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Characterization of siderophore-producing bacteria isolated from plant rhizosphere

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Abstract

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Siderophores are synthesized by plants and microorganisms that thrive in low-iron conditions, serving as biocontrol agents and other applications in agriculture. The aim of the present study was to isolate, characterize, and optimize siderophore-producing bacterial strains DT9, DT10, and DT12. The 16S rDNA nucleotide sequence analysis revealed that DT9 and DT10 were closely related to *Klebsiella pneumoniae*, and DT12 was related to *Acinetobacter bereziniae*. All three strains produced siderophores, crucial compounds aiding plants in acquiring iron when growing in iron-scarce environments. The presented results in this study constituted preliminary findings evaluating the influences of cultural conditions on siderophore synthesis by DT9, DT10, and DT12. They demonstrated efficient siderophores synthesis when cultured at 30°C, with a culture medium pH of 7. Glycerol and $(NH_4)_2SO_4$ served as the carbon and nitrogen sources for the culture medium.

Keywords: Acinetobacter bereziniae; carbon sources; *Klebsiella pneumoniae*; nitrogen sources; rhizospheric bacteria; siderophores

Introduction

Vietnam's economic stability is intricately linked to agricultural production. However, intensive farming has led to the emergence of various plant diseases and insect pests, especially soil-borne diseases. The increasing studies on microbial metabolite production and its application in controlling soil-borne plant diseases have been conducted on developing these microbial metabolites, offering an eco-friendly alternative to chemical fungicides, gaining importance amid concerns about environmental pollution, pathogen resistance, and high plant protection costs (Jayaprakashvel & Mathivanan, 2011).

Siderophores, characterized by low molecular weight organic chelators, exhibit a high and specific affinity for Fe (III) (Butler & Theisen, 2010), are synthesized by many strains of bacteria, actinomycetes, and fungi under iron limitation conditions (Angel Jenifer et al., 2013). These compounds form siderophore-iron complexes, binding to ferric iron, which, in turn, penetrate cell membranes through specific siderophore receptors (Ahmed & Holmström, 2014; Hider & Kong, 2009; Saha et al., 2016). Additionally, si-

derophores could bind with other environmental elements such as Mo, Mn, Co, and Ni, converting them into forms accessible to microbial cells (Bellenger et al., 2008; Braud et al., 2009). Typically synthesized by microorganisms such as bacteria, actinomycetes, and fungi (Bellenger et al., 2008; Goswami et al., 2016; Sandy & Butler, 2009), siderophores boast a diverse range, with over 500 compounds discovered and 270 structurally characterized (Cornelis, 2010). They play a crucial role in agriculture and serve as plant growth promoters, biocontrol agents, antimicrobial agents, and other ecological factors (Postle, 1990). These findings suggested a promising direction in using siderophore-producing bacteria in agriculture production in general and in plant protection in particular.

In the present study, we isolated siderophore-producing bacteria from soil samples and investigated the optimal fermentation conditions. The outcomes revealed the highest siderophore production concentration recorded in Vietnam to date.

Materials and Methods

Isolation and purification of bacterial strains

Ten soil samples were collected from the root zones of maize and soybean plants in the experimental areas of the Faculty of Agronomy, Vietnam National University of Agriculture, located in Hanoi City, Vietnam, in 2020. Bacterial strains were isolated within 48 h on a sterilized LB medium using the spreading plate technique. The soil samples were serially diluted in concentrations of 10^{-1} to 10^{-6}, and 0.1 ml of each dilution was inoculated and grown in LB medium at 28±2°C for 24 h. Purified colonies were selected, purified further, and maintained on LB agar slants. To investigate siderophore production, the isolated strains were streaked on chrome azurol S (CAS) plates following the procedure outlined by Alexander & Zuberer (1991). After spot inoculation, the CAS agar plates were incubated at 28±2°C for 48 h. The development of yellow or orange halos around the growth of each strain was considered a positive indication of siderophore production (Alexander & Zuberer, 1991).

Quantitative and quality assessment of siderophores

The isolated strains were cultured in a Fe-deficient succinate medium and incubated in a shaker incubator at 120 rpm for 48 h at room temperature. Siderophore production of each strain was initially screened and evaluated using spectrophotometry. Subsequent quantitative determination was conducted employing the chrome azurol sulphonate (CAS) assay. For the CAS assay, 0.5 ml of the culture supernatant from the prepared culture was mixed with 0.5 ml of CAS solution and incubated for 20 min. The resulting mixture was then measured at 630 nm. The produced siderophore units (%) were calculated using the following formula:

$$\% \text{ of siderophore units} = \frac{A_r - A_s}{A_r} \times 100$$

where: A_s is the absorbance of the sample at 630 nm; A_r is the absorbance of the reference (CAS reagent) at 630 nm (Kumar et al., 2017; Payne, 1994).

Molecular identification of potential siderophore-producing bacterial strains

The isolated siderophore-producing bacterial strains, displaying highly efficient siderophore production, underwent further molecular identification. They were cultivated in LB broth at 30°C for 48 h. Cultures were centrifuged, and pellets were washed to remove any residual medium. Total DNA extraction employed the CTAB method. PCR assays were conducted to amplify the 16S rDNA gene using the primer pairs 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-AGAAAGGAGGTGATCCAGGC-3') (Weisburg et al., 1991). PCR reactions, in 20-µl volumes, utilized the Mastercycler X50s (Eppendorf, Germany) and GoTaq® Green Master Mix (Cat. # M7123, Promega, USA), comprising 10 µl of GoTaq® Green Master Mix 2X, 1 µl of each primer (10 µmol), and 1 µl of DNA template (20 ng/µl). The PCR assay included 94°C for 3 min, followed by 29 cycles (94°C for 30 s, 53°C for 30 s, and 72°C for 1 min). Negative controls were integrated into all PCR assays. Following PCR, 5 µl of each PCR product was separated onto a 1% (w/v) agarose gel with 1X Redsafe in 1X TAE buffer and visualized using the Chemiluminescence Imagers system ChemiDoc™ XRS+ (BIO-RAD, USA). The PCR amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sequenced bidirectionally with the ABI 3100 Genetic Analyzer using 27F and 1492R primers. Using BioEdit 7.2.6.1 software, the sequenced DNAs were edited, and aligned sequences were compared through the National Centre for Biotechnology Information (NCBI) BLAST search tool, accessible for homology at http://www.ncbi.nlm.nih.gov/BLAST. Sequence alignments performed as the basis for a phylogenetic tree developed through the neighbor-joining method in MEGA 11 with the bootstrap method (1.000 replications) (Tamura et al., 2021).

Effects of culture conditions on siderophore production

Various physical parameters and medium components, including initial pH, temperature, carbon, and nitrogen sources, influenced the selected bacterial strains' siderophore produc-

tion. These parameters were individually studied using a one-factor-at-a-time approach to evaluate their impacts on siderophore production. The effects of temperature on siderophore production were investigated at 30, 35, and 40°C. The influence of the initial pH on the medium was examined at pH levels of 3, 5, 7, 9, and 11. Various carbon sources (0.1% w/w), such as glucose, fructose, maltose, glycerol, mannitol, and lactose); and nitrogen sources (0.1% w/w) including NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, and NH_4NO_3, were employed to assess their effects. Bacterial strains were cultured in a liquid Fe-deficient succinate medium and incubated for 48 hours with constant shaking at 150 rpm. Siderophore production under each culture condition was measured at 630 nm and calculated as described previously (Kumar et al., 2017; Payne, 1994).

Results and Discussion

Isolation, screening, and identification of siderophores-producing bacteria

Twenty-three morphologically distinct bacterial strains were selected from ten soil samples. These strains were subsequently transferred onto a chrome azurol S (CAS) medium to assess their siderophore synthesis capabilities. Based on the size of the induced orange halo observed around the colonies, strains DT9, DT10, and DT12 were identified and selected for further experimentations to determine the optimal conditions for achieving high concentrations of siderophore production. The 16S rDNA gene fragments of DT9, DT10, and DT12 strains were amplified using the primer pair 27F/1492R, resulting in approximately 1.500 bp DNA fragments, and the PCR amplicons were directly sequenced on both sides. A BLAST search revealed that DT9 exhibited 100% identity with *K. pneumoniae* strain C3S3F (Genbank accession number: OQ813779), DT10 showed 99.84% identity with *K. pneumoniae* strain BB-301 (Genbank accession number: MN844878), and DT12 displayed 98.79% identity with *A. bereziniae* strain HPC229 (Genbank accession number: KP765739).

A phylogenetic tree was developed using the DNA sequences amplified from DT9, DT10, and DT12 strains in this study and the sequences from GenBank. The analysis indicated that Dt9 and DT10 strains, *Klebsiella pneumoniae* strain ATCC 13883 (Genbank accession number: NR119278), and *Klebsiella pneumoniae* subsp. *ozaenae* strain ATCC 11296 (Genbank accession number: NR119276) formed a distinct clade. This clade was separate from other *Klebsiella* species, including *K. quasipneumoniae*, *K. aerogenes*, *K. oxytoca*, *K. pasteurii* and other *Acinetobacter* and *Sphingomonas* species. Meanwhile, the DT12 strain was close to the *Acinetobacter bereziniae* strain ATCC 17924 (Genbank accession number: NR117625) and they separated from the *A. guillouiae* strain ATCC 11171 (NR117626), *Acinetobacter* species including *A. gerneri*, *A. junii*, *A. baumanii*, *Sphingomonas yabuuchiae, S. pseudosanguinis* and other *Klebsiella* species. These findings indicated that DT9 and DT10 closely matched *K. pneumoniae*, while DT12 exhibited a high degree of identity with *A. bereziniae* (Figure 1).

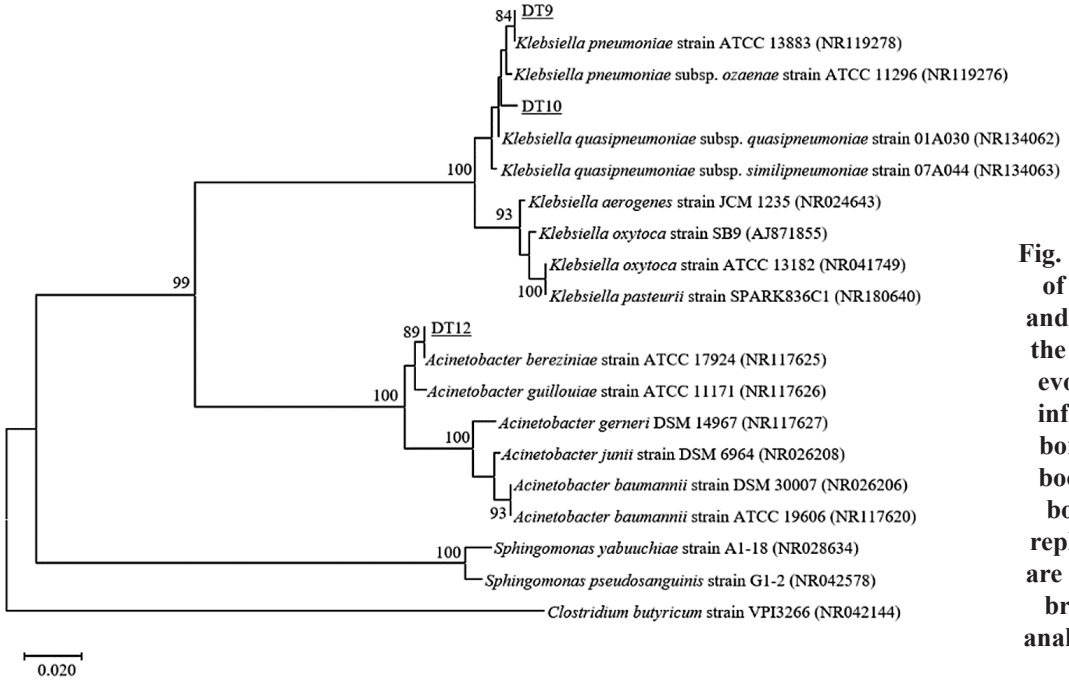

Fig. 1. Phylogenetic analysis of bacterial DT9, DT10, and DT12 strains based on the 16S rDNA region. The evolutionary history was inferred using the neighbor-joining method with bootstrap modeling. The bootstrap values (1,000 replicates) exceeding 80% are indicated alongside the branches. Evolutionary analyses were conducted in MEGA11

Effects of culture conditions on the siderophore production of DT9, DT10, and DT12 strains

Influence of pH

The DT9, DT10, and DT12 strains exhibited growth and synthesized siderophores across a pH range of 3 to 11, shaking at 150 rpm for 48 h at 30°C. Results indicated that these strains produced the highest percentage of siderophores at pH 7.0, with values of 53%, 39%, and 43%, respectively (Figure 2). The study found that the bacterial strains DT9, DT10, and DT12 exhibited optimal growth and siderophore synthesis at pH 7.0, aligning with previous studies highlighting the significance of neutral pH levels in influencing microbial siderophore production, as demonstrated by various comparative studies on different bacterial strains under varying environmental conditions. Given that corn and soybeans, common crops, are typically cultivated in regions with pH levels ranging from 6.5 to 7.0 due to their low tolerance for acidic soils (Baligar et al., 1997). These findings underscore the intricate relationship between iron availability, pH, and microbial siderophore production in nutrient-deficient environments. According to Tailor & Joshi's report (2012), strain S-11 archives maximum siderophore production at pH 7.0, as bacteria thrive optimally in a physiological environment where iron remains insoluble at neutral pH. A previous study on bacteria in heavy metal-contaminated soils investigated *Burkholderia* sp.SX9 and demonstrated that its highest siderophore production was at a pH of 7.4 (Wang et al., 2021). In addition, *Achromobacter* sp. RZS2 showed the highest OD600 value at pH 7.5 (Sayyed et al., 2019). It was revealed that the VITVK5 strain exhibited maximum siderophore production of 60% at pH 4.0 to 7.0. In comparison, the VITVK6 strain achieved peak synthesis of more than 80% at pH 8.0, suggesting that the solubility of iron further decreased when the pH rises, potentially stimulating siderophore production in the soil solution (Kumar et al., 2017). Notably, the NT1, NT3, and Garage 1 strains displayed the highest iron solubility, exceeding 18%, when examined at pH 9.0 (Chaudhary et al., 2017). These findings concluded that iron solubility and its availability to the growing microorganisms depend on the medium's pH (Kumar et al., 2017; Chaudhary et al., 2017).

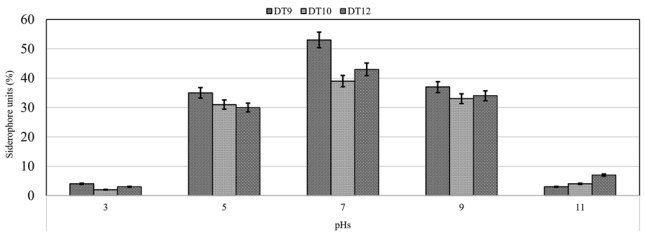

Fig. 2. Influence of pH on siderophore production by bacterial strains DT9, DT10, and DT1

Influence of temperatures

To evaluate the impacts of temperature on siderophore production, the DT9, DT10, and DT12 strains were cultured in a liquid succinate medium with constant pH 7.0, shaking at 150 rpm, 30, 35, and 40°C for 48 h. The optimal temperature for growth and maximum siderophore production by the DT9, DT10, and DT12 strains was 30°C, yielding 36%, 38%, and 39% siderophores, respectively. The production of siderophores gradually decreased as the temperature exceeded 35°C (Figure 3). These findings are with previous studies emphasizing the temperature sensitivity of siderophore biosynthesis in various bacterial isolates. For instance, *Pseudomonas aeruginosa* demonstrated heightened siderophore production at 27°C (Sharma et al., 2016). In another study, the NT1 strain exhibited the highest siderophore production in succinate medium at 37°C, while the NT2, NT3, and Garage 1 strains reached peak production of siderophores at 28°C (Chaudhary et al., 2017). It showed that an increase in incubation temperature from 37°C to 41°C produced a remarkable decrease in the rate and quantity of siderophore production. The elevated temperature could not suppress the growth of *Candida albicans* in either a control culture medium or a deferred culture medium. Significant growth suppression compared to the controls was observed in the deferrated media at 37°C and 41°C (Ismail et al., 1985). Similarly, the bacterial isolates VITVK5 and VITVK6 displayed increased siderophore concentrations up to more than 90% at 25, 37, and 45°C, with optimum production occurring at 35°C suggesting that room temperature might be the optimum temperature for the growth of microorganisms (Kumar et al., 2017). The literature consistently emphasizes the temperature sensitivity of siderophore biosynthesis, with the associated genes regulated by the growth temperature of microorganisms. Optimal siderophore production is often observed when bacteria are incubated at temperatures mimicking natural or sub-optimal conditions. Conversely, incubating bacteria at sub-lethal temperatures may lead to a reduction in siderophore production. These findings un-

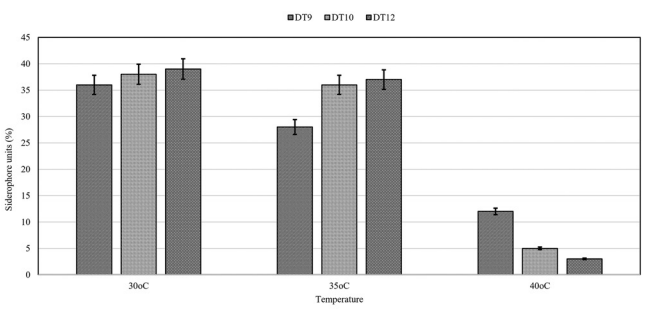

Fig. 3. Influence of temperature on siderophore production by bacterial strains DT9, DT10, and DT12

derscore the intricate interplay between temperature, microbial growth, and siderophore biosynthesis. Our results also suggest that the siderophore production by DT9, DT10, and DT12 also differs in the natural conditions.

Influence of different carbon sources

The DT9, DT10, and DT12 strains were grown in a liquid succinate medium containing various carbon sources, including glucose, fructose, maltose, lactose, glycerol, and mannitol, at 28°C for 48 h. Subsequently, bacterial cultures were assessed at 630 nm to determine the percentage of siderophores. The amount of siderophores produced by the three strains exhibited variations based on the carbon sources in the succinate medium. The choice of carbon source significantly influences medium quality and bacterial metabolism, crucial factors for bacterial growth and siderophore production. In this study, glycerol emerged as a suitable carbon source for three bacterial strains, facilitating rapid metabolism and increased siderophore production. The culture medium containing glycerol demonstrated the highest percentages of siderophore production, with values of 39%, 36%, and 30% for the DT9, DT10, and DT12, respectively (Figure 4). A study on *Achromobacter* sp. RZS2 identified mannitol as the preferred carbon source for the bacteria (Sayyed et al., 2019). Microorganisms often prioritize glycerol as a crucial substrate for triacylglycerols production, considering it an essential energy store in microbial strains (Alvarez & Steinbüchel, 2002). Our study investigated the impact of various carbon sources on siderophore production by the DT9, DT10, and DT12 strains, revealing that glycerol emerged as the most favorable carbon source, promoting rapid metabolism and increased siderophore production, thus highlighting the crucial role of selecting suitable carbon sources for optimizing siderophore production in bacterial strains. The extended utilization of this energy source by the three strains, DT9, DT10, and DT12, contributes to a prolonged growth cycle compared to other carbon sources. These findings underscore the significance of selecting an appropriate carbon source to optimize siderophore production in bacterial strains.

Influence of different nitrogen sources

The nitrogen source is a crucial parameter influencing siderophore production during bacterial growth, similar to carbon sources, as it impacts medium quality and bacterial metabolism. NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, and KNO_3 were utilized to evaluate the influences of different nitrogen sources on siderophore production by the DT9, DT10, and DT12 strains. The percentage of siderophores was determined after two days of culture. Results indicated that all three strains exhibited a high rate of siderophore production when cultured in a medium containing various nitrogen sources. Specifically, the DT9, DT10, and DT12 strains produced siderophores at 24.0%, 26.7%, and 21.0%, respectively (Figure 5), when cultured in a medium containing $(NH_4)_2SO_4$. These findings align with the siderophore production under different nitrogen sources observed in the isolates VITVK5 and VITVK6 (Kumar et al., 2017), as well as in *Pseudomonas fluorescens* strain (Tailor & Joshi, 2012). Interestingly, prior studies have found that *K. pneumoniae* is vital in enhancing plant growth through various mechanisms, including nitrogen fixation, the production of 1-aminocyclopropane-1-carboxylate deaminase, indole-3-acetic acid, gibberellic acid, and siderophores (Iniguez et al., 2004; Ji et al., 2014; Sachdev et al., 2009; Singh et al., 2015). Notably, *Klebsiella* sp. KW7-S06 was found to induce resistance against plant pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani* in rice (Ji et al., 2014); *K. pneumoniae* SnebYK mediated resistance against nematodes *Heterodera glycine* and promoted soybean growth (Liu et al., 2018). A separate study demonstrated that the introduction of *A. bereziniae* IG 2, *Enterobacter ludwigii* IG 10, and *Alcaligenes faecalis* IG 27 strains enhanced the growth parameters of pea seedlings subjected to salinity stress. Furthermore, the inoculation notably impacted vari-

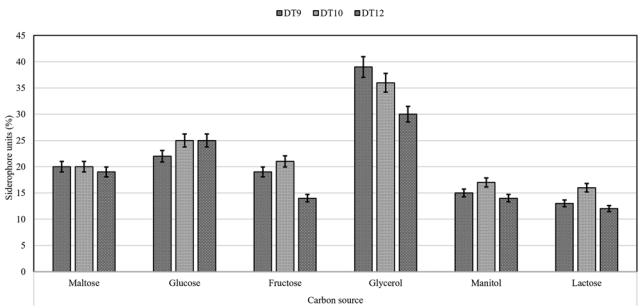

Fig. 4. Influence of carbon source on the production of siderophore by bacterial strains DT9, DT10, and DT12

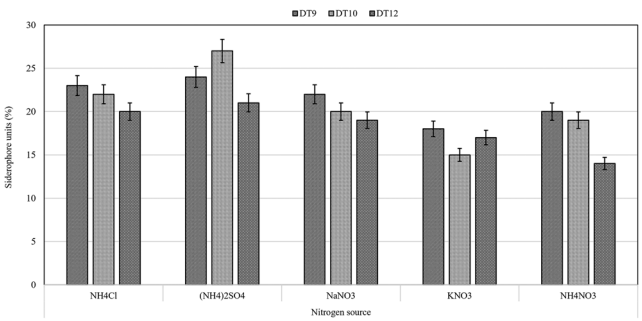

Fig. 5. Influence of nitrogen source on siderophore production by bacterial strains DT9, DT10, and DT12

ous biochemical parameters, including chlorophyll content, proline content, total soluble sugar, electrolyte leakage, and activities of antioxidant enzymes (Sapre et al., 2022).

In the present study, from the twenty-three bacterial strains isolated and identified in rhizosphere soil, DT9, DT10, and DT12 have stood out for their exceptional siderophore production in the CAS medium. These strains exhibited peak siderophore production when cultured in a medium featuring glycerol as the carbon source and $(NH_4)_2SO_4$ as the nitrogen source, maintaining a pH of 7.0 and a temperature of 30°C.

Conclusions

In conclusion, molecular identification revealed that DT9, DT10, and DT12 are close to *K. pneumoniae* and *A. bereziniae*, highlighting their potential for agricultural applications. The identified bacterial strains, DT9, DT10, and DT12, exhibit promising siderophore production capabilities, with glycerol and $(NH_4)_2SO_4$ identified as optimal carbon and nitrogen sources, respectively. They may demonstrate beneficial effects on plant growth and resistance to pathogens, further emphasizing the significance of these findings in agricultural contexts. Moreover, the study acknowledges the need for further optimization and investigation to harness these siderophore-producing strains' potential fully. Future research directions include exploring their interactions with plant systems, environmental factors, and potential genetic modifications to enhance their efficacy as biocontrol agents against plant diseases. The practical implications of this research extend to sustainable and organic farming practices, offering eco-friendly solutions to improve soil fertility and manage soil-borne pathogens in agricultural production.

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