www.ajlifesciences.com / ISSN: 2663-1040 *AJ Life Sci. 2022, 5 (1): 1-10* **Original Research Article**

Abasyn Journal of Life Sciences

Open Access

DOI: 10.34091/AJLS.5.1.1

Characterization and identification of plant growth promoting endophytic bacterial strain IU10

Ihsan Ullah*[1], Muhammad Ali[2], Muhammad Ibrahim[3]

[1]*Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.*
[2]*Department of Biotechnology, Bacha Khan University Charsadda, Pakistan.*
[3]*Department of Forestry, Bamboo Research Institute, Nanjing Forestry University, Nanjing 11210037 China.*

Abstract

Growing evidence has suggested that plant growth-promoting endophytic bacteria can regulate, which can be used as biofertilizers, bio-stimulants, and biocontrol agent. In the present study, *Bacillus subtills* IU10 was isolated from plants and was subjected to advanced chromatography and spectroscopic techniques for the extraction and isolation of indole-3-acetic acid (IAA). Up to 10 µg/mL of IAA was quantified in the bacterial extract using the [$^2H_2$]-IAA internal standards in GC-MS analysis. PVK agar containing $Ca_3(PO_4)_2$ was used to measure the ability of IU10 to solubilize phosphate. The clear zones formed by phosphate solubilization were measured to assess the phosphate solubilization potential. Azurol-S (CAS) medium was used for measuring siderophore production. The orange halo circles were measured to quantify the siderophore production. Endophytic IU10 inoculated plants showed significantly improvement root to shoot length, biomass and chlorophyll as compared to control. The data indicates that IU10 produce phytohormones, siderophore and immobilize nutrients could be used as biofertilizer.

Key words: phytohormones, nutrient immobilization, growth promotion, endophytes.

Article Info:
Received:
August 11, 2021
Received Revised:
September 2, 2021
Accepted:
December 21, 2021
Available online:
September 17, 2022

**Corresponding Author:*
ihsanknu@gmail.com

1. INTRODUCTION

Chemical fertilizers used in agriculture, provide essential nutrients, including nitrogen, potassium, and phosphorus, which make them the basic elements of to plant. However, fertilizer overuse can result in unintended environmental effects and influence the chemical, biological and physical, composition of the soil [1]. There are a number of negative consequences of using chemical fertilizers, including loss of soil fertility, pollution of groundwater [2].

The biocontrol has been considered to an alternative of chemical fertilizer to overcome the damages of the chemical fertilizers [3]. Endophytic microbes are plant associated microbes which increase plant growth through phosphate mobilization, nitrogen fixation, siderophore, and phytohormone production. Phosphorus is an important macro-element and PGP bacteria solubilizes it and converts it into phosphate ions, which can be absorbed by plants[1]. Through phosphate-solubilizing bacteria, plants require less synthetic fertilizer and can thereby grow more efficiently. Biocontrol and disease prevention are essential functions of siderophores. Siderophores chelate iron, preventing it from being accessed by pathogens and reducing their proliferation[2].

An important function of phytohormones is to regulate cell division, thereby promoting the growth of plants. By Gas Chromatography Mass Spectrometry using Selected Ion Monitoring (GC/MS-SIM), the amount of indole-3-acetic acid (IAA) in culture broth can be more accurately measured [4]. Due to its high separation efficiency, it is a good choice for the analysis of metabolomes, as it can resolve highly complex biological mixtures [3]. By combining GC and MS, it is also easy and convenient to identify compounds according to their composition. Sim mode enables detection sensitivity to be increased from ng-levels to pg-levels, an important feature for qualitative analysis [2].

Rhizobacteria produce plant growth regulators, as has been reported in recent research. Such regulators are beneficial to plant growth as well as plant health when applied to plants that are experiencing various environmental stresses. The IAA cause rapid cell division, proliferation, and differentiation [5]. IAA is a secondary metabolite in microbe that serves as plant growth hormones and is important in agriculture and biotechnology [6]. IAA regulates apical dominance and stimulates lateral root formation, increases stunted plants, and stimulates germination processes [7]. Some of the strains of rhizobacteria e.g. *Acetobacter diazotrophicus*, *Rhizobium phaseoli*, *Herbaspirillum seropedicae, Bacillus cereus, B. macroides, B. licheniformis, and B. pumilus, Burkholderia cepacia* SE4, *A. chroococcum* SE370, and have been known to produce IAA which support plant growth promotion [5, 8-11].

In the present study, we aimed to evaluate the plant growth promoting potentials of endophytic bacteria. Bacillus subtilis IU10 with multiple beneficial characteristics was isolated, identified and characterized for IAA, siderophore production and phosphate solubilization. The plant growth promoting characteristics were determined and detected that IU10 could be a better alternative of chemical fertilization.

2. MATERIALS AND METHODS

2.1. Isolation of bacteria

The roots and shoots of rice plants were surface-sterilized using 70% ethanol and then repeatedly rinsed with distilled water. Chopped pieces were placed on petri dishes with LB agar media (1% NaC, 1% tryptone, 0.5% yeast extract and 1.5% agar) and incubated at 30°C for 24 h. During incubation, the endophytic bacteria were induced to grow from leaves, roots, and stems and the colonies were purified by streaking on fresh media. The colonies were identified morphologically i.e. color, colony size, colony texture.

2.2. Bacterial identification and phylogenetic analysis

Genomic DNA was extracted from the bacteria using protocol described by Ullah et al [4]. The 16S-rDNA was amplified using *Taq* DNA polymerase reaction mixture (according to the protocol) under optimized PCR conditions using 16S-rRNA universal primers. A PCR product was purified with a Qiagen kit (USA) and sent to MACROGEN (Korea) for Sanger sequencing. A phylogenetic tree was constructed using MEGA X for the evolutionary analysis. A maximum likelihood method was applied to the phylogenetic analysis.

2.3. Plant growth assessment

The pot experiment was carried out following Ullah et al [4]. The seeds of rice (*Oryza sativa L.*) were surface sterilized with 75% ethanol and repeatedly washed with deionized water for 5 to 7 times. Surface-sterilized seeds were germinated at 25°C for 48 h on pre-soaked filter paper in a Petri dish. Seedlings were transplanted 0.8% agar medium.

Rice seedlings were inoculated with *Bacillus subtills* IU10 to determine their growth dynamics. One liter of LB broth was cultured at 30°C for 36 h. A 0.40 m pore size filter was used to filter the culture supernatant after centrifuging the culture supernatant for 20 minutes at 10,000 × *g*. Rice at two leaf stages was treated with 5 mL of culture filtrate (CF). A cycle of 16 h of light and 8 h of the dark was carried out in a growth chamber at 25°C while plants were grown under 1,600 lux of light. Following a growth period of three weeks, the plants were harvested.

2.4. Solubilization of phosphate by isolates

The phosphate solubilization process was conducted using Pikovskaya's medium containing $Ca_3(PO_4)_2$ as insoluble source of phosphorus following Ullah et al [4]. The bacterial colonies were cultured on Pikovskaya's agar medium and incubated at 30C for 48 h. the halo zones produced by the solubilization of phosphate were measured to evaluate the bacteria's solubilization efficiency.

2.5. Siderophores production of isolates

Using chrome Azurol-S (CAS) inhibitor and hexadecyltrimethylammonium bromide (HDTMA) as indicators, siderophore production of the isolate was assessed. In accordance with the Schwyn and Neilands [13], we prepared the CAS blue agar medium. After being aseptically poured onto sterile plates, the blue agar medium was allowed to solidify. Inoculated bacterial cultures were incubated for 48 h at 30°C on CAS medium. Indications of siderophore production were the yellowish-orange halo that quickly formed around the colonies.

2.6. In vitro IAA analysis of isolates by GC/MS-SIM

Indole-3-acetic acid produced by bacteria was analyzed via GC/MS-SIM following Ullah et al[12]. The IU10 was cultured in 50 mL of LB broth, supplemented with 0 mg/ml and 0.5 mg/ml of L-tryptophan. The culture was centrifuged at 10,000 × *g* at 4°C for 20 min and supernatant was filtered through 0.45 µm filter. As the supernatant of the culture (pH 2.8 ± 0.2) was extracted, organic layers were combined, evaporated at 45°C in an evaporator, and then re-suspended in 0.1 M acetic acid. The filtrate was added with deuterated [$^{13}C_6$] IAA internal standards and analyzed. In order to analyze fractions containing IAA GC-MS was utilized with SIM (6890N network gas chromatography, 5973 network mass selective detector, Agilent, Palo Alto, California, USA). A supplemented [$_2H^2$] IAA internal standard and exogenous IAA were monitored for major ions. Using Kovats retention index, different GAs was quantified.

2.7. Statistical analysis

Microsoft Excel 2019 (Microsoft Inc., USA) was used to calculate the mean and standard deviation. A t-test was performed using Microsoft Excel to analyze the data. Based on the *t*-test, statistical significance was determined. Probabilities of $p < 0.05$ were considered significant.

3. RESULTS AND DISCUSSIONS

3.1 GC-MS-SIM analysis of IAA

The bacterial IAA stimulates root initiation, cell division, gene expression increases and plant phosphate solubility under stress conditions [9]. The endogenous IAA produced by IU10 was quantified through GC by detecting its associated endogenous signals when compared with [$_2H^2$] isotopic standards at retention time = 11.83 min and MS spectra showed the indole dominant ion (*m/z* 130). The concentration of endogenous IAA was calculated by comparing peak areas ratios between internal standards and endogenous IAA. The results showed that isolate IU10 produced 10 µg/mL of IAA (Fig. 1). Several studies have shown that inoculating plants with endophytic bacteria increased plant growth and biomass production [9, 11].

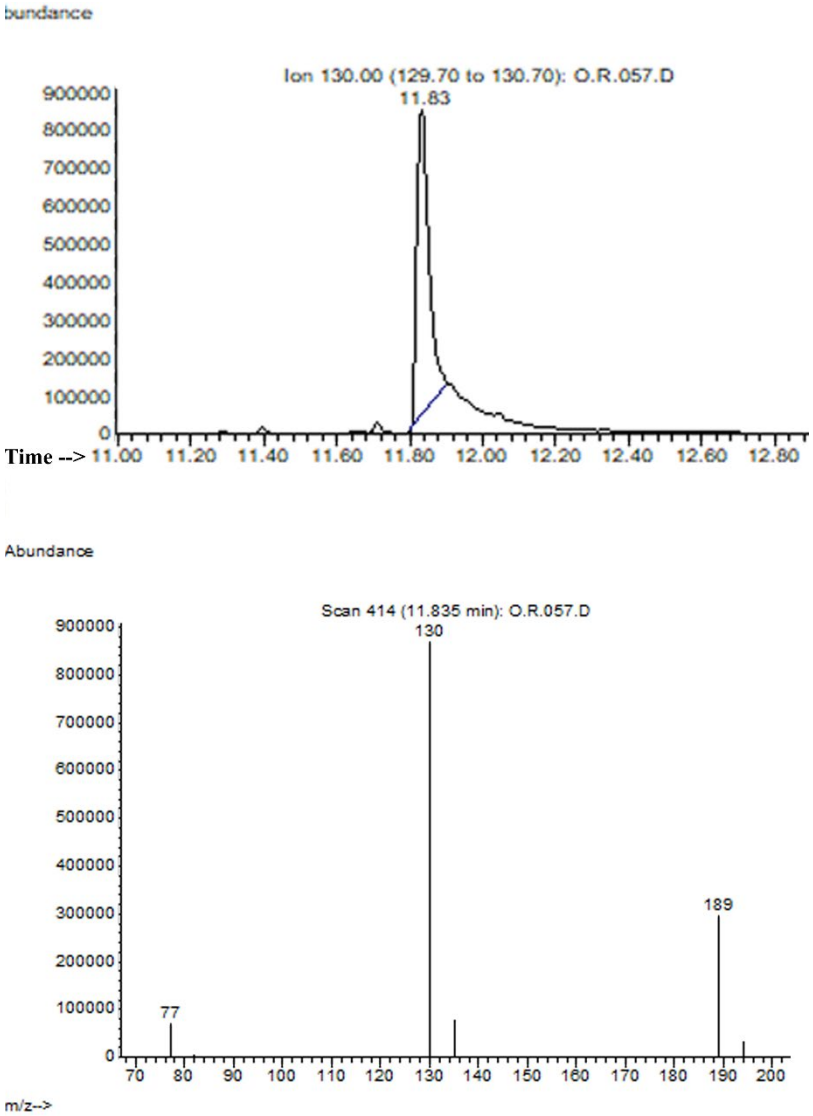

Fig. 1: The GC peak at 11.33 min showed the presence of IAA whereas the major fragment ions located at m/z 130 in MS spectrum confirmed the presence of IAA.

Numerous studies have indicated that both bacteria and plants produce IAA that has multiple functions. Cell division, cell extension, and adventitious root formation are some of these functions [19]. *Serratia* sp. RSC-14 has been shown to significantly increase root and shoot lengths, chlorophyll concentrations, and biomass in plants [14]. *Rhizobium phaseoli, R. tropici, R. leguminosarum*, and *R. etli and Sphingomonas* sp. were reported to produce IAA in the rhizosphere which stimulated plant growth and biomass [9, 15]

3.1. Shoot root length

Bacterial inoculation plays an important role in enhancing plant growth through their interactions with plants[13]. The bacterial isolate was inoculated to estimate the plant growth promoting characters of IU10. Rice plants inoculated with IU100 showed significant improvements in all growth attributes, e.g. shoot and root, fresh and dry biomass, as well as chlorophyll content, as compared to controls. As compared to controls, the IU100 application increased shoot and root length by 24.6% and 54.2%, respectively (Fig. 2).

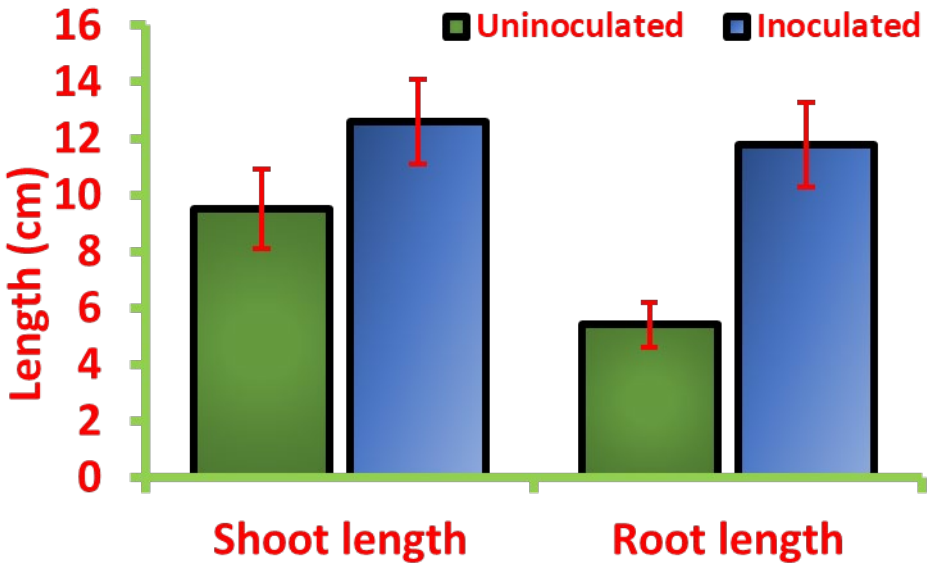

Fig. 2: Effect of bacteria inoculation on shoot length and root length of the plants. The data presented as mean ± SD of three repeated experiment and error bar showed the significant difference ($p < 0.05$) between the control and treated determined by *t*-test.

There is evidence to support the hypothesis that plant-associated microbes contribute to plant growth by secreting an array of metabolites [16]. *Rahnella* sp. isolated from *Polygonum pubescent* showed growth-promoting properties when applied to *Brassica* plants [17]. Among the secondary metabolites produced by endophytic bacteria, Khan et al [14] identified peptides, polyketides, hybrids, and indole derivatives, including auxin. Signalling, biofilm formation, and plant growth have been affected by these metabolites. These metabolites played a significant role in the signalling, biofilm formation, and plant growth promotion [12].

Bacteria have been found to produce IAA that may induce root growth directly by stimulating plant cell elongation and cell division or indirectly by ACC deaminase activity [8, 10]. Microbes that produce IAA can only promote host plant growth to a certain extent, an excess of which can have negative effects, a trait commonly found in pathogenic microbes [5]. A number of previous studies showed that rhizobacteria inoculated in rice seedlings significantly increased growth, root length, shoot length, and yield, while also producing IAA [1-2, 5].

3.2. Fresh and dry biomass

Endophytic bacterial inoculation significantly enhanced plant fresh biomass compared to control. The IU100 treated plants produced 26% more fresh biomass and 39.5% more dry biomass than control plants (Fig. 3). *Pseudomonas* sp. RJ10, *Azomonas* sp. RJ4, *Xanthomonas* sp., RJ3, *Bacillus* sp. RJ16, and *Bacillus* sp. RJ31 effectively promoted plant growth characteristics when inoculated to different agriculturally important was well as model plants [7, 14, 18]. It has been found that IU10 colonization could stimulate plant growth in host plants. Therefore, the isolate could be used to increase plant growth and crop production.

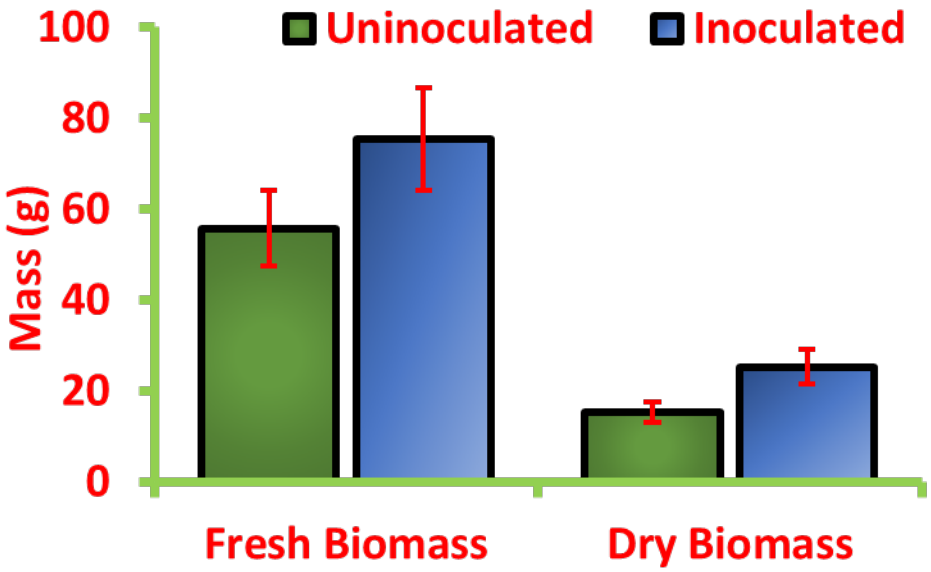

Fig. 3: Effect of bacteria inoculation on fresh and dry biomass of the plant. The data presented as mean ± SD of three repeated experiment and error bar showed the significant difference ($p < 0.05$) between the control and treated determined by *t*-test.

3.3. Chlorophyll content

The chlorophyll content is a good indicator of the bacterial effect on plant growth. Results showed that the chlorophyll content of rice plants increased by 34% following inoculation with the isolate (Fig. 4). As the chlorophyll content increases, photosynthesis increases and therefore production potential and plant vigor increase as well [9, 13, 15]. Many agricultural crop species i.e. tomato, wheat, mung bean, soybean, bell pepper, and rice have been shown to benefit from the inoculation of rhizobacteria and endophytic bacteria [13, 15].

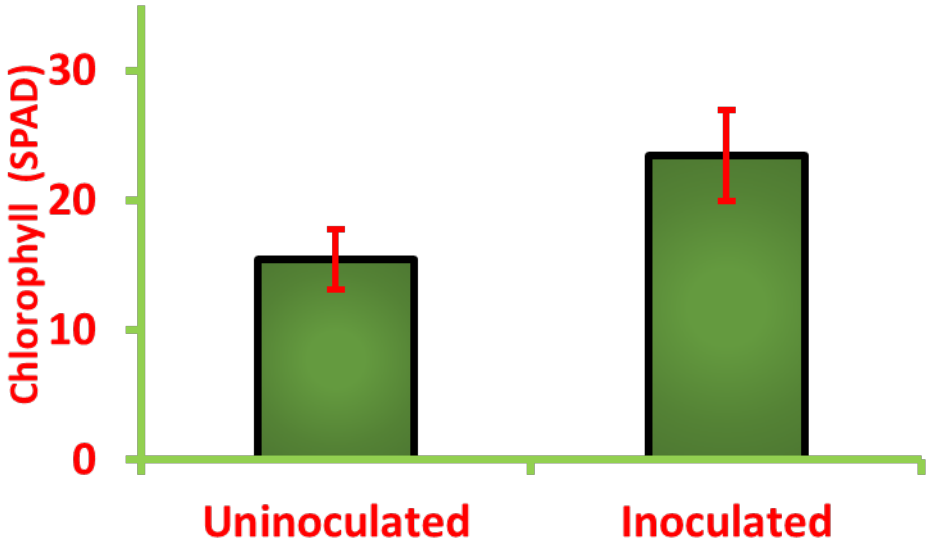

Fig. 4: Effect of bacteria inoculation on chlorophyll contents of the plants. The data presented as mean + SD of three repeated experiment and error bar showed the significant difference ($p < 0.05$) between the inoculated and uninoculated by *t*-test.

A. brasiliense inoculation resulted in an increase in plant growth-promoting photoprotective pigments that benefit the plant. Multiple mechanisms of action may be involved in A. *brasilense* and plant interaction, including nitrogen fixation, mineral absorption, hormonal action [16-17, 19].

3.5. Solubilization of phosphate by isolates

The capacity for phosphate solubilization was measured using PVK containing $Ca_3(PO_4)_2$. As a result of phosphate solubilization by IU10, clear zones were formed the bacterial colonies on plates. Bacteria were able to dissolve $Ca_3(PO_4)_2$ in a clear zone over PVK agar plates, indicating that they were capable of mobilizing phosphate from the medium. For each zone, diameters were measured over a 12 h period, and a graph was plotted against time (Fig. 5).

From an agricultural perspective, all of these aspects are crucial. Treatment with IU10 significantly enhanced plant growth through the production of phytohormones, siderophores, and phosphate solubilization. *Bacillus subtilis* exhibited the greatest capacity for phosphate solubilization [8, 17]. These bacteria improve phosphate availability by secreting enzymes that disrupt the bonds and immobilize phosphate ions, so that the plants are able to better utilize phosphate [8, 10].

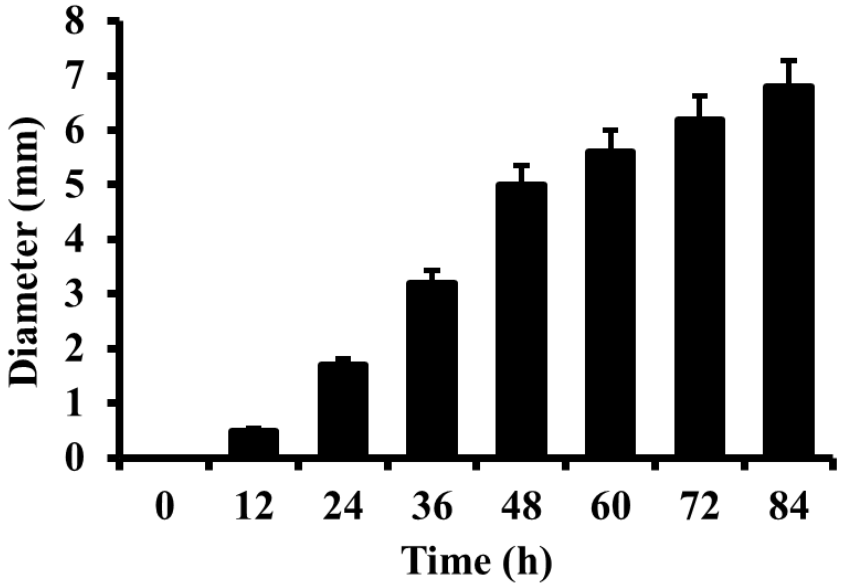

Fig. 5: Phosphate solubilization potential of IU10. The data presented as mean + SD of three repeated experiment and error bar showed the significant difference amount the values.

3.6. Siderophores production of isolates

In CAS blue agar, colonies were stabbed and plates were incubated at 30°C. The plates were observed to have bacterial siderophores after 120 h of incubation. The CAS blue agar developed a pink color and then turned orange during incubation and made haloes. A prolonged halo diameter indicated that the number of siderophores within the medium. For each zone, diameters were measured over a 12 h and graph was plotted (Fig. 6).

Furthermore, bacteria secrete acidic compounds that contribute to soil pH preservation and mobilize phosphate for plant roots [2, 20]. Iron is efficiently scavenged from the soil and its metabolism is enhanced by siderophores produced by microorganisms. Siderophore enables the acquisition of iron by increasing its availability to living organisms since iron is not readily available to them in soluble forms [5, 12].

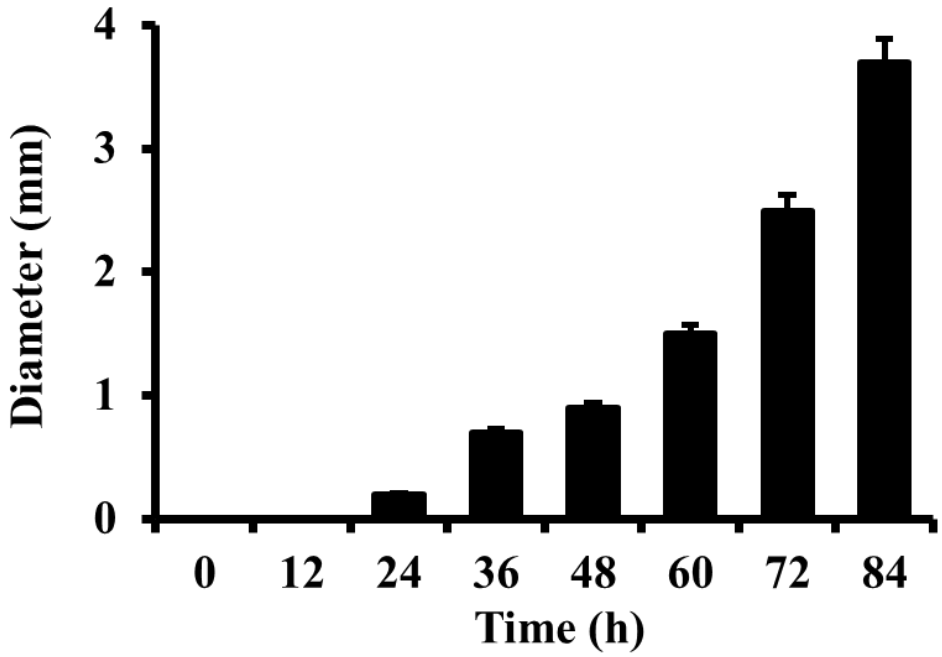

Fig. 6: Siderophore production of strain IU10. The data presented as mean + SD of three repeated experiment and error bar showed the significant difference amount the values.

Ullah et al [12] found that a variety of bacteria produce siderophores on agar plates, confirming that siderophores are produced by a variety of bacteria. The results of previous studies supported the present study and suggested that iron association creates blue in the CAS media, but that color changes to orange when iron chelators are added, since the iron is detached from the dye [10]. Iron uptake in plants is reportedly enhanced by siderophores produced by several microbes e.g. *Photorhabdus* sp., *Bacillus* sp., *Pseudomonas* sp., and many other [2, 21, 12]. Genes such as *exbD* and *ngrA* (PPTase), have been reported to be responsible for encoding a protein complexes in bacteria for siderophores production [8].

3.7. Identification of IU10

Based on the 16S rRNA gene sequence, IU10 was identified. This sequence was aligned with NCBI data using the BLAST algorithm. In the BLAST analysis, IU10 was determined to be Bacillus subtilis, and thus the organism was renamed *Bacillus subtilis* IU10 and the sequences were deposited in GenBank. Based on the sequence homology information obtained from a BLAST alignment, a phylogenetic analysis was conducted (Fig. 7).

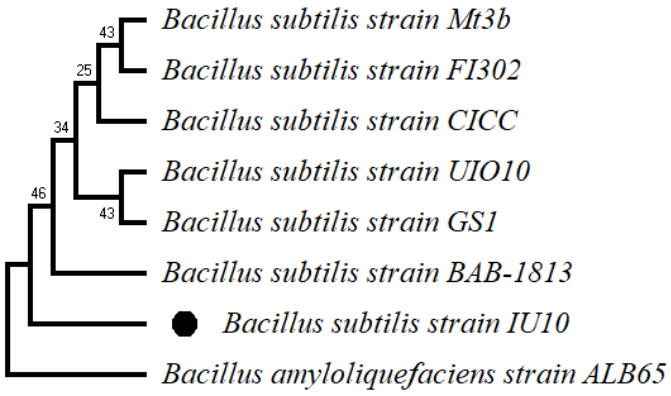

Fig. 7: Phylogenetic tree based on 16S-rDNA sequence of the bacteria. The analysis showed the similarity of the IU10 with *Bacillus* sp.

This approach does not fully account for the high degree of sequence homology in IU10, and the 16S genes prove that it is not complete. We argue, however, that insertions and deletions intragenomic polymorphisms likely play a small role compared to substitutions [20] . As the majority of bacterial isolates, contain multiple or variant copies of the 16S gene, however, the assumption cannot be true and the 16S gene sequences has been the most reliable way to identify the bacteria [19], [21].

4. CONCLUSIONS

Bacillus subtills IU10 isolated in the present study showed the plant growth promotion significantly. The plant growth promoting characteristics such as IAA was detected through GC-MS analysis. other plant growth promoting characteristics such as siderophore production and phosphate solubilization potential were very prominent in IU10. The data indicates that IU10 produce phytohormones, siderophore and immobilize nutrients could be used as biofertilizer.

ACKNOWLEDGMENT

The study has been supported by King Abdulaziz University, Jeddah, Saudi Arabia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Aras, A, Bursal, E, Türkan, F, Tohma, H, Kılıç, Ö, Gülçin, İ Köksal, E. Phytochemical Content, Antidiabetic, Anticholinergic, and Antioxidant Activities of Endemic Lecokia cretica Extracts. Chemistry & biodiversity 2019; 16 (10), e1900341.
2. Ullah, I, Improvement of Cadmium Detoxification Potential and Plant Growth Promotion by Bacterial Endophytes. Preprints.org: 2019.
3. Cetin Cakmak, K Gülçin, İ. Anticholinergic and antioxidant activities of usnic acid-an activity-structure insight. Toxicology Reports 2019; 6, 1273-1280.
4. Gülçin, İ, Gören, AC, Taslimi, P, Alwasel, SH, Kılıc, O Bursal, E. Anticholinergic, antidiabetic and antioxidant activities of Anatolian pennyroyal (Mentha pulegium)-analysis of its polyphenol contents by LC-MS/MS. Biocatalysis and Agricultural Biotechnology 2020; 23, 101441.
5. Gülçin, İ, Scozzafava, A, Supuran, CT, Akıncıoğlu, H, Koksal, Z, Turkan, F Alwasel, S. The effect of caffeic acid phenethyl ester (CAPE) on metabolic enzymes including acetylcholinesterase, butyrylcholinesterase, glutathione S-transferase, lactoperoxidase, and carbonic anhydrase isoenzymes I, II, IX, and XII. Journal of enzyme inhibition and medicinal chemistry 2016; 31 (6), 1095-101.
6. Gülçin, İ, Scozzafava, A, Supuran, CT, Koksal, Z, Turkan, F, Çetinkaya, S, Bingöl, Z, Huyut, Z Alwasel, SH. Rosmarinic acid inhibits some metabolic enzymes including glutathione S-transferase, lactoperoxidase, acetylcholinesterase, butyrylcholinesterase and carbonic anhydrase isoenzymes. Journal of enzyme inhibition and medicinal chemistry 2016; 31 (6), 1698-702.
7. Gulçin, İ, Taslimi, P, Aygün, A, Sadeghian, N, Bastem, E, Kufrevioglu, OI, Turkan, F Şen, F. Antidiabetic and antiparasitic potentials: Inhibition effects of some natural antioxidant compounds on α-glycosidase, α-amylase and human glutathione S-transferase enzymes. International Journal of Biological Macromolecules 2018; 119, 741-746.
8. Turhan, K, Pektaş, B, Türkan, F, Tuğcu, FT, Turgut, Z, Taslimi, P, Karaman, HS Gulcin, I. Novel benzo[b]xanthene derivatives: Bismuth(III) triflate-catalyzed one-pot synthesis, characterization, and acetylcholinesterase, glutathione S-transferase, and butyrylcholinesterase inhibitory properties. Archiv der Pharmazie 2020; 353 (8), 2000030.
9. Rehman, MU, Rashid, S, Arafah, A, Qamar, W, Alsaffar, RM, Ahmad, A, Almatroudi, NM, Alqahtani, SMA, Rashid, SM Ahmad, SB. Piperine Regulates Nrf-2/Keap-1 Signalling and Exhibits Anticancer Effect in Experimental Colon Carcinogenesis in Wistar Rats. Biology 2020; 9 (9).
10. Türkan, F, Huyut, Z, Taslimi, P, Huyut, MT Gülçin, İ. Investigation of the effects of cephalosporin antibiotics on glutathione S-transferase activity in different tissues of rats in vivo conditions in order to drug development research. Drug and chemical toxicology 2020; 43 (4), 423-428.

11. Khan, A, Jahan, S, Imtiyaz, Z, Alshahrani, S, Antar Makeen, H, Mohammed Alshehri, B, Kumar, A, Arafah, A Rehman, MU. Neuroprotection: Targeting Multiple Pathways by Naturally Occurring Phytochemicals. Biomedicines 2020**;** 8 (8).
12. Ullah, I, Khan, AR, Jung, BK, Khan, AL, Lee, I-J Shin, J-H. Gibberellins synthesized by the entomopathogenic bacterium, Photorhabdus temperata M1021 as one of the factors of rice plant growth promotion. Journal of Plant Interactions 2014**;** 9 (1), 775-782.
13. Schwyn, B Neilands, JB. Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry 1987**;** 160 (1), 47-56.
14. Khan, AR, Ullah, I, Waqas, M, Park, GS, Khan, AL, Hong, SJ, Ullah, R, Jung, BK, Park, CE, Ur-Rehman, S, Lee, IJ Shin, JH. Host plant growth promotion and cadmium detoxification in Solanum nigrum, mediated by endophytic fungi. Ecotoxicology and environmental safety 2017**;** 136, 180-188.
15. Rehman, MU Rather, IA. Myricetin Abrogates Cisplatin-Induced Oxidative Stress, Inflammatory Response, and Goblet Cell Disintegration in Colon of Wistar Rats. Plants (Basel, Switzerland) 2019**;** 9 (1).
16. Taslimi, P Gulçin, İ. Antioxidant and anticholinergic properties of olivetol. Journal of Food Biochemistry 2018**;** 42 (3), e12516.
17. Topal, F, Gulcin, I, Dastan, A Guney, M. Novel eugenol derivatives: Potent acetylcholinesterase and carbonic anhydrase inhibitors. International Journal of Biological Macromolecules 2017**;** 94, 845-851.
18. Gülçin, İ, Trofimov, B, Kaya, R, Taslimi, P, Sobenina, L, Schmidt, E, Petrova, O, Malysheva, S, Gusarova, N, Farzaliyev, V, Sujayev, A, Alwasel, S Supuran, CT. Synthesis of nitrogen, phosphorus, selenium and sulfur-containing heterocyclic compounds - Determination of their carbonic anhydrase, acetylcholinesterase, butyrylcholinesterase and α-glycosidase inhibition properties. Bioorg Chem 2020**;** 103, 104171.
19. Sun, D-L, Jiang, X, Wu, QL Zhou, N-Y. Intragenomic Heterogeneity of 16S rRNA Genes Causes Overestimation of Prokaryotic Diversity. Applied and Environmental Microbiology 2013**;** 79 (19), 5962-5969.
20. Earl, JP, Adappa, ND, Krol, J, Bhat, AS, Balashov, S, Ehrlich, RL, Palmer, JN, Workman, AD, Blasetti, M, Sen, B, Hammond, J, Cohen, NA, Ehrlich, GD Mell, JC. Species-level bacterial community profiling of the healthy sinonasal microbiome using Pacific Biosciences sequencing of full-length 16S rRNA genes. Microbiome 2018**;** 6 (1), 190.
21. Yarza, P, Yilmaz, P, Pruesse, E, Glöckner, FO, Ludwig, W, Schleifer, K-H, Whitman, WB, Euzéby, J, Amann, R Rosselló-Móra, R. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nature Reviews Microbiology 2014**;** 12 (9), 635-645.