Cyclic Siloxane Biosurfactant Producing Bacillus Sp. BS14 Biocontrol Charcoal Rot Pathogen Macrophomina Phaseolina and Induce Growth Promotion in Vigna Mungo L.

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Cyclic siloxane biosurfactant producing *Bacillus* sp. BS14 biocontrol charcoal rot pathogen *Macrophomina phaseolina* and induce growth promotion in *Vigna mungo* L.

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Author Contributions

SK designed and conceived the experiment under mentorship of RCD. SD assisted during the experiments and NB helped during manuscript writing. RCD and DKM read the manuscript for scientific correction and approved for communication.

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Ethical statement

This research did not involve any animal and/ or human participants.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Abstract

Rhizobacteria are important component of soil-plant interfaces and help in the management of plant diseases by various means. Precisely, the role of biosurfactant is underscored in biocontrol. The current study showed the exploration of biosurfactant-producing bacteria and its effect in indirect reduction of disease severity in pulse crops. In this study, BS14 was screened as plant growth promoting, biosurfactant producing and biocontrol agent against *Macrophomina phaseolina*. The biosurfactant purified for biocontrol assays and shown inhibition of fungal hyphal growth in dual culture method and cellular-level deformities in mycelia of *M. phaseolina*, as observed under scanning electron microscopic (SEM). The biosurfactant of *Bacillus* BS14 was identified as cyclic siloxane in GC-MS-spectroscopy and FTIR-spectroscopy analysis. In pot-trial studies *Bacillus* sp. BS14 proved their efficiency for the growth-promotion of *Vigna mungo* and significant reduction in disease severity index. Using biosurfactants is a biological alternative to the control of plant diseases.

Keywords: *Bacillus*, Biosurfactant production, Plant growth-promoting rhizobacteria, Biocontrol

1. Introduction

The by-products (enzymes, antibiotics, vitamins, bioorganic acids) secreted by microorganisms showed beneficial effects towards environment, industries, medicine, and agriculture (Lynch et al. 1976; Singh et al. 2017). The products of microbial origin have various utilization in agriculture under the aim to increase crop productivity and soil-fertility management in an eco-safe way (Maheshwari et al. 2013; Maheshwari 2015). Such metabolites like biosurfactants or bio-emulsifiers are precious compounds as they can condition the soil and restrict the growth of phytopathogens for disease control. (Sachdev and Cameotra 2013). The rhizobacteria enhance plant growth via several mechanisms such as phytohormone production, secretion of iron-chelating siderophores, solubilization of insoluble phosphatic salts in the soil, induction of plant-immunity, stress management by ACC-deaminase activity, and controlling fungal phytopathogens in the rhizosphere (Bee et al. 2019).

Besides, some studies talk about certain biosurfactants for the enhancement of rhizobacterial colonization and other means of biocontrol (Sarwar et al. 2018a) *Bacillus* spp. often considered as an elite production source of a comprehensive array of biologically active molecules, such as lipopeptides (LPs) which protect plant from fungal diseases (Agarwal et al. 2017). Earlier, Romano et al. (2013) have purified cyclic lipopeptides from *Bacillus amyloliquefaciens* strain BO5A and checked its antifungal activity against pathogenic fungi *Fusarium oxysporum*, *Aspergillus niger*, *Botrytis cinerea*, and *Penicillium italicum*. Moreover, the production of a wide range of antimicrobial substances by many bacteria, such as lipopeptides (biosurfactants) is consecutively produced by bacilli that demonstrate antibacterial and antifungal activity against a wide array of pathogenic bacteria and fungi (Kumar and Johri 2012). A biosurfactant producing *Bacillus sonorensis* MBCU2 was isolated from vermin compost-amended soil showed potential antagonistic activity against *M. phaseolina in vitro* assays (Pandya and Saraf 2015). A qualitative analysis of biosurfactant from *Bacillus* was carried out along with studies on antifungal properties by Sarwar et al. (2018b). A quantum of reports appeared establishing *Bacillus* as a producer of LPs biosurfactant (Sarwar et al. 2018a, b; Hafeez et al. 2019). On the other hand, Al-Ali et al. (2018) demonstrated that the production of exopolysaccharide (EPS) has the main importance of biofilm formation and rhizosphere colonization. Naturally, exopolysaccharide (EPS) produce silicones, when reacting with siloxanes to form organic siloxane; which is also produced by *Bacillus mucilaginosus* var. siliceous as reported by Avakyan et al. (1986). Cyclic siloxanes are very useful to produce silicone surfactants and foam polyurethane for foam applications (Hil et al. 2002). Recently, a US patent (US20190059385A1) has appeared to increase agronomic yield using the functional approach of siloxanes biosurfactants in agriculture (Hänsel et al. 2019).

The *Vigna mungo*, commonly names black gram, urad bean, black matpe bean grown in the South Asia. It has been in cultivation from the distant past or is one of the utmost prized pulses of India. It is broadly used in Indian food. The plant cultivates 35-110 cm and 4-6 cm seedpods. *V. mungo* is more nutritious as it holds high value of protein, C, K, Fe, thiamine, riboflavin, niacin, folate and essential amino acids. In medieval eyes, this cereal has been used in making crucibles resistant.

Macrophomina phaseolina (Tassi) Goid is the most exterminator phytopathogens of *Vigna mungo* along with almost 500 plant species throughout the world causing charcoal-rot and reducing the yield (Arora et al. 2001; Gupta et al. 2002; Khalil et al. 2016; Shahid and Khan 2019; Pandey et al. 2020) *Vigna mungo* (L.) Hepper is a vital annual pulse crop and valued for its high digestibility and liberty from any harmful effect. Therefore, the use of biosurfactant producing and plant growth-promoting *Bacillus* for an increase in crop production and inhibition of pathogens is the best alternative because they are spore-formers, long survival in hares conditions, and produce toxic metabolites inhibitory to phytopathogens (Bais et al. 2004). The present study was carried out to identify the beneficial roles of biosurfactant-producing and plant growth-promoting *Bacillus* from the rhizospheric soil (Udic Haplustepts) of *V. mungo* and shown their application as plant growth-promoting rhizobacteria (PGPR) for plant growth promotion and biocontrol of *M. phaseolina* in the rhizosphere.

2. Materials and Methods

2.1. Isolation of putative microorganisms

Healthy plants of *V. mungo* were collected from different farmer's fields in district Saharanpur of state Uttar Pradesh, India (29.919°N 77.304°E), carried to the laboratory in sterile polythene bags. Isolates were identified as spore-forming after heat treatment and serial dilution technique as described by Agarwal et al. (2017). Micro-colonies were purified by streaking and designated with laboratory code for prior identification of isolates by cultural and biochemical tests, such as Gram-staining, spore staining, motility test, oxidase test, indole test, catalase test, citrate test, coagulase test, methyl red, and Vogues Proskauer test.

2.2. Screening of biosurfactant production

All the bacterial isolates were initially screened for biosurfactant production via various screening procedure such as mineral salt cetyl-trimethyl-ammonium-bromide (CTAB) - methylene blue agar plate assay (Siegmund and Wagner 1991), hemolytic activity (Youssef et al. 2004), bacterial adherence to hydrocarbons (BATH) assay (Rosenberg et al. 1980), drop collapse assay (Jain et al. 1991), oil spreading assay (Rodrigues et al. 2006),

98 Emulsification stability (E24) test (Das et al. 2008), and measurement of surface tension of cell-free culture broth
99 according to Du Nouy's ring method (Lunkenheimer and Wantke 1981), with the modification as previously
100 carried out in our study (Kumar et al. 2016)

101 **2.3. Isolation of plant growth-promoting bacteria**

102 The determination of IAA production by bacterial isolates was grown on LB broth and incubated at 28 °C for 24
103 h at 120 rpm. Exponentially grown culture (10^8 cfu ml^{-1}) was centrifuged at 10,000 rpm for 20 minutes at 4 °C to
104 collect the supernatant; 2 µl of ortho-phosphoric acid was added to 2 ml of supernatant with the subsequent
105 addition of Salkowski's reagent. The plausibility of pink color confirmed IAA production. Further, HCN
106 (cyanogen) production was determined following the modified method of Bakker and Schippers (1987).
107 Siderophore production was evaluated on Chrome-azurol S (CAS) medium by spot inoculating bacterial culture
108 and incubated at 28 ± 1°C for 48–72 h (Schwyn and Neilands 1987). The formation of orange to yellow halo
109 around the bacterial colonies confirmed siderophore production. Phosphate solubilization ability of all isolates
110 was detected by spotting them separately on Pikovskaya's agar plates (De Freitas et al. 1997). These plates were
111 then incubated at 28 ± 1°C for 3 days and observed for the appearance of the clearing zone around the colonies.
112 The qualitative assay for chitinase production was performed following the method of Dunne et al. (1997). Isolates
113 were separately inoculated by spotting on the plates containing chitin minimal medium (CMM) as the sole source
114 of carbon and incubated at 30 ± 2°C for 7 days. These plates were examined for the development of clear zones
115 around the bacterial colonies.

116 **2.3.1. Biofilm assay**

117 Sterile Muller Hinton broth (MHB) (5 ml) was poured in the pre-sterile test tubes inoculated separately with the
118 test organisms along with proper control and incubated at 37 °C for 24 h. The broth was discarded, washed with
119 0.5 M phosphate buffer saline (PBS) and the internal surface of the tube was stained with 1% crystal violet solution
120 to confirm biofilm formation (O'Toole, 2011).

121 **2.4. Biochemical and physiological characterization**

122 The biochemical characterization of isolates was carried out followed by *Bergey's Manual of Determinative*
123 *Bacteriology* (Holt et al. 1994). Different phenotypic characters of these isolates were compared with the standard
124 strains, such as *Bacillus* sp. (MTCC 297) and *Bacillus subtilis* (MTCC 441) which were procured from the Institute

of Microbial Technology (IMTECH), Chandigarh (India). These eight isolates showed similarity with the species of *Bacillus*.

2.5. Molecular characterization

The 16S rRNA gene sequencing of the isolate BS14 was performed after isolation of bacterial DNA following Sambrook and Russell (2001) and PCR amplified by using universal primer 27F 5′AGAGTTTGATCMTGGCTCAG3′ and 1492R 3′CGGTTACCTTGTTACGACTT5′ gene sequence. The DNA sequences were assembled and subjected to analysis at NCBI. The sequences were deposited to GenBank to get the accession number of the isolate. The phylogeny tree was constructed by employing the neighbor-joining method using MEGA 6.0.

2.6. Production and purification of biosurfactant

The biosurfactant-producing potential isolate BS14 was transferred to 5ml nutrient-rich (NR) broth containing 1% yeast extract 1.5% nutrient broth and 1% ammonium sulfate and incubated at 37 °C for 12 h and 120 rpm as seed culture to get the optical density of 0.5 at 600 nm. Further, 5 ml suspension of BS14 was transferred to a 1000 ml Erlenmeyer flask containing 500 ml of LB medium and incubated on a rotary shaker incubator (150 rpm) at 37 °C. The bacterial cells were removed by centrifugation at 10,000 rpm at 4 °C for 20 min and the supernatant was acidified with 6N hydrochloric acid to get the pH 2.0. The precipitate containing biosurfactant allowed to settle at 4 °C overnight and collected by centrifugation at 15,000 rpm for 20 min. The precipitate was dissolved in distilled water after centrifugation to get pH 7.0 using 1N NaOH. Again, it was centrifuged at 10,000 rpm for 10 min following Sanchez et al. (2007).

2.7. *In vitro* Antagonistic activity

The fungal pathogen *M. phaseolina* was procured from the Culture Collection of the Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar (India) (Singh et al., 2010). Antagonistic properties of bacterial isolates were tested against *M. phaseolina* on potato dextrose agar (PDA) plates following the dual culture technique of Skidmore and Dickinson (1976). Five days old mycelial discs of 5 mm diameter were placed in the center of solidified medium in plates containing modified PDA by adding 2% sucrose. Culture of isolate *Bacillus* sp. BS14 (7×10^6 cfu ml^{-1}) was spotted 2 cm apart from the fungal disc and incubated at 28 ± 1°C for 5 days. Growth inhibition was calculated by measuring the distance between the bacterial and fungal colonies as compared to the control. The fungal growth inhibition (%) was calculated by using the formula: 100 ×C-T/C,

where C = radial growth of fungus in control and T = radial growth of fungus in dual culture. Purified biosurfactant of isolate BS14 was used to check the fungal growth inhibition by Agar well diffusion assay (Nalini and Parthasarathi, 2014).

2.8. Post-interaction events

Fungal mycelia growing towards the zone of interaction were processed for scanning electron microscopic (SEM), available at Wadia Institute of Himalayan Geology, Dehradun. Agar discs of 1 mm thickness were cut from the zone of interaction and placed on cover glasses. These were treated with 2 % glutaraldehyde solution at 20°C for 24 h. Electron microscopic study was done at Wadia Institute of Himalayan Geology, Dehra Dun (India). The samples transferred to copper stubs over double adhesive tape were coated with gold in POLARON, AU/PD sputter coater, and scanned by SEM at 30 kV.

2.9. Chemical characterization of biosurfactant

2.9.1. Fourier transform-infrared spectroscopy (FT-IR) spectra of the dried biosurfactant

FT-IR spectra of the dried biosurfactants were recorded on an 8400S, FT-IR spectrometer (Shimadzu), available at Patanjali Research Institute, Haridwar, and equipped with a mercury–cadmium–telluride (MCT) detector and cooled with liquid nitrogen. About 2 mg of dried biomaterial was milled with 200 mg of KBr to form a very fine powder. The powder was compressed into a thin pellet to be analyzed by FT-IR spectra measurement in wavelength of 400–4000cm^{-1}. The analysis of FT- IR spectra was carried out by using OPUS 3.1 (Bruker Optics) software.

2.9.2. Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis of biosurfactant was done by using a Varian 4000 Mass Spectrometer employing DB5 type capillary column and helium as a carrier gas at a flow rate of 0.5 ml/min. The sample volume was 1µl and the temperature was gradually increased from 40°C to 280°C to identify the compound. The total run time was 45 min. The MS transfer line was maintained at a temperature of 280°C. GC-MS analysis was done using electron impact ionization at 70 eV and data were evaluated using total ion count (TIC) for identification and quantification of the compound. A comparative study was done between the identified compound spectra and that of known compounds of the GC-MS library NIST.

2.10. Pot trial experiments

Pot trial experiments were carried out in triplicate sowing 10 seeds of *V. mungo* var. U-31 (procured from Plant Pathology Department, IARI, Delhi, India) per pot randomly in the rabi season from November to April. A total of 5 treatments were used during pot trials. Treatment 1 was seed dressing with antibiotic-resistant marker strain of *Bacillus* sp. BS14$^{Cam+Ery+}$. For this, healthy socked in lukewarm water overnight and used for seeds biopriming (Dubey et al. 2012). Treatment 2 was designed by infesting the seeds of *V. mungo* with sclerotia of *M. phaseolina* by mixing 0.5 mg sclerotia in 1% CMC or bio-priming. Treatment 3 was a mix of treatment 1 and treatment 2. In treatment 4 crude biosurfactant in 1% CMC (1:1) (for seed bacterization) was used. For Treatment 5, treatment 2 was mixed with treatment 4. Non-bacterized seed acted as a control. Antibiotic marker strain of *Bacillus* sp. BS14 was following the method of Dheeman et al. (2020) using Chloramphenicol and Erythromycin. After all treatments, seeds were sown in sterile pots in triplicates. Rhizospheric soil was sampled and monitored for root colonization from the bacterial treatment for estimation of *Bacillus* sp. BS14$^{Cam+Ery+}$ and indigenous native bacteria at 30, 60, and 90 days after sowing (DAS). *Bacillus* sp. BS14$^{Cam+Ery+}$ strain colonizing *V. mungo* roots was screened on chloramphenicol and erythromycin amended medium. Seed germination and plant growth parameters, such as root/shoot (length) weight (dry/fresh) were measured. As a sample ten fungus-infested plants were taken out after 60 days after plantation and symptoms (defoliation and wilting) of charcoal rot disease in plant roots were measured.

2.11. Statistical analysis

The data were analyzed statistically for the mean differences in values of control and treated plants using Microsoft excel and Graph pad prism 5.0 software. The data were subjected to one-way analysis of variance (ANOVA) to determine the effect of treatment conditions, period, and their interaction on various parameters. The data were analyzed employing the Duncan's Multiple Range Test (DMRT) by taking $p \leq 0.01$ as a significant level.

3. Results

3.1. Isolation of putative *Bacillus*

Based on morphological, physiological, and biochemical characteristics the isolates were found Gram-positive, rod-shaped endospore former, and producers of white, dry and folds, opaque and irregular edged colonies on NAM plates. Eight isolates positively produced catalase, oxidase, and glucose, and were negative for H_2S production, methyl red, glucosamine, and sorbose (Supplementary Table 1).

3.2. Screening of biosurfactant

Eight isolates were screened for biosurfactant production by carrying out several tests. All the isolated exhibited positive β-hemolytic activity, BATH assay, drop collapse test, oil spreading assay, emulsification assay, and surface tension assay. All the isolates except BS21 and BS24 displayed positive CTAB–methylene blue agar plate assay. Among all the isolates BS14 was found to show the best biosurfactant producing properties (Table 1).

A dark blue halo zone with a sharply defined edge around the culture well was observed after 24 h in CTAB–methylene blue agar assay. In BATH assay, the bacterial cells indicated their affinity towards the hydrophobic substrate. BS14 showed β-hemolysis displaying the maximum hemolytic zone of ~2.94 cm. Cell adherence of BS14 to crude oil was 80.23%. Emulsification assay is an indirect method used to screen biosurfactant production. The cell-free culture broth of BS14, BS24, BS27, and BS41 showed more emulsification activity with petrol oil than the other isolates. BS14 showed significant emulsification activity with the emulsification index (E_{24}) of 70.58. In contrast, BS27 and BS41 displayed a better drop collapse test than the other isolates. Furthermore, the isolates BS12 and BS14 showed the maximum reduction in surface tension by 67.14 D/CM than the other isolates. BS14, BS27, and BS41 showed the maximum oil spreading activity forming the clearing zone (Table 1).

3.3. Plant growth-promoting (PGP) activities of isolates

Eight *Bacillus* isolates produced IAA and solubilized phosphate, whereas BS12, BS14, BS24, and BS27 produced HCN. The development of pink color with and without tryptophan in cell-free supernatant indicated IAA production. Change in color of filter paper from yellow to moderate and reddish-brown by adding $FeCl_3$ indicated HCN production by the isolates. Siderophore was produced only by BS12, BS14, BS28, BS40, and BS41. The formation of orange halos around the spots on CAS agar medium indicates siderophore production. However, all the isolates were solubilized phosphate. Formation of clear halos around bacterial spots in Pikovskaya's medium after 48 h displayed phosphate solubilization. Moreover, none of the isolates produced chitinase except BS12 and BS14 (Table 2). All the isolates exhibited biofilm formation except BS12, BS40, and BS41. Bacterial cells adhered to the surface of test tubes which showed biofilm production.

3.4. Molecular Identification

The 16S rRNA gene sequence of the BS14 incorporated 1425bp (NCBI GenBank Accession No. KU991962). It showed 98% sequence similarity with *Bacillus cereus* strain Ks6-15 and *Bacillus cereus* strain X9. Therefore, the isolate BS14 has further been referred to as *Bacillus* sp. BS14.

3.5. *In vitro* Antagonistic activity of *Bacillus* sp. BS14 and pure biosurfactant

The pure culture of *Bacillus* sp.BS14 and its biosurfactant inhibited the radial growth of *M. phaseolina* by 70.10% and 53.6 %, respectively after 7 days of incubation at 28 ± 1°C (Fig. 1A; B). However, fungal inhibition was more pronounced in dual culture as compared to that of pure biosurfactant. Further, fungal growth inhibition corresponded with the incubation period.

3.6. Post-interaction events in mycelia of *M. phaseolina*

Bacillus sp. BS14 in the zone of interaction resulted in halo cell formation and caused mycelial deformities and hyphal degradation of *M. phaseolina*. Formation and development of *M. phaseolina* sclerotia were arrested towards the zone of interaction; consequently, such mycelia and sclerotia lost their vigor. The SEM study shows the dissolution of fungal septa, hyphal fragmentation, and perforation in the cell wall of *M. phaseolina* (Fig. 2A, B, C).

3.7. Chemical analysis of Biosurfactant

The chemical structure of the purified biosurfactant from *Bacillus* sp. BS14 was preliminarily investigated using FT-IR spectroscopy. The peak at 1568.02 cm^{-1} indicates the chemical structure identical to that of a cyclic compound consisting of a hexane ring. The peak at 2329.85 cm^{-1} indicates the Si-H group. The peak between 3394.48 and 3558.42 cm^{-1} shows the relatedness of the Si-NH_2 group. The FT-IR analysis shows the similarity with the cyclic compound produced by *Bacillus* sp. BS14 (Fig. 3). FTIR responsiveness within different absorption regions, absorbance peak heights corresponding to the Si-O bond and Si-CH3 bond were evidenced and indicated a strong correlation with the expected concentration in the siloxane. For external verification of the calibration, FTIR results were compared to those produced by GC-MS. The GC-MS analysis of the concentrated methanol extract resulted in many compounds. The peaks in the chromatogram were integrated and were compared with the database of the spectrum of known components stored in the GC-MS library of NIST to confirm the FT-IR structure analysis. Based on GC-MS analysis, the compound showed the relatedness with cyclic compound 'cyclic siloxane' (Fig. 4).

3.8. Pot trial experiments

Germination was enhanced maximally by the treatment of *Bacillus* sp. BS14, while the germination was significantly decreased in the fungal infested followed by mixed treatment of fungi and *Bacillus* BS14. Following the pattern of robustness, treatment BS14 evidence approximate 25% enhancement in shoot length over control but, significantly lower in the fungal treatment after 90 days of sowing. It is also evidence that the disease severity

index was reached at a peak in the fungal infestation. The development of roots can never be ignored as the attained maximum length in the bacteria treatment similar to the biosurfactant treatment. Enhancement in the shoot weight was observed high in the bacterial treatment again, but the biosurfactant treatment was equally effective in improving plant biomass. *Bacillus* sp. BS14$^{Cam+Ery+}$ strain successfully colonized *V. mungo* roots both in control and *M. phaseolina*-untreated pots and significantly ($p<0.01$) enhanced seed germination, plant growth, and plant biomass over control. (Table 3). *Bacillus* sp. BS14$^{Cam+Ery+}$ plus *M. phaseolina* inoculation displayed disease reduction at 30, 60, and 90 days (DAS). Biosurfactant was able to reduce disease severity index but, not directly support plant growth promotion as compared to bacterial inoculation (Table 3). *Bacillus* sp. BS14$^{Cam+Ery+}$displayed effective root colonization of *V. mungo* as evidenced by the recovery of significant bacteria population from *V. mungo* rhizosphere 90 DAS (Table 4).

4. Discussion

Baliyan et al. (2018) reported that rhizobacteria in the ecological population create a heterogeneous group of bacteria in the rhizosphere. Biosurfactant-producing *Bacillus* with plant growth-promoting traits as a rationale of this study can prove a better alternative for biocontrol agents of phytopathogens, bio-inoculants for plant growth, and crop yield enhancer in eco-balanced ways as compared to the other surfactant (Bais et al. 2004). In this study, *Bacillus* sp. BS14 was found the most doable biosurfactant producing plant growth-promoting rhizobacteria among the other eight isolates. BS14 can establish a new functional niche in the rhizosphere or soil habitat, as able to produce biosurfactants and indigenous farms soil. We observed that, culture filtrate of *Bacillus* sp. BS14 contains many plant growth-promoting compounds, such as IAA, chitinase, siderophore, and volatile compounds. Earlier, *Bacillus* is reported to bear a PGPR's characteristics, as evidence in our study (Kumar et al. 2012). In this trend, plant hormones like indole-3-acetic acid (IAA), gibberellins, cytokinins, and certain volatiles have also been reported in PGPRs (Mehta et al. 2010). Bacillus has been significant effects on the biocontrol of phytopathogenic fungi. (Dheeman et al. 2020). Similarly, BS14 is effective to restrict the growth of phytopathogenic fungi, under this investigation. On the other hand, phosphorus (P) is a very crucial plant growth-limiting nutrient and available in insoluble form in a substantial amount in the soils for plant growth nutrient. The abilities of BS14 in phosphate solubilization was observed, as an important trait of PGPR. Also, Prakash and Arora (2019) evidenced the abilities of *Bacillus* for solubilization of phosphate and other mineral nutrients. Still, the role of biosurfactants in phosphate and other mineral solubilization as the bioremediation approach is

underscored. Further, this study opens a scope to utilize bacterial origin surfactant for soil remediation and enhancement of soil nutrition capacity.

In this sequence, another mineral, iron is a crucial nutrient for almost all forms of life and all microorganisms require iron for their growth and metabolism too (Neilands 1995). *Bacillus* sp. BS14 was found a prominent siderophore-producing bacterium in vitro, producing it can manage iron for their and plants' growth. Many rhizobacteria have been reported to produce antifungal metabolites like HCN (Bhattacharyya and Jha 2012). Production of HCN by *Bacillus* species has earlier been reported by Chen et al. (2010). In the present study, *Bacillus* sp. BS14 shown HCN production, has earlier been reported by Dheeman et al. (2020). BS14 produced a clear zone in the chitin-containing growth medium and showed chitinase producing abilities, as desired mechanisms of the biocontrol agent (Kumar et al. 2012). Chitinase production by rhizospheric bacilli from rice (Chen et al. 2010) and *Phaseolus vulgaris* (Kumar et al. 2012) has been reported earlier. Biosurfactant produced by *Bacillus* sp. BS14 showed antifungal activity against *M. phaseolina*. Mnif et al. (2015) isolated *Bacillus* sp. SPB1 produced a lipopeptide biosurfactant and exhibited antifungal activity against *Rhizoctonia bataticola* and *Rhizoctonia solani*. Cyclic siloxanes are very important and valuable compounds from the industrial application point of view. The cyclic siloxanes are used in the manufacture of silicones, carriers, lubricants, and solvents in a variety of commercial applications (Si 2006).

In the present investigation, biosurfactant produced by the strain *Bacillus* sp. BS14 showed structural similarity with the cyclic siloxanes. Extracellular polysaccharide plays a crucial role in silica release, especially in the case of quartz. Such polysaccharides can react with siloxanes to form organic siloxanes. It can be of bacterial provenance (e.g., from *B. mucilaginosus* var. *siliceous*; Avakyan et al. 1986). Strains of *Bacillus thuringiensis* (*Bt*) produce crystalline proteins (δ-endotoxins) during their stationary phase of growth. Many authors used surfactants (1,2-benzisothiazolin-3-one), of the inert ingredients in Foray 48 B; the siloxane (organosilicone) Triton- X-100, Tween 20, and Latron CS-7 as surfactants for *Btk* formulations (Helassa et al. 2009). Biofilm is a matrix structure of cells adhered to a surface and encased in extracellular polymeric substances. (Bogino et al. 2013). In the present study, *Bacillu*s sp. BS14 produced biosurfactants and formed biofilm. The best biofilm-forming activity was found in other isolates also those produce biosurfactants too. Biosurfactants are also known to play multifarious roles in biofilm-formation.

Beneficial rhizobacteria can promote plant growth not only by facilitating mineral nutrient uptake and phytohormone production but also more indirectly by protecting against the infection of fungal pathogens. They

can antagonize pathogens by producing low-molecular-weight toxin or extracellular lytic enzymes (Haas and Keel 2003) and more indirectly by triggering the defensive capacities in the host plant (Cameotra and Makkar 2004). We found that *Bacillus* sp. BS14 inhibited the growth of *M. phaseolina in vitro* and resulted in several types of abnormalities in mycelia. It may be due to the secretion of many inhibitory compounds leading to multifarious abnormalities in fungal hyphae as observed by scanning electron microscopy (SEM). Similar work on biocontrol of *M. phaseolina* has been carried out by Kumar et al. (2012) Furthermore, fungal inhibition was more pronounced in dual culture as compared to pure biosurfactant

Pot trial experiments illustrated the potential of *Bacillus* sp. BS14 to be developed as an effective commercial biological control agent. Bacterial broth culture of *Bacillus* sp. BS14$^{Cam+Ery+}$ effectively enhanced plant growth and decreased charcoal rot disease. Effect of cyclic siloxane biosurfactant was observed in disease reduction irrespective of a direct impact on plant growth promotion. This may due to living actions and the involvement of other traits of bacteria in the rhizosphere. Thus *Bacillus* sp. BS14 played a great role in declining charcoal-rot disease along with plant growth promotion of *V. mungo*. Earlier, *Bacillus* spp. has been reported for the biological control of fungi including species of *Fusarium*, and *Macrophomina* (Cavagliery et al. 2005; Kumar et al. 2012). The capacious spectrum antagonistic activity of bacilli is executed by the secretion of several metabolites including antibiotics (Haas and Keel 2003), volatile HCN (Chen et al. 2010). and siderophores (Gupta et al. 2002).

As per evidence from the results obtained above, the isolate was putative to produce biosurfactants which already been reported by various scientists (Ayed et al. 2019; Nalini et al., 2020; Khedher et al. 2020), but still in its infancy, besides bacteria were able to show biosurfactant production as an indirect approach, similar to enzyme production, antibiotic production for biocontrol. Also, in the pot-trial, disease severity index was reduced significantly in the biosurfactant treatment, therefore, we postulate the effects of biosurfactant in biocontrol of *M. phaseolina* and their other PGP traits in plant growth promotion

5. Conclusions

Based on the above studies it may be concluded that biosurfactant-producing bacteria are also available in the rhizosphere of legume crops. *Bacillus* sp. BS14 of our study exhibited strong plant growth-promoting properties and biocontrol potential against *M. phaseolina* causing charcoal rot in *V. mungo* further explore the use of biosurfactants(s) or biosurfactant producing bacteria for biocontrol of charcoal rot disease. Future studies can

include the production of bio inoculants in various carrier materials and further, use such bacteria in the production of bio inoculants is a future requirement of agricultural and environmental sustainability.

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492 **Legends Figures**

493 **Fig. 1** Antagonistic effect of (A) *Bacillus* sp. BS14 against *M. phaseolina* in dual culture media (B) purified
494 biosurfactant of *Bacillus* sp. BS14 against *M. phaseolina* in fungal culture media

495 **Fig. 2** Scanning Electron Microscopy (SEM) showing post-interaction events in the hyphae and sclerotia of *M.*
496 *phaseolina* caused by *Bacillus* sp. BS14; (A) Control; (B) hyphal lysis and cell shrinkage; (C) loss of vigour and
497 mycelia fragmentation; (D) hyphal fragmentation, shrinkage, loss of vigour, and loss of sclerotia integrity

498 **Fig. 3** FTIR spectroscopy analysis of biosurfactant produced by *Bacillus* sp. BS14

499 **Fig. 4** GC-MS spectra of purified biosurfactant produced by *Bacillus* sp. BS14.

500 **Legends Table**

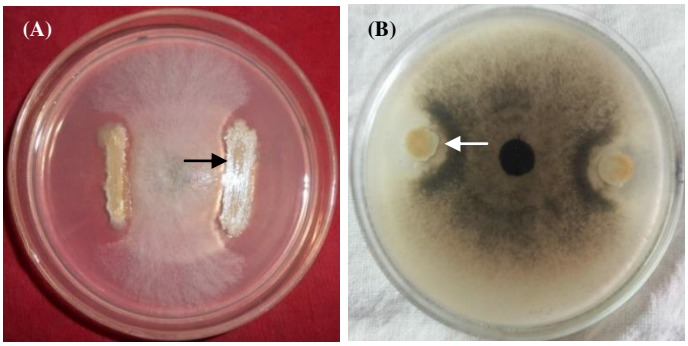
501 **Table 1** Biosurfactant producing assays of rhizospheric bacilli from *Vigna mungo*

502 **Table 2** Plant growth promoting attributes, chitinase production and biofilm assay of rhizospheric bacilli from
503 *V. mungo in vitro*

504 **Table 3** Effect of cyclic siloxane biosurfactant and *Bacillus* sp. BS14$^{Cam+Ery+}$ under various treatments on the
505 growth of *V. mungo* under pot assays after 90 days

506 **Table 4** Root colonization of *V. mungo* by *Bacillus* sp. strain BS14$^{Cam+Ery+}$ at 30, 60 and 90 days

507



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509 **Fig. 1**

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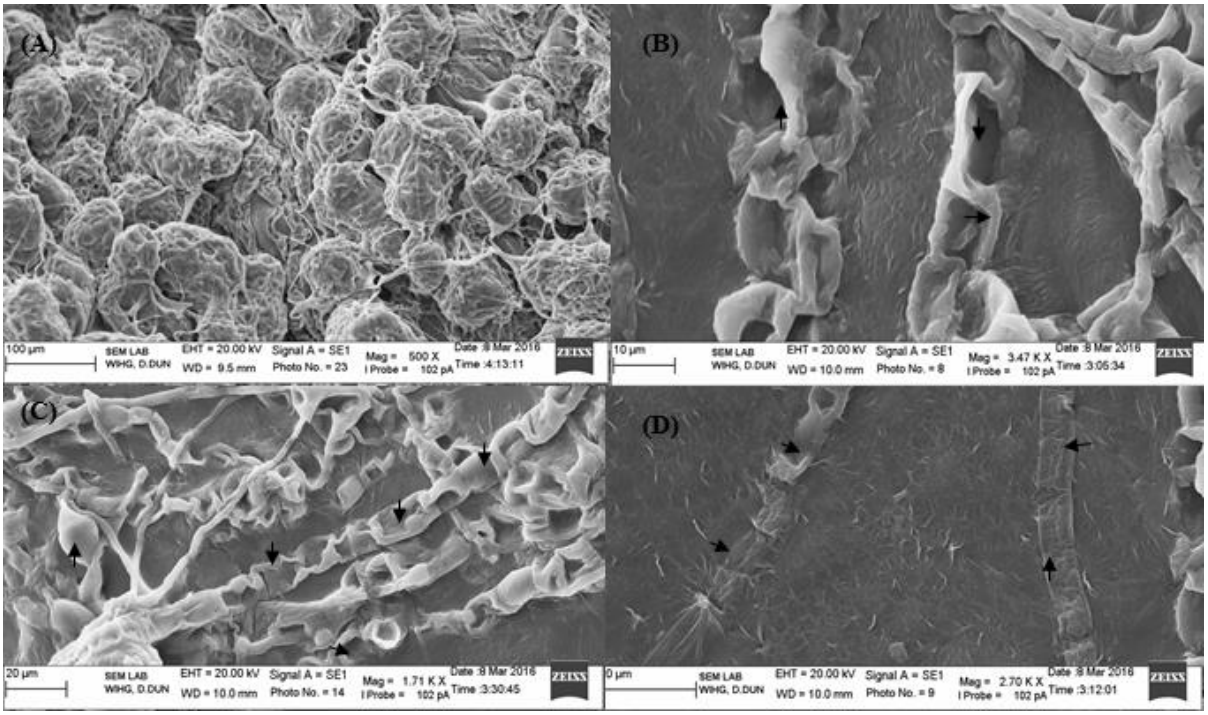
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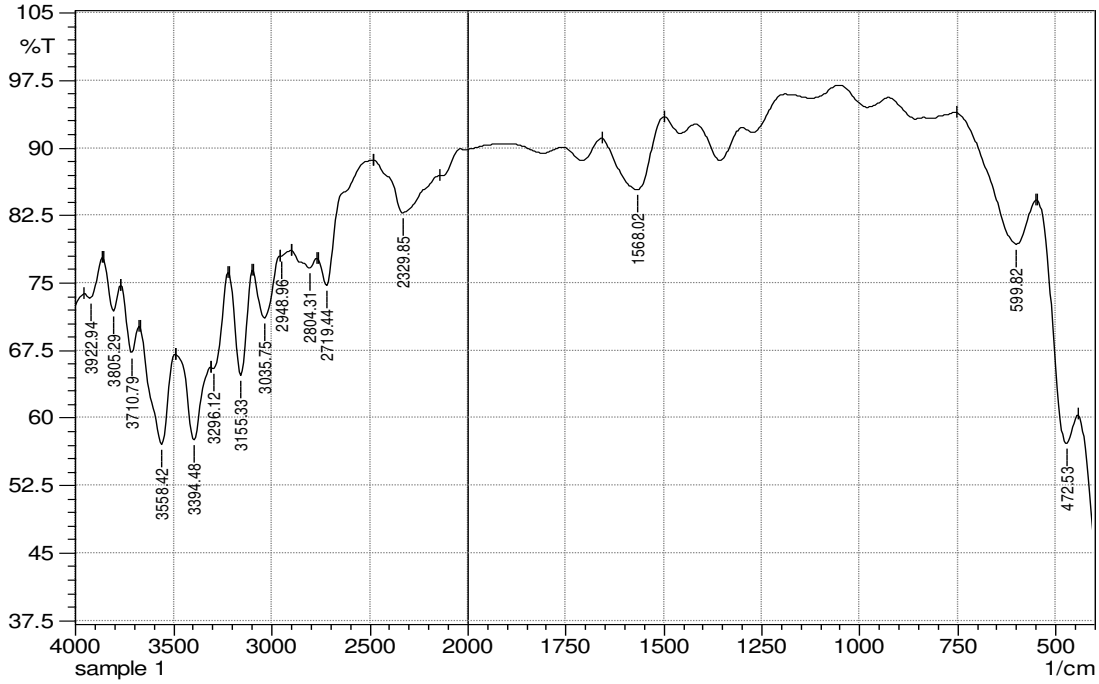
Fig. 2



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525 **Fig. 3**

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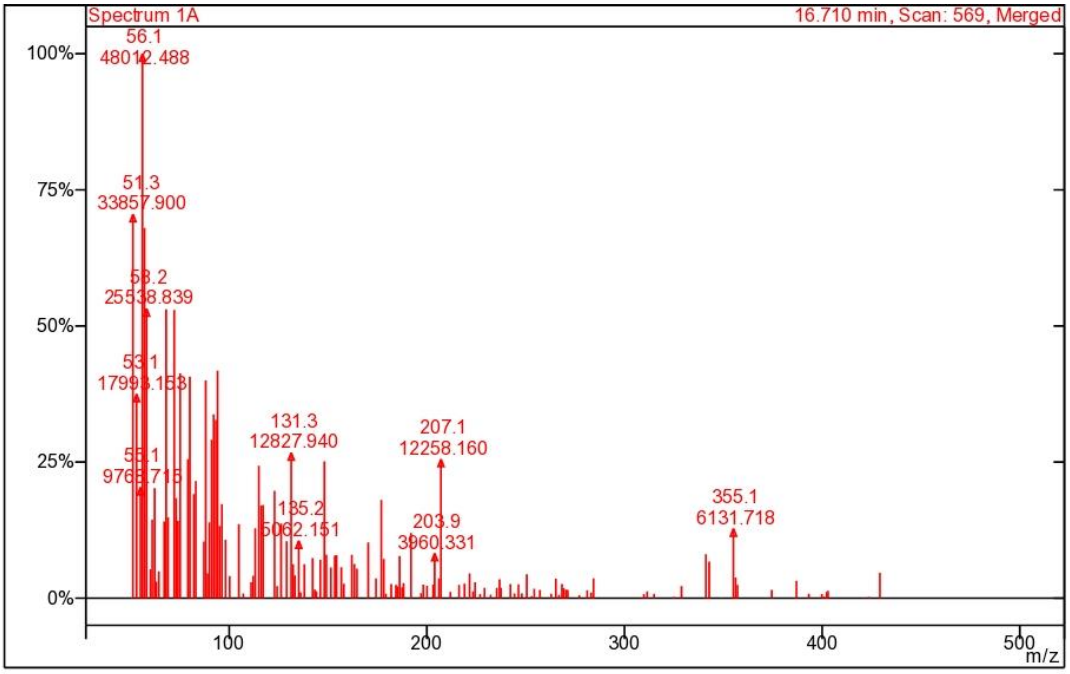
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527

528 **Fig. 4**

Table 1

Isolates	CTAB methylene blue agar plate assay	β-hemolytic activity	BATH assay	Emulsification assay	Drop collapse test	Surface Tension measurement (D/CM)	Oil spreading assay
BS12	++	++	++	+	+	52.12	+
BS14	+++	+++	++	++	+	67.14	++
BS21	-	+	+	+	+	31.23	+
BS24	-	++	++	++	+	28.32	+
BS27	+	+	++	++	++	32.31	++
BS28	+	+	+	+	+	21.12	+
BS40	+	+	+	+	+	40.13	+
BS41	++	+	+	++	++	24.12	++

Abbreviations: (-): Absence of halo formation or no activity; (+): Small halo < 0.5 cm; (++): Medium halo formation > 0.5 cm wide surrounding to colonies; (+++): Large holo >1.0 cm wide surrounding to colonies.

Table 2

Isolates	Siderophore	HCN	IAA	Chitinase production	Phosphate solubilization	Biofilm assay
BS12	+	+	++	+	+++	-
BS14	+++	++	+++	++	+++	+
BS21	-	-	+	-	+	+
BS24	-	+	+	-	+	+
BS27	-	+	+	+	+	+
BS28	++	-	+	-	+	+
BS40	+	-	+	-	+	-
BS41	++	-	+	-	++	-

Abbreviations: IAA: indole-3-acetic acid; HCN: hydrogen cyanide; (-): Absence of halo formation or no biofilm former; (+) Small halo < 0.5 cm or biofilm forming; (++): Medium halo formation > 0.5 cm wide surrounding to colonies; (+++) Large holo >1.0 cm wide surrounding to colonies.

Table 3

Treatments	Germination (%)	Shoot length (cm)	Root length (cm)	Shoot Weight		Root Weight		Disease Severity Index
				Fresh (g)	Dry (g)	Fresh (g)	Dry (g)	
***Bacillus* sp. BS14$^{Cam+Ery+}$**	93.3	49.0**	19.0**	5.530**	0.954**	1.932**	0.090*	2.0
M. phaseolina	53.3	31.8**	11.4**	3.329**	0.710**	0.754**	0.066**	9.0
Bacillus* sp.BS14$^{Cam+Ery+}$ +*M. phaseolina	73.6	46.8**	17.7**	5.241**	0.906**	1.808**	0.085**	7.0
Biosurfactant (Pure)	93.3	49.0**	19.0**	5.530**	0.954**	1.932**	0.090**	1.0
Biosurfactant (Pure) + *M. phaseolina*	81.5	36.4**	12.6**	4.173**	0.718**	1.741**	0.073**	5.0
Control	63.3	38.9	13.9	3.998	0.793**	1.173	0.075	8.0

Values are the mean of triplicates; ** significant at 1% level of LSD by two-way ANOVA. Duncan Multiple range test employed differentiate each parameter with different alphabetic superscripts.

Table 4

Treatment	Bacterial population (log cfu)		
	30 days	60 days	90 days
***Bacillus* sp. BS14$^{Cam+Ery+}$**	6.14±0.12	7.22±0.17	7.12±0.11
Bacillus* sp. BS14$^{Cam+Ery+}$+*M.phaseolina	7.13±0.08	7.42±0.19	7.67±0.07

Values are the average of three replicates. Bar ± represents standard deviation (SD) from the mean value.

Supplementary Material

Cyclic siloxane biosurfactant producing *Bacillus* sp. BS14 biocontrol charcoal rot pathogen *Macrophomina phaseolina* and induce growth promotion in *Vigna mungo* L.

Table 1

Characteristics	BS12	BS14	BS21	BS24.	BS27	BS28	BS40	BS41	MTCC 441 *B. subtilis*	MTCC 297 *Bacillus* sp.
Gram Reaction	+	+	+	+	+	+	+	+	+	+
Rod	+	+	+	+	+	+	+	+	+	+
Endospore Former	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+
Growth at 4ºC	+	+	+	+	+	+	+	+	+	+
at 45ºC	-	-	-	+	-	-	+	-	-	-
Growth in 4%Nacl	-	+	+	+	+	+	+	-	-	-
5%Nacl	+	+	+	+	+	+	+	+	+	+
6%Nacl	+	+	+	+	+	+	+	+	+	+
7%NaCl	+	+	+	+	+	-	+	-	+	-
Oxidase Reaction	-	-	-	+	-	+	-	-	-	+
Catalase Reaction	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-
Indole Production	-	-	-	-	-	-	-	-	-	-
VP test	+	+	-	-	+	-	+	-	+	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+
Lipid Hydrolysis	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	+	+	+	+	+	+	+	+	+	+
Casein Hydrolysis	+	+	+	+	+	+	+	+	+	+
Gelatine Hydrolysis	+	+	+	+	+	+	+	+	+	+
Urease production	+	+	+	+	+	+	+	+	+	+

Abbreviations: (-): Absence of activity; (+): Presence of activity.

Figures

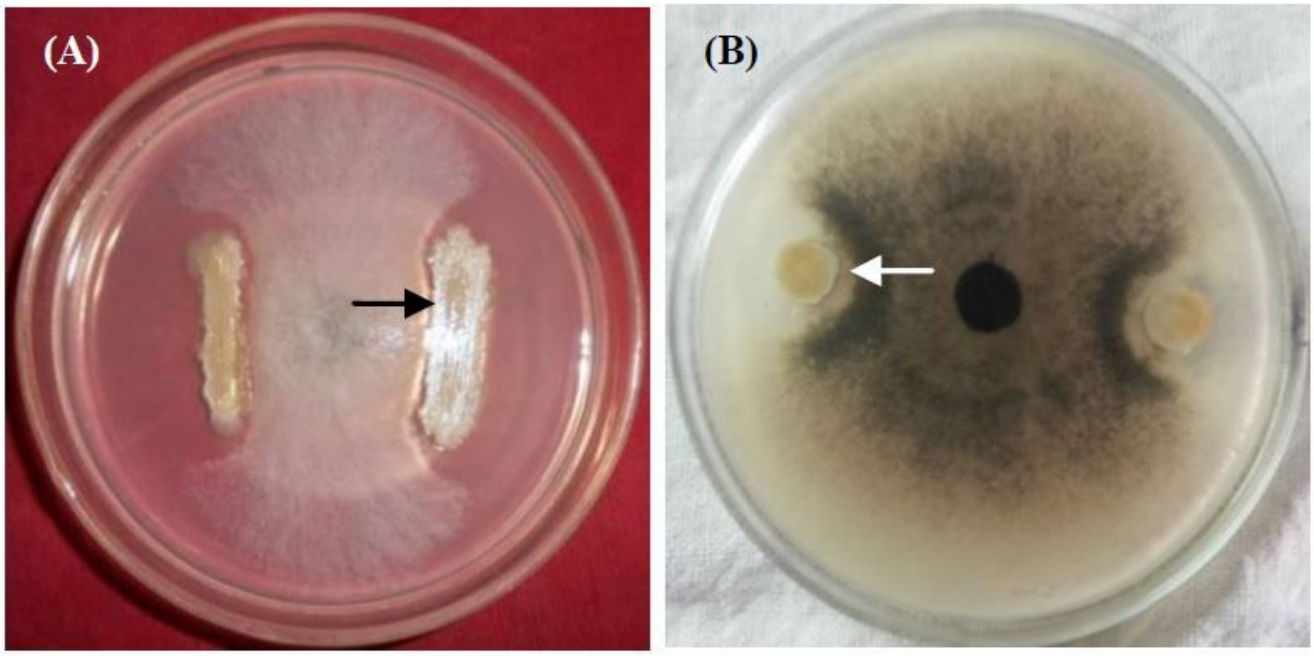

Figure 1

Antagonistic effect of (A) Bacillus sp. BS14 against M. phaseolina in dual culture media (B) purified biosurfactant of Bacillus sp. BS14 against M. phaseolina in fungal culture media

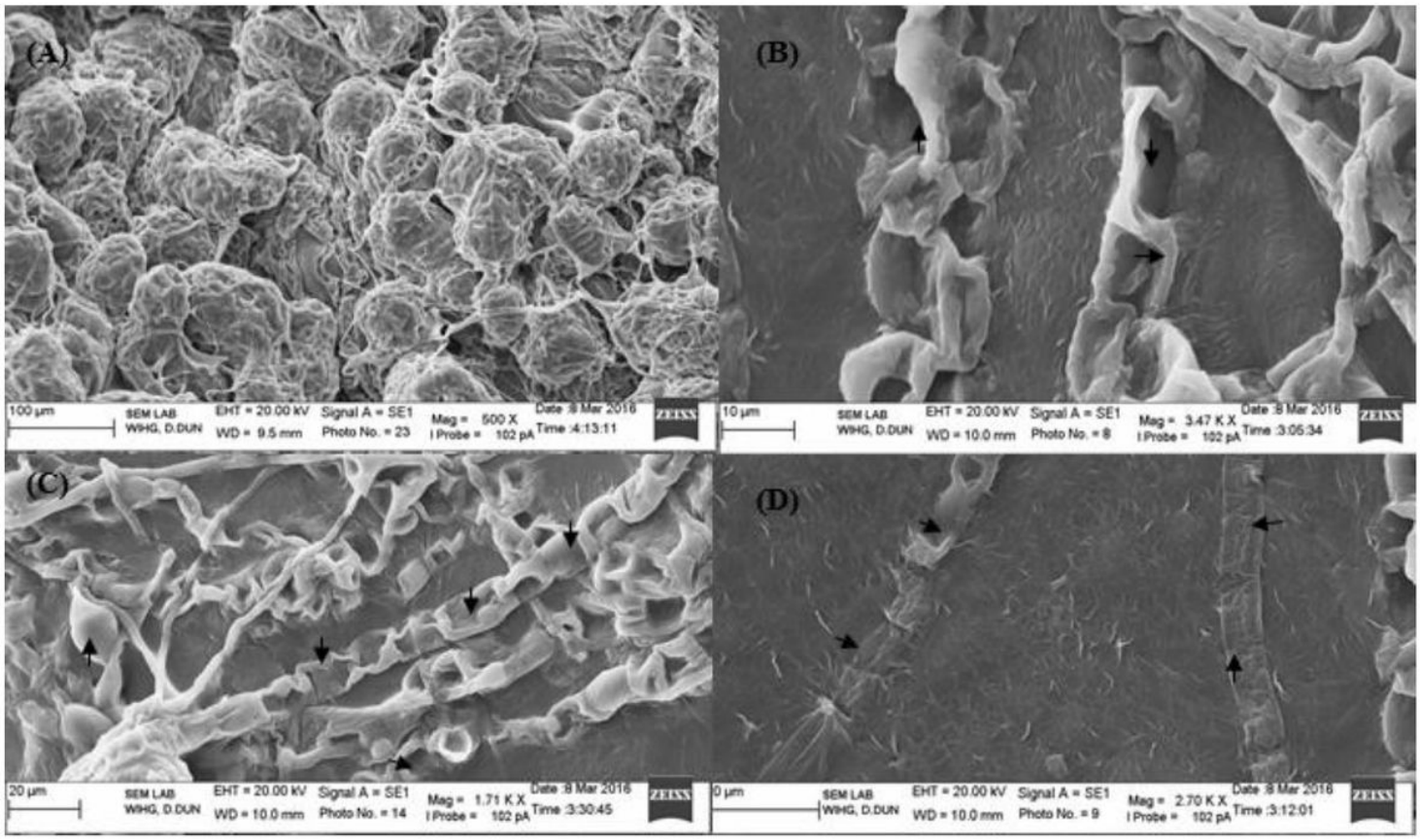

Figure 2

Scanning Electron Microscopy (SEM) showing post-interaction events in the hyphae and sclerotia of M. phaseolina caused by Bacillus sp. BS14; (A) Control; (B) hyphal lysis and cell shrinkage; (C) loss of vigour and mycelia fragmentation; (D) hyphal fragmentation, shrinkage, loss of vigour, and loss of sclerotia integrity

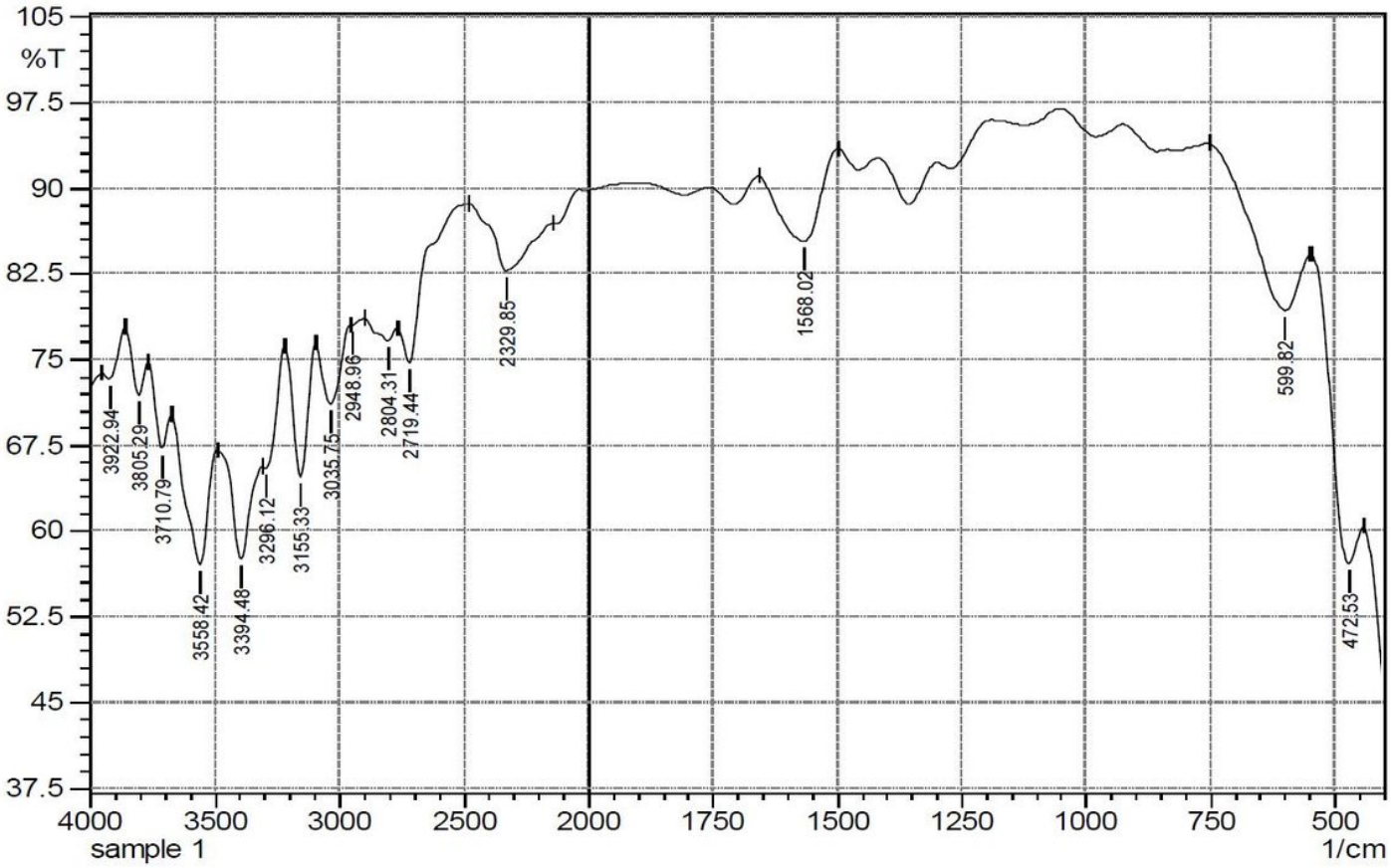

Figure 3

FTIR spectroscopy analysis of biosurfactant produced by Bacillus sp. BS14

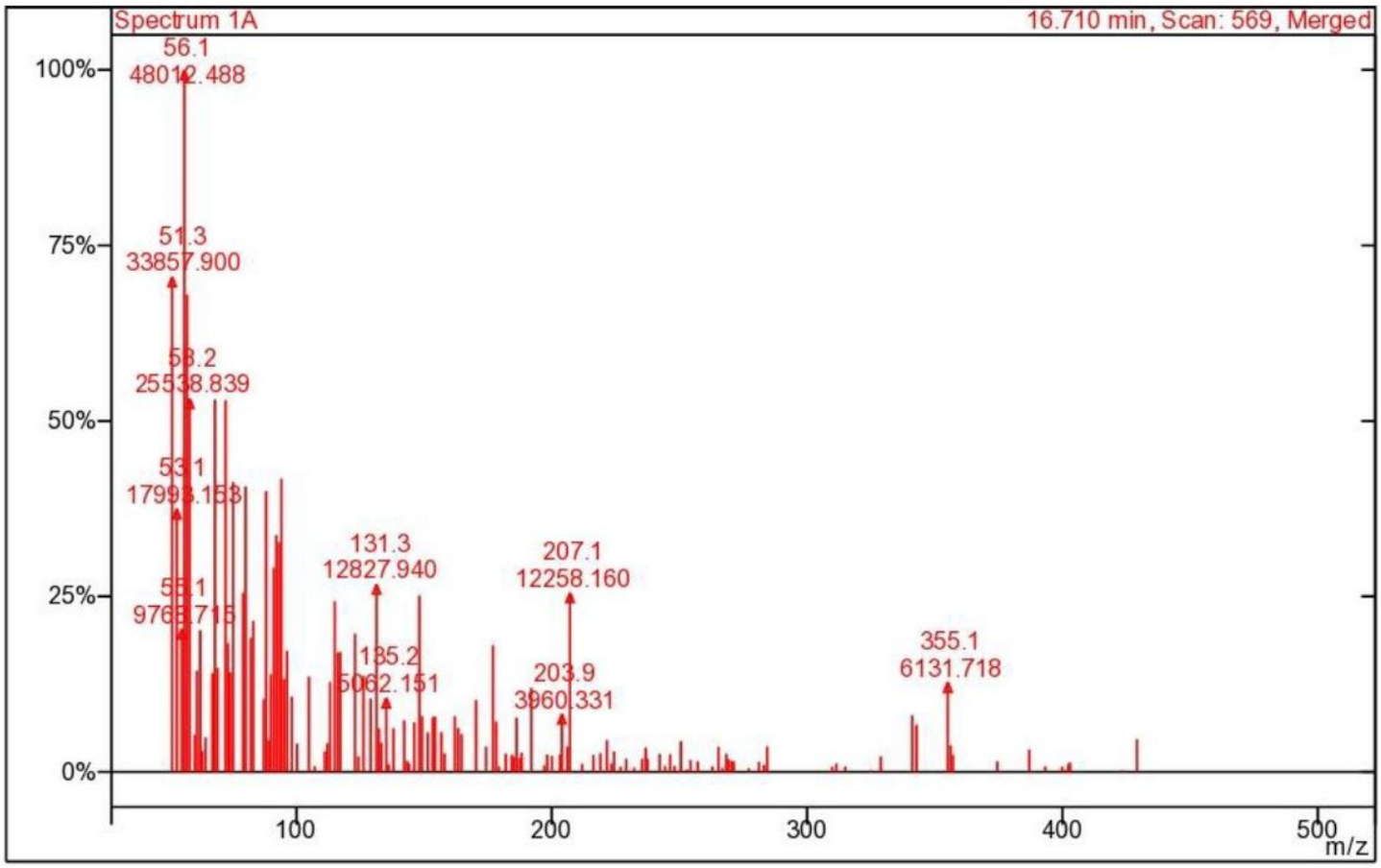

Figure 4

GC-MS spectra of purified biosurfactant produced by Bacillus sp. BS14.