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Biosurfactant-Mediated Biocontrol of *Macrophomina phaseolina* Causing Charcoal Rot in *Vigna mungo* by a Plant Growth Promoting *Enterococcus* sp. BS13

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Abstract

A potential bacterial isolate *Enterococcus* sp. BS13 screened from rhizospheric soil of *Vigna mungo* was identified as *Enterococcus* sp. based on morphological, biochemical and genomic characterization. The biosurfactant producing activity of BS13 was based on many tests such as blood heamolysis test, CTAB agar, emulsification stability (E_{24}) test, oil spreading/displacement assay, drop collapse assay, bacterial adhesion to hydrocarbons (BATH) assay and surface tension (ST) measurement after 72 h of growth. GC-MS and FT-IR analyses established the similarity of biosurfactant with glycolipid type biosurfactant. Furthermore, *Enterococcus* sp. BS13 displayed plant growth-promoting ability, HCN production and antagonistic activity against *Macrophomina phaseolina*. Scanning electron microscopic study of fungal mycelia from zone of inhibition showed hyphal degradation, halo cell formation and mycelial deformities in the pathogen. *M. phaseolina* sclerotia formation and development were arrested towards the zone of interaction; consequently, such mycelia and sclerotia lost the vigour. In pot trials *Enterococcus* sp. BS13 increased the growth of *V. mungo* with considerable diseases reduction. Hence, *Enterococcus* sp. BS13 bears ability of biosurfactant production, plant growth promotion and biocontrol of *M. phaseolina*. Therefore, the exogenous application of BS13 can be a potential strategy to accelerate plant growth promotion and biocontrol of *M. phaseolina*.

Keywords: **Biosurfactant;** *V. mungo;* *Enterococcus* **sp. BS13;** *Macrophomina phaseolina;* **Biocontrol**

Introduction

The naturally occurring plant growth promoting rhizobacteria (PGPR) aggressively colonize plant roots and benefit plants by providing growth nutrients and hormones [1]. They produce many plant growth-promoting metabolites, solubilize insoluble phosphates, and survive under various stresses environmental conditions. Plants cannot always meet their demand of minerals from natural soils [2]. The environmental enterococci are a taxonomically diverse group of bacteria but the plant-associated enterococci represent the lesser-known bacterial group. A little work has been done on enterococci of rhizospheric soil. The enterococci are temporary resident of agricultural plants and grasses [3]. Ulrich and Muller [4] studied the effect of *Enterococcus faecalis* and *Enterococcus faecium* on plants. The application of non-pathogenic soil bacteria as biofertilizers can be environmentally safe for increasing crop productivity and soil fertility owing to their ability to produce auxin, gibberellins and cytokinin [5]. The European Food Safety Authority recognized some of the strains *E. faecium* as feed additives [6]. The ability of *E. faecium* to produce phytohormones has been reported by Lee et al. [7].

A variety of soil microorganisms demonstrated the biocontrol of various soil-borne phytopathogens. But less work on biocontol potential of enterococci has been done. Enterococci produce several compounds that inhibit the growth of many phytopathogens [8,9]. Biocontrol potential of *Enterococcus faecium* against phytopagens was studied by Fhoula et al. [10].

Several enterococci have been screened during the isolation of biosurfactant producing and plant growth promoting rhizobacteria of *V. mungo*. *V. mungo* is a major Indian pulse crop grown throughout India. Annual production of urdbean in India is about 1.3 million tonnes. It has very high nutritious value possibly due to presence of protein (25 mg/100 g), potassium (983 mg/100 g), calcium (138 mg/100 g), iron (7.57 mg/100 g), niacin (1.447 mg/100 g), Thiamine (0.273 mg/100 g), and riboflavin (0.254 mg/100 g) [11]. *M. phaseolina* is most destructive phytopathogens. It infects more than 500 plant species [12]. It caused charcoal rot in *V. mungo* and reduces the crop yield. Therefore, the present work was designed to elucidate biosurfactant production, PGP activity and biocontrol of *M. phaseolina* by *Enterococcus* sp. BS13 as well as purified biosurfactant *in vitro*.

Material and Methods

Sample collection

The young plants of *Vigna mungo* were randomly collected from different locations of a farmer's field in Saharanpur (India), put in to a sterile poly bag and brought to the laboratory for further work.

Isolation and selection of bacteria

Rhizospheric soil (1 g) was suspended in 10 ml of 0.85% (w/v) NaCl (saline) water, vortexed vigorously, serially diluted and plated on nutrient agar medium. The plates were incubated at 37°C for 24 h. Colonies appearing with dissimilar morphology on the surface of growth medium were purified and selected for further tests [13].

Screening for biosurfactant production

Blood heamolytic test: Log phase growing culture of isolated bacteria was streaked on blood agar plates and incubated at 37°C for

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24-48 h. The plates were then observed for the presence of clear zone around the bacterial colonies. The clear zone indicates the presence of biosurfactant producing microorganisms [14].

Oil displacement method: Distilled water (20 ml) was poured in the Petri plates and 1 ml oil was added in the centre of the plates. Then 20 µl of the culture supernatant of the isolates as added to it. The biosurfactant producing organism displaced the oil and spread in the water [15].

CTAB agar plate method: All the bacterial isolates were initially assayed for biosurfactant production using mineral salt cetyl-trimethyl-ammonium-bromide (CTAB) - methylene blue agar plate method (0.2 mg/ml CTAB and 5 µg/ml methylene blue). Isolates were grown for 24 h in mineral salt medium under appropriate culture conditions. Shallow wells were cut on the surface of the indicator plates. Ten microliters of the appropriate culture was placed into each well and incubated at 30 ± 1°C and growth was observed regularly for 24 - 48 h [16].

Drop collapse method: A drops of culture supernatant are placed on an oil coated solid surface. The polar water molecules repelled from the hydrophobic surface and the drops remained static in the absence of biosurfactants in the liquid. If the liquid contains surfactants, the drops spread or even decadence [17].

Emulsification stability (E_{24}) test: Kerosene (2 ml) was added to 2 ml of supernatant culture in a borosil glass test tube (15 mm diameter) and mixed for 2 minute using a vortex mixer. Kerosene emulsion was formed in the test tube which was then covered with para-film and allowed to stand at room temperature for 24 h. The height of the emulsion in the tube was noted after 1 minute of aging and periodically for about 24 h to monitor the stability of the emulsion. E24 (%) was calculated by using the following formula: [18].

$$E_{24}(\%) = \frac{H1}{H2} \times 100$$

Where, H_1= height of emulsified layer; H_2=height of total solution.

Bacterial adhesion to hydrocarbons (BATH) assay: Cell hydrophobicity was measured by bacterial adherence to hydrocarbons (BATH) assay. The cell pellets collected in the above culture medium and bacterial growth section were washed twice and suspended in a buffer salt solution (g l^{-1}, 16.9 K_2HPO_4 and 7.3 KH_2PO_4) and diluted using the same buffer solution to an optical density (OD) of ~ 0.5 at 610 nm. To the cell suspension (2 ml) in test tubes (10 ml volume with 10 × 100 mm dimension) 100 µl of crude oil was added and vortex-shaken for 3 min. After shaking, crude oil and aqueous phases were allowed to separate for 1 h. Then OD of the aqueous phase was measured at 610 nm in a spectrophotometer (UV VIS 1600 Shimadzu, Japan) and bacterial cells attached to crude oil were calculated using the following formula: [19].

Bacterial cell adherence (%) = [1-(OD shaken with oil/OD original)] × 100

Where, OD with oil = OD of the mixture containing cells and crude oil, OD original = OD of the cell suspension in the buffer solution (before mixing with crude oil).

Surface tension measurement: Measurement of surface tension of cell-free culture broth from isolated strain was determined according to Du Nouy ring method. Deduction in surface tension by cell free culture was compared with a standard biosurfactant CTAB solution (1 mg/ml) [20].

Production and purification of biosurfactant: The isolate BS13 was transferred to nutrient rich (NR) broth containing 1% yeast extract, 1.5% nutrient broth and 1% ammonium sulfate. The culture was incubated at 37°C for 12 h and 120 rpm as seed culture to get optical density of 0.5 at 600 nm. Then, 5 ml suspension 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 g at 4°C for 20 minutes and supernatant was acidified with 6N hydrochloric acid to get the pH 2.0, and incubated overnight at 4°C. The precipitated biosurfactant was collected by centrifugation at 15,000 g for 20 min and precipitate was dissolved in distilled water to get pH 7.0 by using 1N NaOH. Further, it was centrifuged at 10,000 g for 10 min following Sánchez et al. [21].

Chemical characterization of biosurfactant

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, Japan) was 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 at wave length of 400–4000 cm^{-1}. FT- IR spectra were analyzed by using OPUS 3.1 (Bruker Optics) software.

Gas chromatography-mass spectroscopy (GC-MS) analysis: GC-MS analysis of biosurfactant was performed 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; temperature was gradually increased from 40°C to 280°C to identify the compound. 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 compound. A comparative study carried out was between the identified compound spectra and that of known compounds of the GC-MS library NIST.

Biofilm formation *in vitro*: Biofilm formation was tested during bacterial growth in borosilicate glass tubes. Sterile Muller Hinton broth (MHB) (5 ml) was poured in the pre-sterile test tubes inoculated separately with BS13 culture along with proper control, and incubated at 37°C for 24 h. Broth was discarded and BS13 biomass attached to the glass surface was observed by staining with crystal violet to confirm biofilm formation [22].

Biodegradation of polycyclic aromatic hydrocarbon (PAH): Hydrocarbon-utilizing bacteria were screened for their ability to utilize PAH as sole source of carbon and energy using Bushnell-Haas fortified with 2% agar medium. In the present study mainly two PAHs such as kerosene and diesel were used for degradation.

Plant growth promotion (PGP) traits of isolate BS13

Phosphate solubilization: Phosphate solubilization ability of isolate was detected by spotting them separately on Pikovskaya's agar plates [23]. A loopful of bacterial culture was spot inoculated on the plate and incubates at 28°C for 7 days. The presence of clear zone around the bacterial colonies indicates the phosphate solubilization.

Indole-3-acetic acid (IAA) production: IAA production by the isolate BS13 was determined following the methods of Gordon and Weber [24]. BS13 was grown at 37°C for 72 h in LB broth and centrifuged at 8, 000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent (50 ml of 35% of perchloric acid plus 1 ml of 0.5 M $FeCl_3$). Appearance of pink colour confirmed the production of IAA.

Siderophore production: Siderophore production by BS13 was tested using chrome azurol S (CAS) agar plate method of Schwyn and Neilands [25]. Overnight culture (10 µl) was spot inoculated on CAS agar plate and incubated 28 ± 1°C for 48–72 h. Orange to yellow halo formation around the bacterial colonies confirmed siderophore production.

Chitinase production: Chitin plates were prepared using M9 agar medium amended with 1% (w/v) colloidal chitin. The plates were divided into equal sectors; spot inoculated with 10 µl of overnight grown BS13 culture and incubated at 37°C for 48–96 h. Zone of clearance around bacterial colonies indicated chitinase production.

HCN production: HCN production was determined following modified method of Bakker and Schippers [26]. For detection of HCN production, exponentially grown culture of BS13 was streaked on agar plates supplemented with or without 4.4 g glycine l^{-1} with simultaneous addition of filter paper soaked in 0.5% picric acid in 1% Na_2CO_3 in the upper lids of plates along with uninoculated control. The plates were incubated at 28 ± 1°C for 72 h along with a control. The changes in colour from yellow to light brown or moderate brown to strong brown was examined for putative HCN production.

16S rRNA gene sequencing of isolate BS13: Pure culture of BS13 was grown in LB broth until log phase achieved. The genomic DNA was extracted according to Bazzicalupo and Fani [27]. PCR amplification of genomic DNA was carried out following Kumar et al. [28]. The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R primer (5′CGGTTACCTTGTTACGACTT 3′) and 27F (5′AGAGTTTGATCMTGGCTCAG 3′) under the conditions described by Brown and Balkwill [29]. The PCR product was sequenced by Aakar Biotech Pvt. Ltd., Biotech Park, Lucknow (U.P.) India. Sequence search in the EMBL/Gen Bank/DDBJ/PDB data libraries was performed using the BLAST (blastn) [30] search algorithm in order to establish the identity of the strain. Sequences of the closest matched reference strains were retrieved and aligned using ClustalW (multiple and pairwise) with the newly determined sequence. Phylogenetic analysis was performed using MEGA version 6 and phylogenetic tree was constructed by neighbour-joining method with 1500 replicates to produce bootstrap value and to validate the reproducibility of the branching pattern of the tree. 16S rRNA gene sequence was submitted to the NCBI GenBank and to get the accession number.

Antagonistic activity against phytopathogenic fungi: Antagonistic activity of the isolate BS13 against *M. phaseolina* was determined by dual culture technique of Skidmore and Dikinson [31]. A fungal disc of 1 cm diameter was placed in the centre of potato dextrose agar plate. Then overnight grown bacterial culture was spot inoculated on the surface of agar plate 2 cm away from the central fungal disc. The plates were incubated at 28°C for 72 h. Growth inhibition was calculated by using the formula: 100 × (C–T)/C (T, treatment; C, control). Petri plates without bacterial spotted culture served as control.

Post interaction events by scanning electron microscopy (SEM): For preparation of SEM samples, the fungal mycelia disc was cut with the help of a sterile cork borer from the zone of interaction. The discs were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed thrice in phosphate buffer (10 min each). Then the samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min each) and re-suspended thrice in 100% ethanol at room temperature. Ethanol was then replaced by liquid CO_2 and the samples were air dried. Then samples were mounted on stubs and coated with gold. These coated specimens were observed at 15 kV in a LEO 485 VP SEM. Photo-micrographs were recorded by the same microscope.

Pot trial

Development of antibiotic-resistant marker in strain BS13: Antibiotic resistant marker strain of BS13 was developed followed by Bhatia et al. [32]. For development of an antibiotic strain marker, *Enterococcus* sp. BS13 was subjected to antibiotic sensitivity test. Antibiotic discs (5 mm diam) of different concentrations were placed at four corners over the surface of *Enterococcus* sp. BS13 seeded plate and incubated at 28 ± 1°C for 24 h to measure the inhibition zone. Resistance marker strains were developed by subjecting the culture successively to low concentration to high concentration of methicillin and fusidic acid.

Preparation of fungal inoculum: Inoculum of *M. phaseolina* was prepared by multiplying the pathogen on potato dextrose broth medium. Fungal culture was filtered after 7 days incubation at 28°C. Filtered mycelial mat and sclerotia were dried at 85°C to evaporate moisture content and crushed to make powder. Fungal powder (500 mg/kg) was mixed in the pre-steriled soil (1 kg) in pots.

Mass production of bacteria and seed bacterization: Antibiotic resistant marker *Enterococcus* sp. BS13 $^{Met+Fus+}$ (100 mg/l) was used for mass production**.** Starter culture of *Enterococcus* sp. BS13$^{Met+Fus+}$ was prepared by inoculating 50 ml LB broth in flask and incubated at 30°C for 24 h at 150 rpm [33]. Then the culture was separately transferred in 1 L flask containing sterilized broth and incubated at 30°C at 150 rpm. The pH of the medium was maintained at 6.8. Culture broth was periodically excluded to check the cfu (colony forming unit) for detection of any contamination. When cell concentration increased to 1×10^8 cells/ml in pure form, it was used for seed bacterisation and pot trials.

Healthy and viable seeds of *V. mungo* were washed in sterile water and surface-sterilized with 95% alcohol for 30 second followed by 0.1% (w/v) $HgCl_2$ for 1 min. Seeds were washed 5-6 times with sterile distilled water. The bacterial culture introduced as above was centrifuged at 8000 g for 15 min at 4°C. The pellets obtained were suspended in sterile distilled water to obtain a population density of 1×10^8 cfu/ml.

Seed bacterization was carried out following the method of Weller and Cool [34]. The cell suspension was mixed with 1% carboxymethyl cellulose (CMC). Slurry was coated separately on the surface of healthy seeds. The seeds coated with 1% CMC slurry without bacteria served as control [32]. The fungal culture in the form of powder was mixed in sterile sandy loam soil (sand 56%, silt 22%, clay 27%, WHC 41.10%, pH 8.29) put in the earthen pots (25 × 25 × 25 cm). Healthy seed of *V. mungo* inoculated with the *Enterococcus* sp. BS13$^{Met+Fus+}$ strain alone and in combination with *M. phaseolina* separately were examined for their ability to enhance the overall growth of plants. In the pot trial, bacterial inoculated and non-inoculated seed were sown in triplicate along with control. Seed germination was recorded after 15 days of the sowing. Vegetative growth parameters were recorded after 30 days and bacterial root colonization was recorded at an interval of 30 days up to 90 day after sowing (DAS).

Statistical analysis

All data of pot trial statically were analyzed by GraphPad Prism 5. Differences among treatments were assessed using one-way analysis of variance (ANOVA).

Results

The isolate BS13 was found most potent biosurfactant producing and PGP activity bearing isolate. On the basis of morphological,

physiological and biochemical characteristics the isolate BS13 was found Gram-positive, coccus, nonspore former, catalase negative, oxidae positive, and producers of white, dry and irregular edged colonies on NAM plates (Table 1). The isolate BS13 was identified as *Enterococcus* sp. based on microscopic and biochemical analysis according to Bergey's manual of determinative bacteriology [35]. Different phenotypic characters of isolate was compared with the standard strains of *Enterococcus faecium* (ATCC 700221) *Enterococcus faecium* (ATCC 51858), *Enterococcus faecium* (ATCC 51559) and *Enterococcus faecium* (ATCC 27270).

Screening of biosurfactant

BS13 was further tested for biosurfactant production employing haemolytic test, oil displacement assay, CTAB agar plate method, drop collapsing, emulsification index, and Surface tension measurement. The isolate BS13 showed β-haemolytic activity and caused the maximum zone of ~1.66 cm. It also showed a zone of displacement in the oil. The flat drop appearance in microtiter plate confirmed the positive result of drop collapse test, proving the use of drop collapse method as a quick and simple method for detection biosurfactant production. A prominent emulsification activity with the emulsification index (E_{24}) of 70.58 was also exhibited by BS13. Production of dark blue halo zone in the CTAB methylene blue agar plate confirmed the presence of anionic biosurfactant. The formation of insoluble ion pair precipitates in the agar plate containing methylene blue exhibited dark blue colour against the light blue background. In BATH assay, the bacterial cells indicated their affinity towards hydrophobic substrate. The isolate caused the maximum reduction in surface tension by 68.974 D/CM.

FTIR analysis

The molecular composition of biosurfactant manifest displayed the most prominent adsorption bands located at 3120 cm^{-1}, 2771 cm^{-1} and 3001 cm^{-1} (C–H stretching bands of CH_2 and CH_3 groups), 1735 cm^{-1} (C = O stretching vibrations of the carbonyl groups), 1278 cm^{-1} (C–O stretching bands; formed between carbon atoms and hydroxyl groups in the chemical structures) and 794 cm^{-1} (CH_2 group) (Figure 1).

GC-MS analysis

The fatty acid composition of biosurfactant was analyzed by GC-MS and compared with the library data. The biosurfactant comprised of long chain fatty acids, mainly C-10 long fatty acids. The major fatty acid of biosurfactant was C-10 decanoic acid. On the basis of FTIR and gas chromatography the structure of biosurfactant produced by *Enterococcus* sp. BS13 was predicted as glycolipid type (Figure 2).

Biosurfactant properties		PGP Properties	
CTAB	++	Siderophore	+
Haemolysis	++	HCN	+
BATH Assay	++	IAA	++
Emulsification assay	+	Chitinase production	+
Drop collapse test	+	Phosphate solubilization	++
Surface Tension measure	+++	Biofilm assay	+
Oil	+	Antagonistic activity against *M. phaseolina*	+++

Note: -: Absence of halo formation or no activity; +: small halo < 0.5 cm, biofilm forming HCN, hydrogen cyanide; Chitinase producing; Chitinase producing; ++: medium halo formation > 0.5 cm wide surrounding colonies; medium IAA production; +++, large holo > 1.0 cm wide surrounding colonies, high surface tension reducing, +++: 51% to 75% inhibition against *Macrophomina phaseolina*.

Table 1: Biosurfactant screening test and plant growth promoting attributes of *Enterococcus* sp. BS13.

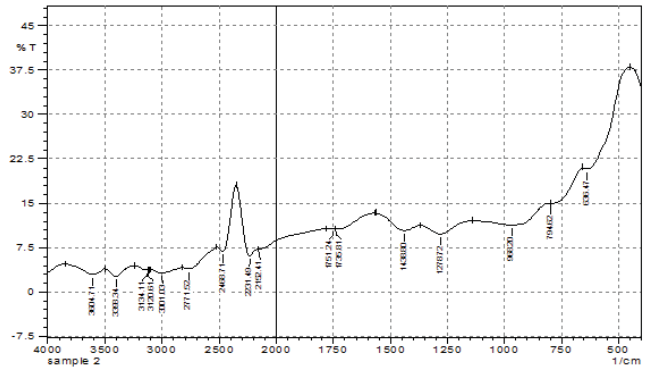

Figure 1: FTIR spectroscopy analysis of biosurfactant produced by *Enterococcus* sp. BS13.

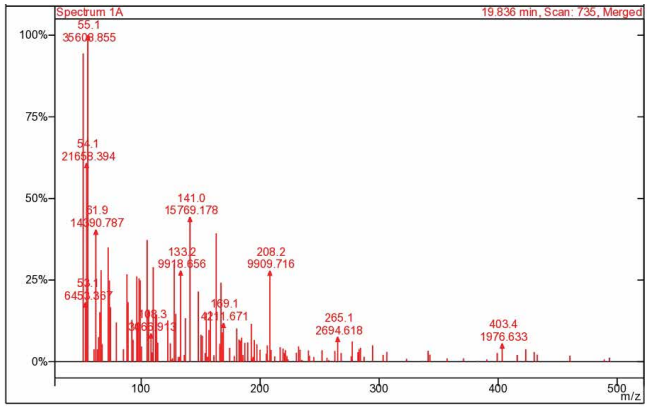

Figure 2: GC-MS spectra of purified biosurfactant produced by *Enterococcus* sp. BS13.

Biofilm forming ability of BS13: BS13 formed biofilm in the test tube assay. Bacterial cells adhered on the internal surface of test tubes which eluded the biofilm production.

Biodegradation of polycyclic aromatic hydrocarbon (PAH): The isolate grew in the Bushnell Haas agar medium containing kerosene and diesel used as carbon source; it developed clear zone in Petri plate showing it's PAH degradation activity.

Plant growth promoting traits of BS13: The isolate BS13 showed IAA production. Similarly BS13 showed marked phosphate solubilization as visualized by a clear zone around the colony after 48 h. The zone of hydrolysis gradually increased with the increase the incubation time reaching to 3.4 mm after 7 days. Change in the color of the chrome-azurol S medium from blue to orange-red confirmed the production of siderophore. The isolate BS13 changed colour of filter paper from yellow to reddish-brown after 2-3 days of inoculation confirming HCN production. BS13 isolate produced a wider zone of chitin hydrolysis in chitin minimal medium plate, indicating the chitinase production.

16S rRNA gene sequencing and phylogenetic analysis: The 16S rRNA gene sequence of the BS13 comprised of 1431 bp (NCBI Gen-Bank accession No. KU99196). It showed 94% sequence similarity with *Enterococcus faecium* L3 (accession No. KJ728981) and *Enterococcus faecium* KCL1 (Accession No. KM497512). The generated 16S rRNA sequences were analyzed with Basic Local Alignment Search Tool (BLAST) available on NCBI website. A multiple sequence was produced followed by a guide-tree generation, phylogram and cladogram

formation [36]. Therefore, the isolate BS13 has further been referred to as *Enterococcus* sp. BS13 (Figure 3).

***In vitro* antagonistic activity of BS13 and pure biosurfactant:** The pure culture of *Enterococcus* sp. BS13 and its biosurfactant inhibited the radial growth of *M. phaseolina* by 72.3% and 53.2%, respectively after 7 day of incubation at 28 ± 1°C (Figures 4A and 4B). However, fungal growth inhibition was more pronounced in dual culture as compared to that of pure biosurfactant. Inhibition in fungal growth corresponded with incubation time.

Post-interaction events in mycelia of *M. phaseolina*: *Enterococcus* sp. BS13 in the zone of interaction resulted in halo cell formation, mycelial deformities and hyphal degradation of *M. phaseolina*. Formation and development of sclerotia of the pathogen were also arrested towards the zone of interaction; consequently such mycelia and sclerotia lost their vigour (Figures 5A-5C).

Pot trials: *Enterococcus* sp. BS13$^{Met+Fus+}$ inoculated seeds showed significantly ($P<0.01$) increased seed germination, plant length and biomass over the control (Table 2). *Enterococcus* sp. BS13$^{Met+Fus+}$ also resulted in reduction of disease caused by *M. phaseolina* after 30, 60 and 90 DAS (66.6%, 37.5%, and 42.5%) (Figure 6). *Enterococcus* sp. BS13$^{Met+Fus+}$strain showed effective root colonization as evidenced by the prominent population recovery from *V. mungo* rhizosphere 90 DAS (Table 3). The strain successfully colonized *V. mungo* roots, alone and in combination with *M. phaseolina*, and increased its population in rhizosphere.

Discussion

The isolate *Enterococcus* sp. BS13 was found more potential to secrete biosurfactant and promote plant growth. The biosurfactant had similarity with glycolipid type surfactant. Sharma et al. [37]

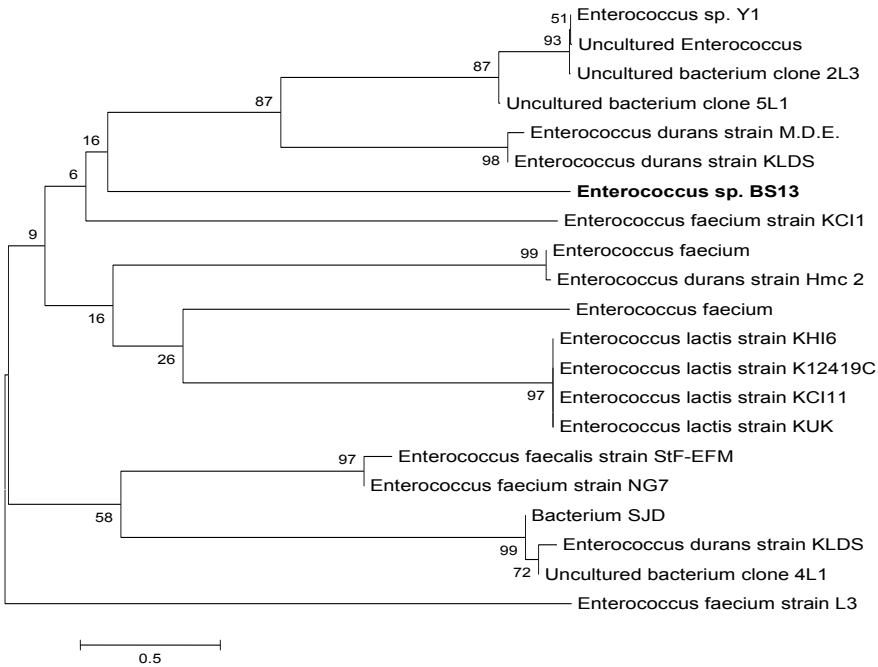

Figure 3: Phylogenetic analysis of rhizobacteria *Enterococcus* sp. BS13 (KU99196) and presenting species based on16S rDNA sequences using cluster algorithm. Distance and clustering with neighbor-joining method was performed by using the MEGA 6.06 software.

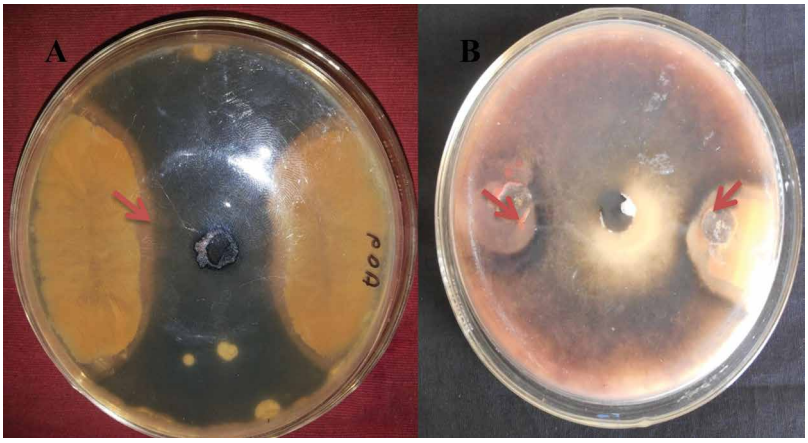

Figure 4: Antagonistic effect of *Enterococcus* sp. BS13 against *M. phaseolina* in dual culture (A); effect of pure biosurfactant on fungal growth (B).

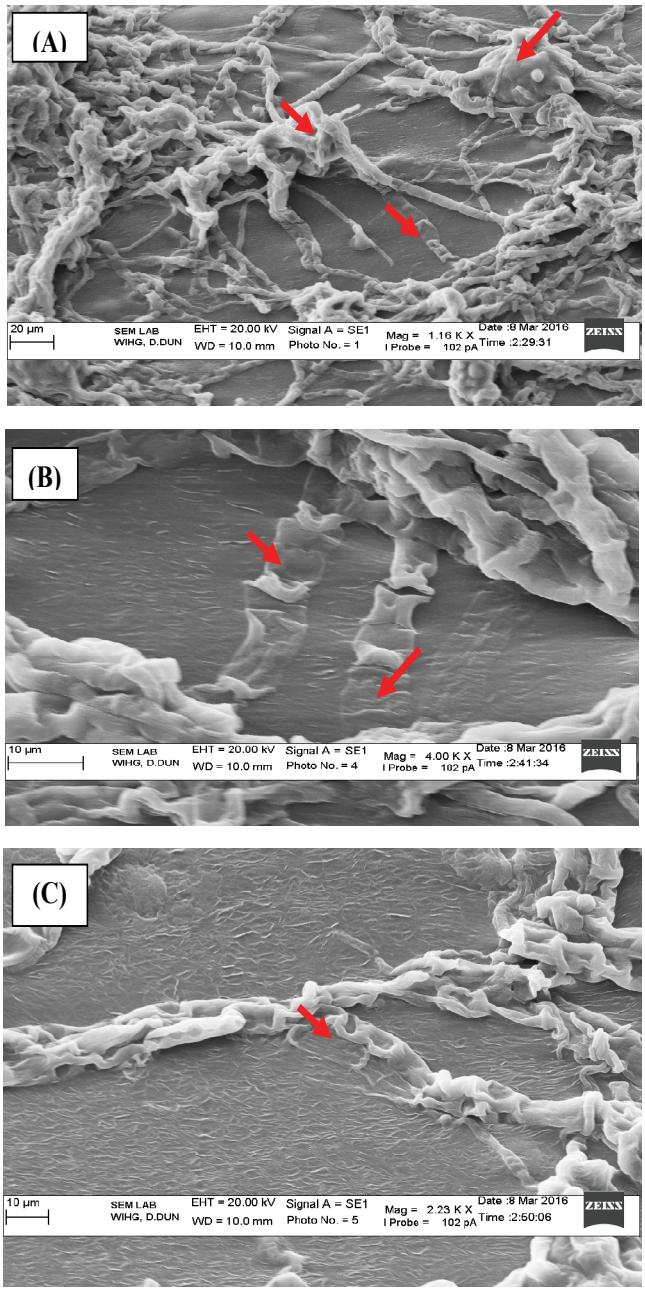

Figure 5: Scanning electron micrograph showing post-interaction events in the hyphae and sclerotia of *M. phaseolina* caused by *Enterococcus* sp. BS13; A- shrinkage, loss of sclerotia integrity (see arrows), B- hyphal lysis and fragmentation cell (see arrows); C- cell shrinkage and loss of viability (see arrows);

have also reported a novel glycolipid biosurfactant producing *Enterococcus faecium*. Besides, the biosurfactant also displayed *in vitro* antifungal activity against *M. phaseolina*. Presence of antifungal activity of glycolipid against phytopathogenic fungi has also been reported by Teichmann et al. [38] and Kulakovskaya et al. [39]. Biofilms are comprehensive colonies of single or multispecies of microbial cells adhered to surfaces or communicate contact with each other, encased in a self-produced matrix of extracellular polymeric substances (EPS). The isolate *Enterococcus* sp. BS13 formed biofilm and produced biosurfactant. The importance of biofilm formation in PGP rhizobacterial action has been studied by Seneviratne et al. [40]. In the present investigation *Enterococcus* sp. BS13 was found to degraded hydrocarbon. In agricultural soil increased contamination by different hydrocarbons is a serious environmental problem due to their persistence in nature for a long time. The use of biosurfactants is an alternative over the chemical surfactant as the former is a better biodegradable and ecofriendly [41]. Biodegradation of hydrocarbon has also been studied by Zhang et al. [42].

Auxins called indol acetic-acid (IAA) are plant hormones which originate from an amino acid tryptophan. IAA production is widespread among plant associated bacteria. In the present study the *Enterococcus* sp. BS13 was found to produce IAA *in vitro*. IAA production by *Enterococcus* sp. has also been reported by Lee et al. [7]. Phosphorus plays a major role in many biological processes such as cell division, photosynthesis, sugar break down, energy and transfer of nutrient in crop plant. *Enterococcus* sp. BS13 efficiently solubilized phosphate *in vitro*. Ghosh et al. [43] also isolated phosphate solubilizing bacteria from the rhizosphere soil of seagrass. Iron is a necessary nutrient for all living organisms. In the soil it is directly assimilated by microorganisms because ferric iron (Fe III) which predominates in nature is only thriftily soluble and in very low concentration to support microbial growth. *Enterococcus* sp. BS13 was found siderophore producing in CAS medium. Similar study on siderophore production by *Enterococcus faecium* LKE12 has been performed by Lee et al. [7]. *Enterococcus* sp. BS13 showed the HCN production. HCN is a major chemical weapon to depress the growth of phytopathogen. HCN indirectly plays a major role in plant growth promotion by biological control of phytopathogen [44]. Similarly, HCN production has also been carried out by Gupta et al. [45] and Bhatia et al. [46]. Chitin is one of the most natural renewable polysaccharides present in cell wall of fungi, algae, insects and marine invertebrates. *Enterococcus* sp. BS13 showed the most significant chitinolytic activity in chitin minimal medium. Chitin is hydrolysed by chitinase enzyme [47]. Vaaje-Kolstad et al. [48] have also chitinase enzyme production by *Enterococcus* sp.

Several pesticides and herbicide are being used to control of phytopathogens in routine agriculture by farmers. These are not biodegradable and persist for long duration in the soil. Hence, biocontrol is the most vital alternative of this problem to maintain agricultural sustainability. In the present study, *Enterococcus* sp. BS13 displayed strong antagonistic properties and proved as a good biocontrol agent against *M. phaseolina*. Similar work on biocontrol efficiency of *Enterococcus faecium* has been carried out by Fhoula et al. [10].

Bacterial broth culture of *Enterococcus* sp. BS13$^{Met+Fus+}$ applied as seed treatments the most effective method for plant growth promotion of *V. mungo* and biocontrol of charcoal rot disease. *Enterococcus* spp. has earlier been reported for the biological control of different fungi including species of *Aspergillus niger, Penicillium expansum, Botrytis cinerea*, *Vercticillium dahliae* [10]. Plant growth potential of *E. faecium* for enhancing the growth of rice plant has been reported by Lee et al. [7], which significantly enhanced the length and biomass of rice shoots in both normal and dwarf cultivars.

Conclusion

Based on our findings it may be concluded that *Enterococcus* sp. BS13 produces glycolipid type biosurfactant and bear PGP and biocontrol properties which may be exploited to develop an effective commercial biocontrol agent in future.

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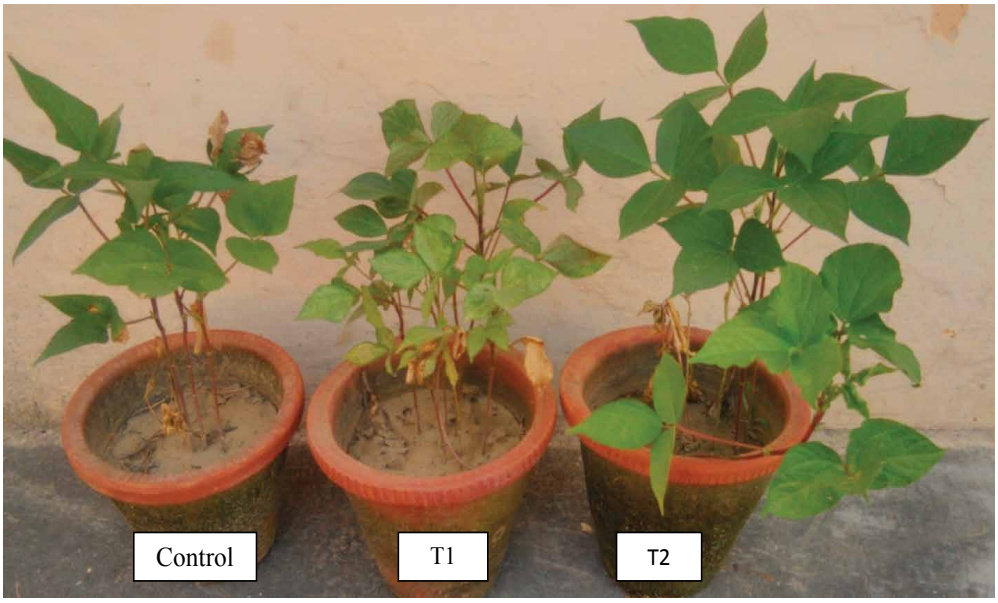

Figure 6: Effect of *Enterococcus* sp. BS13$^{Met+Fus+}$ growth of *V.mungo* in Pot experiment. T1, *Enterococcus* sp. BS13$^{Met+Fus+}$ +*M. phaseolina*; T2, *Enterococcus* sp. BS13$^{Met+Fus+}$.

Treatment	Germination	Shoot length	Root length	Shoot Weight		Root Weight	
	(%)	(cm)	(cm)	Fresh	Dry	Fresh	Dry
Enterococcus sp. BS13$^{Met+Fus}$	80.0	48.1**	17.7**	5.433**	0.912**	1.857**	0.087**
Enterococcus sp.BS13$^{Met+us+}$+ *M. phaseolina*	76.66	45.2**	16.5**	5.109**	0.889**	1.819**	0.085**
Control	63.33	38.9	13.9	3.998	0.793	1.173	0.075
*Values are the mean of triplicates; ns-non significant; ** Significant at 1% LSD as compared to control.							

Table 2: Effect of *Enterococcus* sp. BS13$^{Met+Fus+}$ on the growth of *V. mungo* under pot assays after 90 days*.

Treatment	Bacterial population (log10 cfu)*		
	30 days	60 days	90 days
Enterococcus sp. BS13$^{Met+Fus+}$	6.38	7.19	7.96
Enterococcus sp. BS13$^{Met+Fus+}$+ *M. phaseolina*	7.20	7.50	7.52
Values are mean of three replicates.			

Table 3: Root colonization of *V. mungo* by *Enterococcus* sp. strain BS13$^{Met+Fus+}$ at 30, 60 and 90 days*.

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