients in the control group. Altering root morphology by increasing the number of lateral roots and root hairs or extending root hair length and thereby regulating plant growth, is the common growth-promoting mechanism of many plant growth-promoting bacteria[47-48].

SOD and POD are important reactive oxygen species (ROS) scavengers in plants. They also participate in the induced plant resistance by producing toxic substances, activating other defense mechanisms, or enhancing physical barriers such as lignin and suberin deposition[49]. PAL is a key enzyme in the phenylpropanoid biosynthesis pathway, while PPO is involved in the oxidation of phenolic compounds. The phenolic compounds, lignin and quinones synthesized through their catalytic action protect plants from pathogen damage[49-50]. Treatment with strain PB-2 significantly enhanced the activities of SOD and POD ($P < 0.05$) in mulberry seedling leaves, while the activities of PAL and PPO also increased to varying degrees. It indicated that the strain PB-2 could mitigate pathogen damage to plants by reducing oxidative stress caused by stress, producing antimicrobial substances, or inducing the formation of physical barriers, thereby indirectly promoting plant growth.

In this study, a bacterial strain (PB-2) producing catechol-type siderophores at a high yield was isolated and screened from the root system of *S. divaricata*. It was preliminarily identified as *Paenibacillus* sp. Strain PB-2 exhibited efficient siderophore secretion after 3 d of fermentation using the MKB medium with 15 g/L glycerol as the carbon source and 6 g/L glycine as the nitrogen source at the original pH value. Through the combined effects of multiple factors, strain PB-2 demonstrated the growth-promoting effect on mulberry seedlings by modifying root morphology, increasing biomass, reducing oxidative stress, and mitigating pathogen damage.

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