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Submission date: 03-Apr-2023 12:28PM (UTC+0700)

Submission ID: 2054321274

File name: Production_Purification.pdf (1.95M)

Word count: 9906

Character count: 57685

Article

Production, Purification, and Characterization of Bacillibactin Siderophore of *Bacillus subtilis* and Its Application for Improvement in Plant Growth and Oil Content in Sesame

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Citation: Nithyapriya, S.; Lalitha, S.; Sayyed, R.Z.; Reddy, M.S.; Dailin, D.J.; El Enshasy, H.A.; Suriani, N.; Herlambang, S. Production, Purification, and Characterization of Bacillibactin Siderophore of *Bacillus subtilis* and Its Application for Improvement in Plant Growth and Oil Content in Sesame. *Sustainability* **2021**, *13*, 5394. <https://doi.org/10.3390/su13105394>

Academic Editor: Weilan Zhang

Received: 13 March 2021

Accepted: 9 May 2021

Published: 12 May 2021

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Abstract: Siderophores are low molecular weight secondary metabolites produced by microorganisms under low iron stress as a specific iron chelator. In the present study, a rhizospheric bacterium was isolated from the rhizosphere of sesame plants from Salem district, Tamil Nadu, India and later identified as *Bacillus subtilis* LSBS2. It exhibited multiple plant-growth-promoting (PGP) traits such as hydrogen cyanide (HCN), ammonia, and indole acetic acid (IAA), and solubilized phosphate. The chrome azurol sulphonate (CAS) agar plate assay was used to screen the siderophore production of LSBS2 and quantitatively the isolate produced 296 mg/L of siderophores in succinic acid medium. Further characterization of the siderophore revealed that the isolate produced catecholate siderophore bacillibactin. A pot culture experiment was used to explore the effect of LSBS2 and its siderophore in promoting iron absorption and plant growth of *Sesamum indicum* L. Data from the present study revealed that the multifarious *Bacillus* sp. LSBS2 could be exploited as a potential bioinoculant for growth and yield improvement in *S. indicum*.

Keywords: bacillibactin; bioinoculant; P solubilization; plant growth promotion; *Sesamum indicum*; siderophore

1. Introduction

Sesame (*Sesamum indicum* L.), an oilseed crop of the family *Pedaliaceae*, is one of the oldest cultivated crops of the world with a total production of 3.3 million tons. It is an economically important crop for its edible oil, fatty acids, antioxidants, and its nutritive additive values, medicinal, and industrial purposes [1]. On the other hand, the demand for edible oil is increasing gradually with the increase in world population; therefore, the cultivation of oilseed crops is expanding all over the world. The efforts are needed to increase the sustainable production of vegetable oils in an ecofriendly and effective manner. Sustainable measures are being explored to improve the quality and quantity of edible oil for human consumption. The utilization of multifarious plant-growth-promoting rhizobacteria (PGPR) as biofertilizers is increasingly being reported as one

of the best practices of complementing conventional agrochemical inputs in agricultural systems [2–4]. These PGPRs promote plant growth by facilitating nutrient and nitrogen fixation [5,6], providing plant hormones such as including indole acetic acid (IAA) etc., phosphate solubilization [7,8] and iron nutrition through siderophore. Siderophores are iron scavenging ligands produced by a wide range of PGPR including *Bacillus* spp. to help the solubilization and transport of iron through the formation of soluble Fe^{3+} [9,10].

Bacillus sp. is one of the most studied PGPRs. Members of this species produce a wide range of plant-growth-promoting (PGP) traits such as antibiotics, hydrolytic enzymes, phytohormones, antioxidant enzymes, and siderophores etc. [11]. *Bacillus* sp. produces the catecholate siderophore 2,3-dihydroxy benzoyl glycine. Bacillibactin is the archetypal triscatecholate siderophore. Owing to the incorporated threonine, bacillibactin is known for its highest affinity for iron (Fe^{3+}) of natural siderophores, and bacillibactin has been stated as the dominant extracellular ferric iron scavenger of *Bacillus subtilis* under iron limitations [12]. Inoculation of PGP cells suspension of *Bacillus* sp. promotes plant growth, plant nutrition and control plant pathogens. Addition of suspension of the purified siderophore also promotes plant growth and control phytopathogens; however, the effect is less as compared to the whole cell suspension [11]. Despite the plant growth and nutrition potential, the bioefficacy of *Bacillus* sp. has not been much studied in sesame. Therefore, the present study was aimed to isolate the siderophore and the other plant-growth-promoting metabolite PGPR from sesame rhizosphere, to produce, purify and characterize the siderophore, and to evaluate the bioefficacy of the siderophore producing *Bacillus* sp. as well as the iron nutrition and plant growth promotion in sesame plants.

2. Materials and Methods

2.1. Soil Sample and Sesame Seeds

Soil samples were collected in five replicates, from the rhizosphere of sesame plant in Salem (11.8386° N, 78.0723° E), Tamil Nadu, India and subjected to further analysis [13]. *Sesamum indicum* (sesame) seeds were procured from the local market of Salem district of Tamil Nadu, India.

2.2. Soil Analysis

The pH of the soil samples was measured with a pH meter (Elico, Mumbai), and electrical conductivity (EC) was measured using an electrical conductivity bridge and expressed as dS/m [14]. The available nitrogen content in the soil was measured by the alkaline permanganate method [15,16]. Soil phosphorus was estimated according to Olsen's method [17]. The potassium and iron content of the soil were estimated according to Jackson's [18] and Emsens's method [19], respectively. The sulfur, zinc, and copper content of the soil samples were measured according to the method of Jackson [17].

2.3. Isolation of *Bacillus* sp.

The *Bacillus* sp. was isolated from the collected soil samples by serial dilution techniques. The isolate was subcultured and maintained on nutrient agar slants and incubation at 28 ± 2 °C for 24 h [20]. Each soil sample was processed in triplicate.

2.4. Screening of PGP Traits

2.4.1. Production of Hydrogen Cyanide (HCN)

Hydrogen cyanide production of the isolate was tested as described by Castric [21]. The isolate was streaked on a nutrient agar medium amended with glycine (4.4 g/L). Plate was covered with a Whatman filter paper previously soaked in a mixture of 0.5% picric acid and 2% Na_2CO_3 , and incubated at 28 ± 2 °C for 72 h. Following the incubation, the plate was observed for the development of dark brown color on the filter paper. For quantification of HCN production, LSBS2 was inoculated in glycine-nutrient broth and the OD values were measured at 625 nm.

2.4.2. Production of Ammonia

To test the production of ammonia, the isolate was grown in peptone broth (10 mL) at 28 ± 2 °C for 48 h. After incubation, 0.5 mL of Nessler's reagent was added to the bacterial suspension and observed for the development of brown to the yellow color [22].

2.4.3. Production of Indole Acetic Acid (IAA)

For this purpose, each NB broth containing L-tryptophan (100 mg/L) was separately inoculated with the isolate and incubated at 28 ± 2 °C for 48 h in the dark followed by centrifugation at $9730 \times g$ for 15 min, and cell-free supernatant was assayed for IAA content according to Salkovski's method [23].

2.4.4. Estimation of Phosphate Solubilization Activity

For this purpose, 5 μ L (10^6 cells/mL) of each isolate was grown on Pikovskaia's agar medium at 28 ± 2 °C for 72 h. Following the incubation, the zone of P solubilization was measured [24].

2.5. Screening and Production of Siderophore

For siderophore production, 5 μ L (10^6 cells/mL) of each isolate was spotted onto the center of each chrome azurol sulphonate (CAS) agar plates. Following the incubation at 30 °C for 48 h, plates were observed for the formation of orange or yellow halo zone around the colonies [25].

Quantitative estimation of siderophore production was performed by growing the isolate in a succinate medium at 28 ± 2 °C for 48 h [26]. This was followed by centrifugation at 10,000 rpm for 15 min. Cell-free supernatant was assayed for the amount of siderophore according to CAS assay [25]. Percent siderophore unit (PSU) was calculated according to the following formula [27].

$$\text{Siderophore production (psu)} = \frac{A_r - A_s}{A_r} \times 100$$

where

A_r = absorbance of reference (CAS solution and un-inoculated broth), and

A_s = absorbance of the sample (CAS solution and cell-free supernatant of the sample).

2.6. Determination of Type of Siderophore

The catecholate type of siderophore was determined by the Arnow's test [28]. In this method, 1.0 mL of culture supernatant was added to 0.5 mL of HCl (5 M), 0.1 mL NaOH (10 N), and 1.0 mL of nitrite-molybdate reagent and observed for color change. The catecholate siderophore was confirmed by the formation of orange-red color.

Detection of hydroxamate type siderophore was carried out by tetrazolium test [29]. In this, 100 μ L of culture supernatant was added to 1–2 drops of NaOH (2 N) mixed with a small quantity of tetrazolium salt and observed for the deep red color formation.

2.7. Polyphasic Identification of Potential Isolates

2.7.1. Biochemical Characterization

Physiological and biochemical characteristics of potent isolates were carried as per Bergey's Manual of Systematic Bacteriology [30] using pre-sterilized biochemical kits (Hi-Media, Mumbai, India). The ability of isolates to oxidize different carbon sources and the production of various enzymes was investigated.

2.7.2. 16S rRNA Gene Sequence Analysis

The sequencing of 16S rRNA genes of the isolate was carried out as per the method described by Gangurde et al. [31]. Genomic DNA of the isolate was extracted by using Hi-PurATM Plant Genomic DNA Miniprep Purification Spin Kit (HI-Media, Mumbai, India) as per the method of Sambrook and Russel [32]. Amplification of 16S rRNA gene

sequencing was performed using the primers fd1 (50-ACAGTTTG ATCCTGGCTCAG-30) and rP2 (30-ACGGCTACCTTGTTACGACTT-50) [33]. The amplified sequences were analyzed with gapped BLASTn (<http://www.ncbi.nlm.nih.gov>, 2 March 2020), and the evolutionary distances were computed using the neighbor-joining method [34].

2.8. Purification of Siderophore

For this purpose, *B. subtilis* LSBS2 (5×10^{-5} cells/mL) was grown in 2 L of SM medium in a 5 L flask at 28 ± 2 °C for 24 h followed by centrifugation at 9370 for 15 min. After confirming the CAS test, the pH of the cell-free supernatant was adjusted to 6.0 with 12 N HCl. The acidified supernatant was concentrated (10 times, i.e., to obtain about 200 mL) on a rotary vacuum evaporator (Buchi, R-124, Flawil, Switzerland) at 100 rpm at 50 °C. The concentrated supernatant was then subjected to purification [35].

2.8.1. Solvent Purification

Concentrated supernatant was subjected to the extraction of siderophore by using various solvents like chloroform–phenol, ethyl acetate, water, etc. were used for extraction of siderophores [35].

2.8.2. Purification on Sep Pack C C18 Column

A 5 mL of concentrated supernatant was passed through Sep-Pak C18 cartridge (Waters, Milford, MA, USA); the cartridge was washed with 2 mL of distilled water followed by elution with acetonitrile (20% *v/v*) [36]. The fractions from elute were separately collected and subjected to the CAS test.

2.8.3. Purification on Amberlite-400 Resin Column

Fractional purification of the siderophore was performed using Amberlite IR120 (Na⁺) ion-exchange chromatography [35]. Amberlite XAD-400 resins were dissolved in distilled water, soaked overnight at 8 °C, loaded in a sintered glass column; pre-washed with distilled water; methanol, and then water. The concentrated siderophore-rich broth was loaded on Amberlite XAD-400 resin column. The loading was continued until the saturation of the columns, indicated by the browning of the column. The loaded supernatant was allowed to flow through the column at a flow rate of 5 mL/min. The column was then washed with distilled water to remove all unbound material and eluted with 50% (*v/v*) methanol [37]. Different fractions were separately collected and subjected to the CAS test. CAS-positive fractions were used for further analysis.

2.9. Characterization of Purified Siderophore

2.9.1. Thin Layer Chromatographic (TLC) Analysis

Crude siderophore supernatant was spotted drop by drop on TLC plates (Merck, thickness 0.25 mm of Silica gel G) using mobile phase isopropanol: acetic acid: distilled water (12:3:5) following the procedure of Neilands, [38]. Fully developed TLC plates were sprayed with 0.1 M of FeCl₃ reagents to detect the siderophore and their types.

2.9.2. Fourier Transform Infra-Red (FTIR) Spectroscopic Analysis

Purified siderophore fraction was processed for FTIR in the range of 4000–400 cm⁻¹. The infrared spectrum wavelengths were determined based on their functional groups [39].

2.9.3. High-Performance Liquid Chromatography (HPLC) Analysis

The purified siderophore was subjected to HPLC analysis using the stationary phase pinnacle II C18 reverse-phase column (5 μM integrated pre-column, 250 × 4.6 mm) (Waters 2190 system, Milford, MA, USA) and methanol: water (8:2 *v/v*) as mobile phase [40]. Siderophore sample was injected at the flow rate of 1 mL/min at 25 °C at 400 nm. The preparatory separation of siderophore was done using the same mobile phase. The retention times (RT) of peaks with comparable heights were analyzed [41].

2.9.4. Nuclear Magnetic Resonance (NMR) Spectroscopic Analysis

The NMR spectrum of the purified siderophores was recorded using NMR spectrometer (Bruker AM-500, 500 MHz, Basel, Switzerland) equipped with a 5 mm triple resonance HCN probe with Z-axis pulsed-field gradients [42]. Samples and reference compounds were dissolved in 90% H₂O/10% D₂O or 100% D₂O and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Carbon and nitrogen chemical shifts were referenced using indirect chemical shift ratios to calculate the frequencies at 0 ppm for the respective nuclei. Spectra were recorded at 25 uC [42].

2.10. Plant Growth-Promotion Studies under Greenhouse Conditions

Seeds of *Sesamum indicum* were surface sterilized with 0.1% HgCl₂ for 5 min, washed three times with sterile distilled water, and air-dried at 28 °C and seeded in pots containing 500 g of a sterile mixture of garden soil, sand, and manure (2:1:1). Each pot was seeded with five sterilized seeds and treated as follows. For each treatment, five pots were used.

T0—Control uninoculated nutrient broth

T1—with 200 mL of *B. subtilis* LSBS2 broth

T2—with 200 mL of pure siderophore suspension (296 mg/L)

Pots were incubated under greenhouse conditions (24/20 °C and 70% relative humidity, with the alternate cycle of day and night photoperiod of 18/6 h) for 60 days. Pots were arranged in randomized complete block design (RBD). The plants were irrigated with nitrogen-free sterile tap water every two days. Seedlings were harvested 60 days after sowing and plant growth parameters were measured. After harvest, the fresh weight, dry weight, and root and shoot lengths of the plants were measured. The shoots and roots were separated and dried in an oven at 68 ± 2°C for 48 h and weighed. There were five pots for each treatment and three seedlings per pot.

2.10.1. Estimation of Photosynthetic Pigments

The sesame leaves were harvested from the treated seedlings 60 DAS and were used to estimate the total carotenoids and chlorophyll a, and chlorophyll b contents according to the procedure of Wellburn [43].

2.10.2. Estimation of Total Iron Content in Sesame Seedling

For the estimation of total iron content, sesame leaves, stem and seeds were collected after 60 DAS. About 0.5 g of each sample was separately digested with a mixture of 65% HNO₃ and 30% of H₂O₂ (5:3, (v/v)). The iron content was measured according to the method of Bansal et al. [44].

2.10.3. Determination of Oil Content in Seeds

For the estimation of the oil content of sesame seeds, 100 g of dried ground seeds were used to extract the oil using chloroform followed by extraction of oil with the help of Sokule device [43–45]. Then the oil content was determined on a percentage basis based on the following formula:

$$\text{Ether extract (\%)} = \frac{(\text{Weight of the flask + Extract}) - \text{Tare weight of the flask}}{\text{weight of the sample}} \times 100$$

2.10.4. Estimation of Soil Nutrients

Soil samples were collected from the untreated and treated pots. Total nitrogen was calculated by the method of Labconco [46]. The amount of P from the soil was calculated according to Sims [47] and the amount of potassium was measured according to the method of Upadhyay and Sahu [48]. While calcium (Ca), copper (Cu), zinc (Zn), iron (Fe), magnesium (Mg), manganese (Mn), and sodium (Na) were analyzed by the method of Sahrawat [49].

2.11. Statistical Analysis

All the experiments were performed in five replicates and the data is represented as mean \pm standard error (SE). The data was analyzed by One Way Analysis of Variance (ANOVA) and Tukey's test. All the data were analyzed using SPSS software 20.0 version (SPSS Japan Inc., Tokyo, Japan). The significant differences in the means were analyzed based on Tukey's multiple comparison test ($p < 0.05$).

3. Results

3.1. Soil Analysis

Soil sample was analyzed for various physicochemical properties, such as EC, pH, available macro and micronutrients such as phosphorus, nitrogen, and potassium, iron, zinc, calcium, magnesium, sulfur, and organic carbon contents (Table 1).

Table 1. Physical and chemical properties of soil sample used in this study.

| Properties Measured | Rhizosphere Soil from <i>Sesamum</i> |
|------------------------------|--------------------------------------|
| Sand (%) | 37.9 |
| Silt (%) | 19.86 |
| Clay (%) | 21.4 |
| pH | 7.2 |
| Organic carbon (%) | 0.315 |
| Electric conductivity (dS/m) | 0.17 |
| Water-holding capacity (%) | 41.4 |
| Nitrogen (mg/ha) | 48.5 |
| Phosphorus (mg/ha) | 12.3 |
| Potassium (mg/ha) | 107 |
| Magnesium (me/100 g) | 2.52 |
| Sulphur (mg/ha) | 8.1 |
| Calcium (mg/ha) | 6.1 |
| Zinc (ppm) | 0.37 |
| Iron (ppm) | 1.26 |
| Manganese (ppm) | 0.58 |
| Sodium (me/100 g) | 0.77 |
| Copper (ppm) | 1.52 |

Values are the average of triplicates and significant at $p > 0.5$.

3.2. Isolation of *Bacillus* sp.

A bacterium initially labeled as LSBS2 was isolated from the agriculture soil from Salem district, Tamil Nadu, India (Table 1).

3.3. Characterization of Plant Growth Promoting (PGP) Activities

3.3.1. Production of Hydrogen Cyanide (HCN)

The isolate LSBS2 produced HCN as evidenced by the change in the color of filter paper from yellow to moderate brown. In the presence of glycine and FeCl_3 , the deep brown color of filter paper was observed as an indication of HCN production by bacterial strain (Figure 1A). In quantitative estimation, LSBS2 showed a strong OD value of 0.094.

3.3.2. Production of Ammonia

LSBS2 produced copious amounts of ammonia in peptone water. The addition of 0.5 mL of Nessler's reagent in LSBS2 inoculated peptone water resulted in the development of brown to yellow color (Figure 1B). The isolate produced 8 $\mu\text{g}/\text{mL}$ of ammonia.

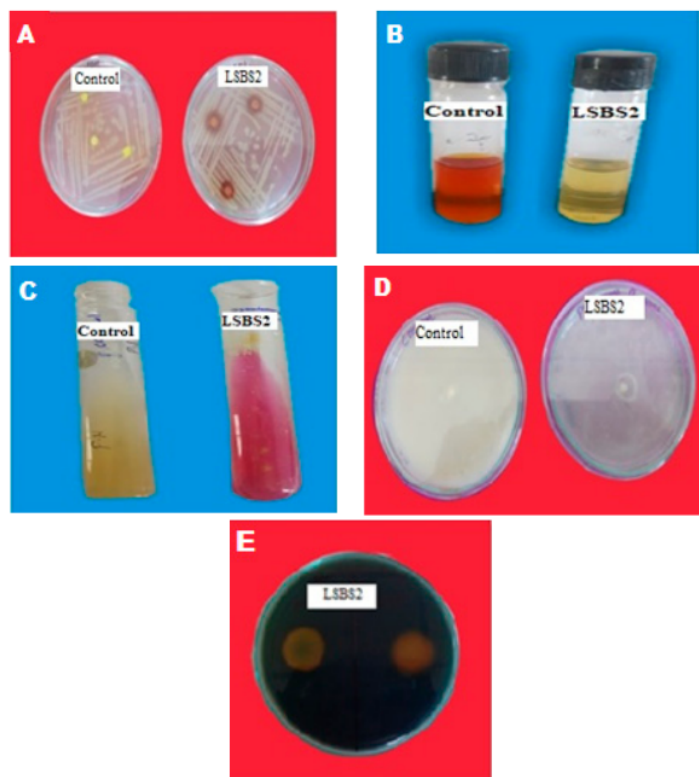


Figure 1. (A) Production of HCN indicated by deep browning of filter paper in test. (B) Production of ammonia as indicated by yellowing of peptone water in LSBS2 inoculated medium. (C) IAA production in NB indicated by pink coloration in LSBS2 inoculated medium following the addition of Nessler's reagent. (D) Phosphate solubilizing activity of LSBS2 on Piko agar. (E) Formation of orange halos on CAS agar indicating siderophore production.

3.3.3. Production of Indole Acetic Acid (IAA)

The addition of Salkowski's reagent in the cell-free supernatant produced pink color indicating the production of IAA by LSBS2 (Figure 1C). Quantitative analysis revealed the production of $78.45 \pm 1.9 \mu\text{g/mL}$ IAA.

3.3.4. Phosphate Solubilization Analysis

A clear zone of phosphate solubilization was observed around the colonies of LSBS2 on Pikovskaya agar LSBS2. A maximum zone of P solubilization of 0.5 mm was observed. (Figure 1D). The isolate solubilized $540 \mu\text{g/mL}$ of P.

3.3.5. Screening and Production of Siderophore

In CAS assay LSBS2 showed a positive reaction (orange color zone) for siderophore production. The strain LSBS2 produced a 10 mm-sized orange color zone in the CAS plate. (Figure 1E). CAS shuttle assay estimated the production of $296 \mu\text{g/mL}$ of siderophore.

3.4. Qualitative Detection of Siderophore

In the Arnow's assay, the addition of 0.5 mL of HCl (5 M) followed by the addition of 0.1 mL of NaOH (10 N), and 1.0 mL of nitrite-molybdate reagent resulted in the formation

of a red-colored solution. This assay confirmed the production of catecholate siderophore by LSBS2.

3.5. Polyphasic Identification of LSBS2

Biochemical test followed by 16S rRNA gene sequencing analysis using BLAST search of NCBI revealed 98.2% similarity of LSBS2 with the sequence of *Bacillus subtilis* available in the GenBank. Thus, the siderophore-producing isolate LSBS2 was identified as *Bacillus subtilis* LSBS2, and its 16SrRNA gene sequencing was deposited in NCBI GenBank under the accession number MH4832621 (Figure 2).

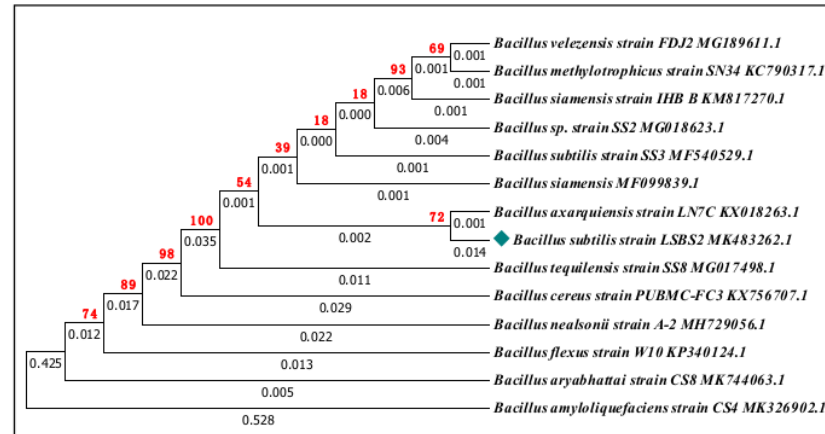


Figure 2. The phylogenetic tree on *Bacillus subtilis* 16S rRNA gene sequencing. The data of type strains of related species were submitted to Genbank under accession number MK483262.

3.6. Purification of Siderophore

3.6.1. Solvent Purification

No extraction was obtained with any of the solvents used. None of the solvents extracted the siderophore from the concentrated broth.

3.6.2. Purification on Sep-Pak C18 Column

All the fractions obtained from the Sep-Pak C18 column were CAS negative, while the filtrate was strongly CAS positive, indicating the presence of siderophores. The CAS-positive filtrate and water-wash yielded 200 and 20 mg/L siderophore, respectively (Table 2).

Table 2. Purification of siderophore on Sep-Pak, C18 column.

| Fraction | Color | CAS Test | Absorbance | Absorbance Maxima (nm) | Yield (mg/L) |
|------------|---------------|----------|---------------------|------------------------|--------------|
| 1 | Brown | Negative | 3.011, 2.911, 3.211 | 291, 195, 305 | 00 |
| 2 | Dark Yellow | Negative | 2.911, 1.871, 3.013 | 295, 201, 311 | 00 |
| 3 | Faint Brown | Negative | 1.931, 1.951, 2.401 | 271, 232, 301 | 00 |
| Filtrate | Golden Yellow | Positive | 3.161 | 272 | 200 |
| Water Wash | Yellow | Positive | 3.161 | 272 | 20 |

3.6.3. Purification on Amberlite-400 Resin Column

Siderophore-rich concentrated supernatant when passed through Amberlite XAD-400 resins yielded five fractions. The first fraction showed a single peak with absorption

maxima of 224 nm. This fraction contained a major amount of a single type of siderophore; the second CAS positive fraction (λ_{max} 264) contained the minor siderophore (Table 3).

Table 3. Profile of siderophore purification on Amberlite-XAD-400 column.

| Fraction | Color | CAS Test | Absorbance (nm) | λ max (nm) | Yield (mg/L) |
|----------|---------------------|----------|-----------------|--------------------|--------------|
| 1 | Dark Golden yellow | Positive | 4.010 | 224 | 296 |
| 2 | Light Golden yellow | Positive | 3.957 | 224 | 50 |
| 3 | Yellow | Negative | 2.112, 1.782 | 298, 302 | 00 |
| 4 | Faint yellow | Negative | 0.975, 0.893 | 404, 396 | 00 |
| 5 | Greenish-yellow | Negative | 0.243, 0.0471 | 462, 664 | 00 |

3.7. Characterization of Purified Siderophore

3.7.1. Thin-Layer Chromatographic (TLC) Analysis

The siderophore produced by crude *Bacillus subtilis* (LSBS2) extract upon being loaded on the TLC showed a red color spot when sprayed with 0.1 M FeCl_3 in 0.1 N HCl reagent. The red color spots developed reveal the presence of a catecholate type of siderophore in the tested extract of LSBS2 isolate.

3.7.2. Infra-Red (IR) Spectroscopic Analysis

The partial purification of siderophore resulted in a yield of 20 mg/L. Further, the FTIR spectrum of LSBS2 shows the adsorption bands at 3445, 2951, 1652, 1455, and 1143 cm^{-1} , respectively, which indicates the presence of (-OH), aromatic (-CH), (-C=O), (-CH₂) and (C-O-C) linkage (Figure 3).

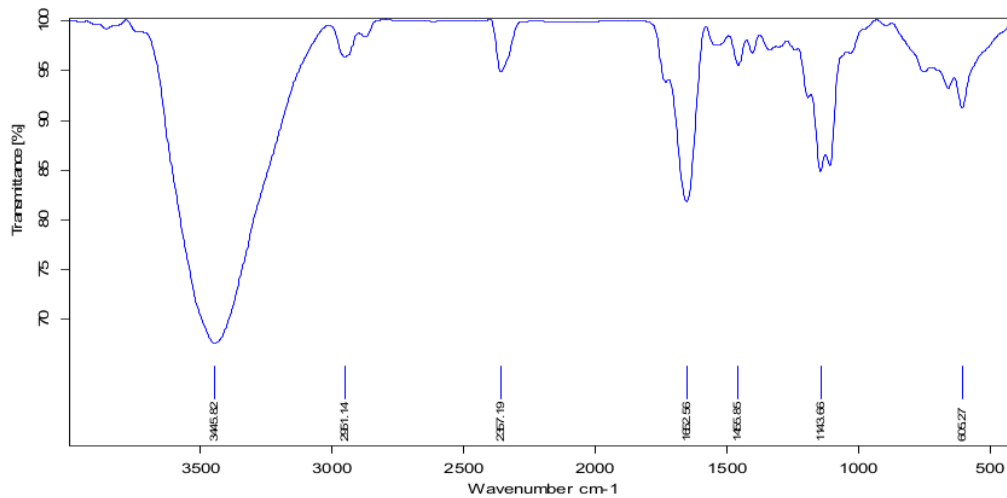


Figure 3. FTIR analysis of functional bands associated with catecholate type siderophore produced by *Bacillus subtilis* LSBS2.

These functional groups are present in the catecholate type of siderophores so that the purification of siderophore production in FTIR analysis conformed to catecholate type that are specific to compound 2,3-dihydroxybenzoic acid are present in LSBS2 (Figure 3).

3.7.3. High-Performance Liquid Chromatography (HPLC) Analysis

The confirmation of purified siderophore was done by HPLC analysis using a mixture of methanol:water (80:20 *v/v*) as a solvent system. In HPLC analysis, the peaks appeared at retention time 1.621 min, 2.188 min, 2.473 min. The peaks appeared at 1–0.767 min; 2–2.033 min was used as a standard. From these results, the presence of purified 2–3 dihydroxy benzoic acid siderophore in the sample is revealed (Figure 4). By performing preparative HPLC, all three peaks were separated and checked again for the presence of no additional peak of any degraded product or impurity visible in the chromatogram supporting its purity.

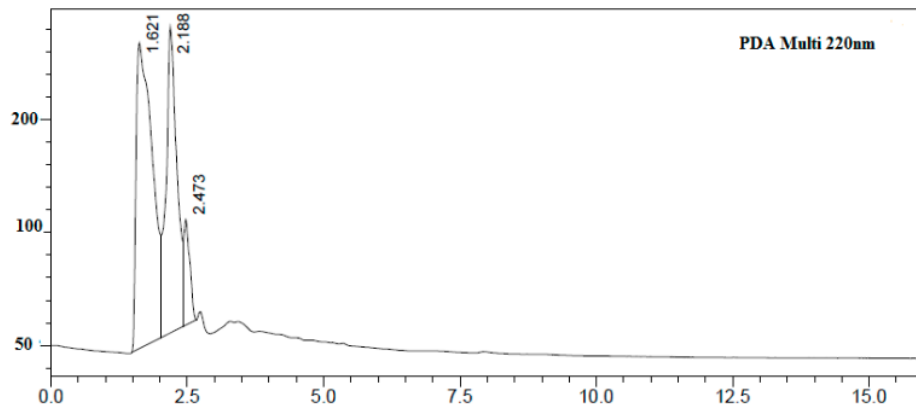


Figure 4. HPLC analysis of purified catechol type siderophore produced by *Bacillus subtilis* LSBS2 showing resemblance with the standard 2–3 dihydroxy benzoic acids.

3.7.4. Nuclear Magnetic Resonance (NMR) Spectroscopic Analysis

In ^1H NMR aliphatic protons appeared at 1–4 ppm aromatic protons appeared at 6–8 ppm respectively (Figure 5).

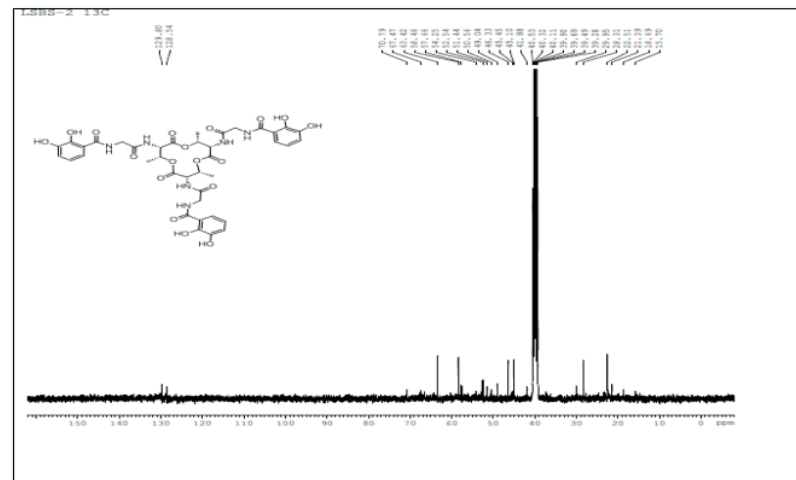


Figure 5. ^{13}C NMR analysis of purification of siderophore produced by *Bacillus subtilis* LSBS2129 which shows the presence of aromatic carbons which also confirms the presence of aliphatic carbons including NHCCH_2 linkage.

In ^{13}C NMR 129.80 and 128.54 ppm, which shows the presence of aromatic carbons, this also confirms the presence of aliphatic carbons including NHCOCH_2 linkage (Figure 6)

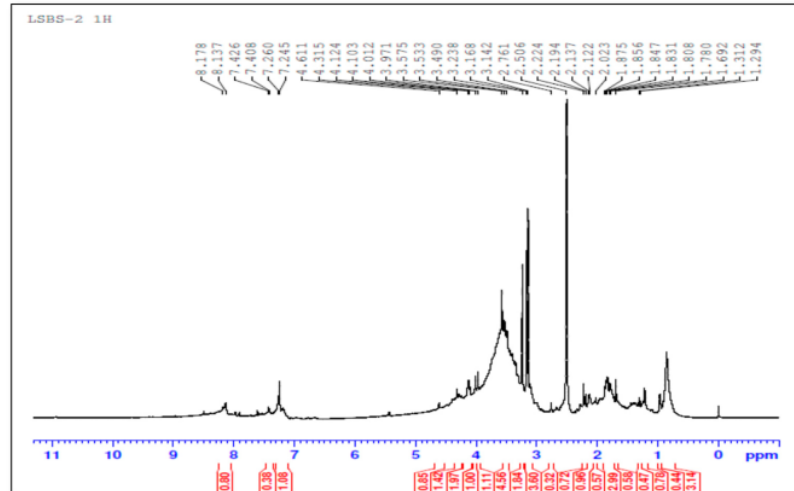


Figure 6. ^1H NMR analysis of purification of siderophore produced by *Bacillus subtilis* LSBS2. Aliphatic protons appeared at 1–4 ppm, aromatic protons appeared at 6–8 ppm respectively.

Overall, the accumulated peak values determine the chemical shifts as noticed from the NMR confirms the presence of catecholate type of siderophore in the tested compound (LSBS2). Furthermore, the chemical hydrolysis in the NMR is considered as the most authentic evidence for the bacillibactin structure, chelation of ferric iron which is a catecholate type of siderophore (Figure 7) produced by the isolated *Bacillus subtilis* LSBS2.

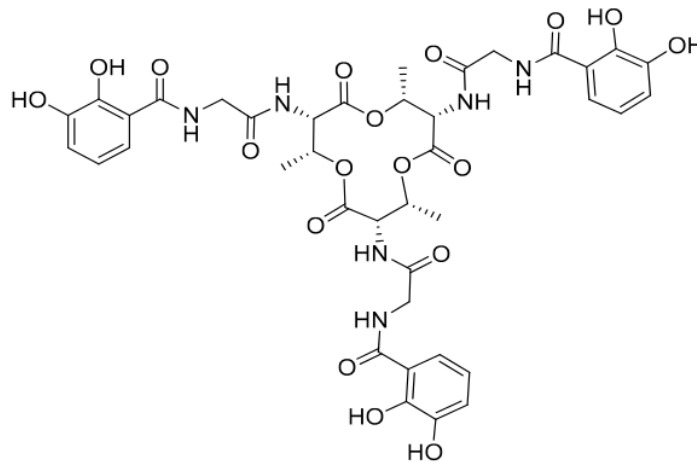


Figure 7. Elucidation of bacillibactin structure based on NMR spectral analysis.

3.8. Plant Growth-Promotion Studies under Greenhouse Conditions

3.8.1. Measurement of Plant Growth Parameters

Sesame plants treated with the *B. subtilis* LSBS2 broth significantly enhanced the leaf length by 32%, shoot length by 39%, root length by 43%, fresh weight by 27%, and dry

weight 24% as compared to the untreated control plants (Figure 8) (Table 4). The application of pure siderophore solution also improved the growth parameters in sesame. However, the plant-growth-promoting effect of a pure siderophore was comparatively less. It resulted in a 17% increase in leaf length, 24% increase in shoot length, 31% improvement in root length 19% increase in fresh weight 16% increase in dry weight 24% over the control plants.



Figure 8. Influence of *B. subtilis* LSBS2 inoculation on sesame growth and pods under greenhouse conditions after 60 days of inoculation.

Table 4. Effect of *B. subtilis* LSBS2 inoculation on growth parameters of sesame (60 days after soil treatment).

| Treatment | Leaf Length (cm) | Shoot Length (cm) | Root Length (cm) | Fresh Weight (g) | Dry Weight (g) | Number of Pods |
|------------------|------------------|-------------------|------------------|------------------|-----------------|----------------|
| | Mean \pm SD | Mean \pm SD | Mean \pm SD | Mean \pm SD | Mean \pm SD | Mean \pm SD |
| Control | 7.3 \pm 0.9 | 29.6 \pm 1.24 | 6.66 \pm 0.47 | 2.6 \pm 0.1 | 1.3 \pm 0.6 | 7 \pm 1.0 |
| LSBS2 | 9.2 \pm 0.7 | 35.4 \pm 1.24 | 8 \pm 0.81 | 3.2 \pm 0.3 | 2.0 \pm 0.4 | 12 \pm 0.5 |
| Pure siderophore | 8.2 \pm 0.5 | 31.3 \pm 0.93 | 7.1 \pm 0.81 | 2.9 \pm 0.2 | 1.9.0 \pm 0.3 | 9 \pm 0.4 |

Values are means of five replicates. \pm S.E. The data was analyzed by Turkey's test.

3.8.2. Estimation of Total Iron, Seed Oil, and Photosynthetic Pigments

The iron content of *B. subtilis*-LSBS2-treated sesame plants was significantly higher. The iron content in the leaf, shoot, and seeds was 34%, 29%, and 47.0% higher as compared to the control (un-inoculated) plants. The application of pure siderophore solution also resulted in a substantial increase in iron. It resulted in a 1%, 11%, and 19.0% increase in iron content over the control. LSBS2-treated seeds showed 47% more oil content as compared to the control. Plants treated with pure siderophore solution showed a substantial improvement of 11% in iron content as compared to control treatment. Application of LSBS2 improved the photosynthetic pigments over the control and pure siderophore solution application; it resulted in a 50% and 42% increase in chlorophyll and carotenoids content in treated as compared to the control (un-inoculated), and 36% and 29% increase in chlorophyll and carotenoids content over the pure siderophore-treated plants (Table 5).

Table 5. Estimation of iron, total seed oil content, and photosynthetic pigments in sesame plants treated with or without *B. subtilis* LSBS2.

| Treatment | Iron Content * | | | Total Seed Oil Content (%) * | Photosynthetic Pigments * | |
|------------------|--------------------|--------------------|-------------------|------------------------------|---------------------------|--------------------|
| | Leaf (μ g/g) | Shoot (μ g/g) | Seed (μ g/g) | | Carotenoids (mg/g) | Chlorophyll (mg/g) |
| Control | 420.46 \pm 5.76 | 512.4 \pm 4.34 | 433.38 \pm 2.45 | 36.16 \pm 2.75 | 0.04 \pm 0.04 | 0.056 \pm 0.056 |
| LSBS2 | 484.02 \pm 5.28 | 563.00 \pm 2.31 | 562.60 \pm 4.30 | 47.3 \pm 2.06 | 0.26 \pm 0.02 | 1.078 \pm 0.229 |
| Pure siderophore | 441.02 \pm 4.078 | 537.00 \pm 1.92 | 541.51 \pm 2.21 | 39.9 \pm 1.24 | 0.17 \pm 0.02 | 0.901 \pm 0.231 |

* Values are means of five replicates. \pm S.E. The data was analyzed by Turkey's test.

3.8.3. Measurement of Soil Nutrients

Soil analysis was done after the experiment on 60DAS of treated seeds. The soil treated with LSBS2 broth showed a high amount of nitrogen content of 63.5 (mg/kg), followed by 19.5 mg/kg of phosphorus and potassium content with 154 mg/kg, whereas the control soil recorded a low amount of nitrogen phosphorous and potassium. In the case of iron, the treated plants recorded a 3.5-fold increase (5.22 ppm) in comparison with untreated

control plants which recorded 1.37 ppm. Other nutrients such as Mg, Mn, Zn, Na, and Cu recorded significant uptake in treated plants in comparison with untreated plants (Table 6).

Table 6. Measurement of nutrients in untreated and soil treated with or *B. subtilis* LSBS2.

| Nutrients Measured | Untreated Soil | Soil Treated with <i>B. subtilis</i> LSBS2 |
|--------------------|----------------|--|
| Nitrogen (mg/kg) | 48.5 ± 0.5 | 62.96 ± 0.55 ** |
| Phosphorus | 12.36 ± 0.3 | 19.2 ± 0.15 * |
| Potassium | 107 ± 1.0 | 153 ± 1.0 ** |
| Magnesium | 2.52 ± 0.03 | 3.89 ± 0.60 * |
| Calcium | 6.1 ± 0.1 | 11.37 ± 0.45 ** |
| Zinc (ppm) | 0.37 ± 0.45 | 0.21 ± 0.01 |
| Iron (ppm) | 1.26 ± 0.01 | 6.41 ± 0.05 *** |
| Manganese (ppm) | 0.58 ± 0.01 | 0.6 ± 0.1 * |
| Sodium (mg/kg) | 0.77 ± 0.01 | 3.72 ± 0.04 *** |
| Copper (ppm) | 1.52 ± 0.02 | 2.96 ± 0.15 ** |

* Values are means from five replications. The data were analyzed by the Tukeys test. Significant difference *, **, *** = Extent of Significance LSD ($p < 0.05$).

4. Discussion

Plant-growth-promoting beneficial characters are produced by various groups of microorganisms [49,50]. These rhizosphere organisms are soil inhabitant, nonpathogenic capable of plant growth promotion [51]. Considering their ability in crop improvement, they are used as a substitute for chemical application in agriculture. Most of the rhizosphere bacteria secrete secondary metabolites called siderophores which act as chelating agents for ferric iron, produced under low iron stress [51–55].

In the present study, a bacterium (LSBS2) was isolated from the agricultural soil, based on biochemical and plant-growth-promoting traits and 16SrRNA gene the isolated bacterium was identified as *Bacillus subtilis* LSBS2. In the past, various reports are documented for the isolation of *B. subtilis* from rhizosphere soil from India by biochemical and plant growth-promoting (PGP) traits such as hydrogen cyanide (HCN), ammonia, and indole acetic acid (IAA), and solubilized phosphate and molecular characterization [56]. As a next step, the qualitative assessment of siderophores production was demonstrated from the isolated *B. subtilis* LSBS2 under nutrient agar medium with iron limiting stress. In earlier reports, *B. subtilis* can produce higher yields of siderophores under iron stress conditions [57]. In the current investigation, the siderophore was positively isolated using the CAS reagent which revealed a change in blue to orange color which resembled similar observations of siderophore production [58–61]. Mehri et al. [62] reported that different doses of zinc and magnesium also play a major role in the induction of siderophore production by the formation of yellow-green fluorescent pigment around them on nutrient agar medium.

To detect the type of siderophore, we have used the tetrazolium test for the estimation of siderophore type using spectrophotometric assay, the subjected extract showed the appearance of red color indicating the presence of catechol type of siderophores. This result is well supported by previous findings of Santos [61] in which the authors described the biocontrollability of siderophore producing *Burkholderia cepacia* XXVI against *Colletotrichum gloeosporioides*.

It was noticed that a clear red spot was developed on the TLC plates which revealed the positive response of catechol type siderophores in the test extract. In the same manner, the detection of siderophores was confirmed in the bacillus culture supernatant obtained from rhizosphere soil [63]. The partial purification of siderophores on the XAD Amberlite column yielded 20 mg/L. Sayyed and Chincholkar [35] purified siderophores of *Alcaligenes faecalis* on Amberlite XAD-400 resins. Further, the FTIR spectrum of LSBS2 showed the adsorption bands at 3445, 2951, 1652, 1455, and 1143 cm^{-1} , respectively, which indicates the presence of (-OH), aromatic (-CH), (-C=O), (-CH₂) and (C-O-C) linkage. These functional groups are present in the catechol type of siderophore so that the purification of siderophore production in FTIR analysis confirmed the presence of a catechol type

of siderophore that is specific to 2,3-dihydroxybenzoic acid [64]. In HPLC analysis, the peaks appeared at retention time 1.621 min, 2.188 min, 2.473 min. The peaks appeared at 1–0.767 min; 2–2.033 min was used as a standard. The results of the present study revealed the presence of purified 2–3 dihydroxy benzoic acid siderophore in the sample [65]. Microbial siderophores directly enhance the availability of iron present in the rhizosphere soil, thereby stimulating plant growth by supplying low iron [56,57,66–69].

Inoculation of siderophore producing *B. subtilis* LSBS2 significantly enhanced plant biomass, pigment content, iron, and oil content in the bioinoculant-treated sesame plants as compared to the control (uninoculated) plants. Several investigations on the application of different species of *Pseudomonas* provided evidence of siderophores directly involved in the stimulation of plant growth and also protecting the plants against various biotic stresses [58,70–73]. Likewise, an increased carotenoids level was observed in siderophores producing strain (Ros2) treated to wheat plants in the presence of pesticide stress [70]. Kusale and co-workers [74] isolated a potential siderophores-producing organism from the rhizosphere; this bacterium treated to sesame plants showed a drastic increase in the seed germination, shoot, root length and also recorded enhanced levels of chlorophyll. Furthermore, in this study, the pH and electrical conductivity, NPK, and iron content in the soil are influenced by the presence of siderophores secreting microorganisms, this is in agreement with the results obtained by Kumar et al. [75]. The present findings also provide evidence of enhanced oil content, chlorophyll, carotenoids pigments, and micro and macronutrients in siderophore-producing bioinoculant-treated sesame plants. These enhanced beneficial characters are correlated with the previous studies [60,75,76]. In an independent study [60], a significant increase in the rice grain oil content after application of siderophore-producing rhizobacteria.

Improvement in plant growth by *Bacillus* sp. is well documented and several mechanisms of plant growth promotion are known for this group of PGP. *Bacillus* sp. is known to stimulate plant growth and prevent pathogen infection [77] and also plays a significant role against biotic stresses through the production of phytohormones, volatile organic compounds, exopolysaccharides, siderophores, and P solubilization [78–80]. Application of *B. subtilis* has been reported to improve crop productivity. *Bacillus* species have been reported as the most promising PGPR, which is an ecologically sound and economically feasible alternative to pesticide practice agriculture [78]. Tahir al [80] reported the production of a wide variety of volatile organic compounds in *B. subtilis* SYST2. They found these VOCs responsible for triggering hormone activity and promoting plant growth. Qiao et al. [81] reported the plant-growth-promoting ability of *Bacillus subtilis* PTS-394. Trivedi [82] reported the promotion of plant growth in maize and wheat by using broth, coal and alginate beads formulation of *Bacillus* sp. Saxena et al. [83] reported *Bacillus* species as a natural soil resource for plant health and nutrition. Verma et al. [84] isolated various *Bacillus* species from the sesame rhizosphere and reported improved seed germination and plant growth promotion in sesame due to inoculation with these Bacilli.

5. Conclusions

The results from the present investigation provided evidence of a positive correlation between the production of various plant growth parameters, nutrient uptake, and improvement in the iron and oil content in sesame plants inoculated with *Bacillus* sp. LSB2. This inoculation also improves soil nutrients. The results of the present study suggest the role of LSBS2 as multifarious PGPR for improvement in the growth parameters and nutrient content in sesame as well as soil nutrients. Such multifarious PGPR strains can be used as effective bioinoculants for sesame farming. However, field trials under different agroclimatic zones are needed to assure the bioefficacy of the isolate.

Author Contributions: Supervision and Conceptualization, S.L.; Investigation and Methodology, S.N.; Formal Analysis D.J.D.; N.L.S. and S.H.; writing original draft—S.N.; writing—review and editing—M.S.R., R.Z.S., H.A.E.E. fund Acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by All Cosmos Industries Sdn. Bhd. through project No. RJ130000.7344.4B200, and Arif Efektiv Sdn. Bhd. Project No. RJ130000.7646.4C110.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: H.E.E. would like to thank All Cosmos Industries Sdn. Bhd. and Arif Efektiv Sdn. Bhd. for financial support through project No. RJ130000.7344.4B200, and RJ130000.7646.4C110, respectively.

Conflicts of Interest: The authors declare no conflict of interest.

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