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Research Article

Biocontrol potential and molecular characterization of lipopeptides producing *Bacillus subtilis* against *Sclerotinia sclerotiorum*

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ABSTRACT: *Bacillus subtilis* is a Gram-positive and endospore producing bacterium. Limited studies have shown that lipopeptides produced by *B. subtilis* can be inhibitory to phytopathogens. *Sclerotinia sclerotiorum* is a plant pathogenic fungus which causes various diseases like cotton rot, watery soft rot, stem rot, crown rot and blossom blight in vegetable crops. The objective of the study was to isolate lipopeptides from *B. subtilis* and study their inhibitory potential against *S. sclerotiorum*. So, the *B. subtilis* isolates were extracted from the collected soils of Western Ghats of India. They were initially characterized through morphological parameters followed by PCR amplification of the 16S rDNA gene and confirmation through BLAST algorithm in NCBI database. The lipopeptides produced by these isolates were tested against *S. sclerotiorum*. *B. subtilis* strains were effective against *S. sclerotiorum* and exhibited 18.33 to 29.5 % inhibition under dual culture bio-assay. The antagonistic activity of lipopeptides extracted from *B. subtilis* strains showed 21.56 to 88.89 % inhibition of *S. sclerotiorum* in the lowest to highest concentration of lipopeptide tested and was found to be significantly higher than the control. The present study has shown that *B. subtilis* strains vary in the production of lipopeptides and some of them could produce lipopeptides that are highly inhibitory to *S. sclerotiorum*. *B. subtilis* strain NBAIR BSWG1 showed the highest inhibition for *S. sclerotiorum*. Lipopeptide based poison food technique and the dual culture bioassay results showed that *B. subtilis* strain NBAIR BSWG1 has immense potential for use in the biological control of *S. sclerotiorum*. Further studies are being carried out in formulating the lipopeptides for field application.

KEYWORDS: Antimicrobial property, biopesticide, PCR, soft rot of vegetable, Western Ghats

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INTRODUCTION

Bacillus subtilis is an aerobic, endospore forming, rod-shaped, Gram-positive soil bacterium. It is a remarkably versatile bacterium and is capable of thriving under different environmental conditions (Kumbar *et al*., 2017). *B. subtilis* strains are easily formulated and stored due to their ability to produce endospores that are resistant to dynamic physical and chemical treatments, such as heat, desiccation, organic solvents and UV radiation and hence bioformulations of *B. subtilis* can have a long shelf life (Gao *et al*., 2016).

The Western Ghats of India are biologically rich and unexplored for flora and fauna (Kumbar *et al*., 2017). Western Ghats is considered a biodiversity hotspot, hence novel bacterial isolates, isolated from the soil of Western Ghats of India, can have high potential for use as biological control agents due to its natural ecosystem and rich nutrient. Hence, we collected soil samples from the Western Ghats of India for the isolation of *B. subtilis*.

Bacillus subtilis as a biocontrol agent plays a fundamental role in the field of biopesticides. Numerous strains of *B. subtilis* have shown effectiveness against a variety of plant diseases. Some of the antimicrobial compounds isolated from *B. subtilis* have been employed as biocontrol agents. Among the thousands of microbial derived antimicrobial compounds, lipopeptides are one such emerging class of molecules that are produced by rhizosphere colonizing *B. subtilis* (Biniarz *et al*., 2017).

Lipopeptides are commonly produced by plant-associated bacteria, especially by the *Bacillus* group. Few studies have shown that these lipopeptides can suppress

plant diseases (Li *et al*., 2012; Sicuia *et al*., 2008). Based on structural relationships, the lipopeptides identified in *Bacillus* spp. are classified into three different groups: the surfactin group, fengycin group and iturin group. These molecules are important for the functioning and survival of lipopeptide producing bacteria. Lipopeptides are biologically surface-active agents produced by various microorganisms. In the biocontrol activity, they are mentioned to interfere with microbial quorum sensing, cell motility and biofilm formation (Sicuia *et al*., 2008; Penha *et al*., 2020). Hence in the present study, we isolated and characterized the lipopeptides from *B. subtilis* to demonstrate their biocontrol potential against the important plant pathogen *S. sclerotiorum* which is known to cause cottony rot, watery soft rot, stem rot, crown rot and blossom blight in vegetables.

MATERIALS AND METHODS

Collection of soil samples

The soil samples for isolation of *B. subtilis* were collected from different regions of the Western Ghats of India, details of the soil samples collected are presented in Table 1. The soil samples were collected randomly from a depth of 0-15 cm in each location and placed in sterile polythene bags. The samples were brought to the laboratory within 24 h and stored at -4 °C (BPL frost) in the refrigerator (Gautham *et al*., 2012). We extracted the lipopeptides from *B. subtilis* strains as per procedures of Kaur *et al*. (2017) and Jiang *et al*. (2016) with slight modifications to get a three-layer column comprising of upper solvent layer, middle peptide/lipopeptide layer and lower aqueous layer.

Isolation and purification *B. subtilis* from soil samples

For isolation, 10 grams of soil sample was suspended in 100 mL of double distilled water (stock). Two mL of this stock suspension was taken in an Eppendorf tube mixed well and incubated at 80 °C in a heating block for 10 minutes to select spore-forming bacteria and to eliminate most of the vegetative cells of Gram-positive and Gram-negative bacteria. After cooling, the suspension was serially diluted and suitable dilutions were plated onto Luria Bertani Agar (LB agar) by spread plate method, distinct colonies resembling Bacilli were selected and streaked onto LB agar plates using an inoculation loop and incubated at 30 °C in BOD incubator (Scientek India Private Limited) (Zaccardelli *et al*., 2020). Gram-positive and spore-forming colonies were purified by re-streaking every single colony on LB agar. The plates were incubated at 30 °C for 1 day and then kept at -4 °C (BPL frost) refrigerator for further studies. All isolates were stored on slants at -4 °C in the refrigerator and also in glycerol stocks (50 % v/v) at −80 °C in a biofreezer (Model Thermo Fischer).

Molecular characterization by 16S rRNA sequence analysis

DNA was isolated from *Bacillus* isolates using Qiagen kit as per manufacturer protocol and 16S rRNA was amplified in PCR using universal primers (FD2 and RP1). PCR products were separated on 1.5% agarose gel for the confirmation of amplification of the 16S rRNA gene. Amplified products were purified using Qiagen purification kit as per manufacturer protocol and purified samples were sequenced by Sanger sequencing method. The obtained sequences were analyzed using the BLAST algorithm in the NCBI database for species identification. Further 16S rRNA sequences were submitted to the NCBI database.

Extraction of lipopeptides produced by *B. subtilis* isolates

A starter culture of *B. subtilis* isolates was prepared by inoculating a loopful of pure culture of *B. subtilis* from the Petri plate into 100 mL of LB broth medium and kept in an incubator cum shaker (Orbitek) overnight at 37 °C and 150 rpm for further growth.

The main culture for extracting lipopeptides was prepared from the starter culture by inoculating 15 mL of each strain into 1.5 litres of LB Broth in two litres Erlenmeyer flasks (added glycerol 15 mL/1.5 L) and kept in an incubator cum shaker (Orbitek) for 4 days at 37 °C with 150 rpm for further growth.

The crude lipopeptide from the culture broth was extracted by solvent extraction followed by acid precipitation. The culture broth was centrifuged in a refrigerated centrifuge (Kubota, model 6500) at 7500 rpm for 20 min to separate the cell mass and the supernatant. The supernatant was acidified by adding 2 N concentrated HCl and allowed overnight to precipitate low molecular weight proteins (Jiang *et al*., 2016).

The acid-precipitated supernatant was mixed with an equal proportion of chloroform and methanol (2:1 ratio) and stirred in a magnetic stirrer for 15 min. The mixture was added into a separating funnel and left for four hours for the separation of layers. The mixture was separated into three distinct layers viz, top solvent layer, middle lipopeptide layer and lower aqueous layer. The lipopeptide layer which was white in colour and semi-liquid was collected. The upper and lower layer were again re-extracted thrice in the same way for the collection of remaining lipopeptides. The extracts were pooled into a sterilized tube (Kaur *et al*., 2017).

Purification and quantification of lipopeptides

The crude extract of lipopeptides was obtained after repeated solvent extraction from the culture broth from five different *B. subtilis* isolates were pooled in separate

falcon tubes and quantity was measured in volume per liter of culture broth. The crude extracts were dissolved in an equal quantity of methanol, syringe filtered and stored in a deep freezer at -20 °C for further use. The methanol was evaporated using a vacuum rotary evaporator at 50 °C to concentrate the lipopeptides and the resulting sample was subsequently dissolved in the required volume of milli Q water for quantitative analysis (Ma *et al*., 2016).

Bio-efficacy of *B. subtilis* strains by dual culture assay

A dual-culture test was conducted to examine the growth of *S. sclerotiorum* (obtained from the Department of Plant Pathology, University of Agricultural Sciences, Bengaluru) against five different isolates of *B. subtilis***.** A mycelial disk of 0.5 cm in diameter of a pure culture of *S. sclerotiorum* was placed on one side of a Potato Dextrose Agar (PDA) plate and the test *B. subtilis* strains NBAIR BSWG1, NBAIR BSWG2, NBAIR BSWG3, NBAIR BSWG4 and NBAIR BSWG5 were streaked perpendicular to mycelium disk. The plates were incubated for five days at 28 °C, monitored for the formation of an inhibition zone. Three replications were maintained. The width of the inhibition zones was measured by using the formula $(R_1-R_2)/R_1$, where R_1 is radial growth of control, R_2 is radial growth of treatment (Jeyaseelan *et al*., 2012) and statistical analysis was done using ANOVA in Opistat software (Yu *et al*., 2011).

Poison food technique

Antimicrobial activity of lipopeptides isolated from *B. subtilis* strain NBAIR BSWG1 was tested against *S. sclerotiorum* using Poison food technique. Syringe filtered lipopeptides extract from *B. subtilis* strains NBAIR BSWG1 were spread onto PDA in aseptic conditions at 12.5 µl, 25 µl, 37.5 µl, 50 µl, 62.50 µl, 75.0 µl, 87.50 µl and 100 µl per ml of media. After solidification, agar plugs of *S. sclerotiorum* were placed at the center of the plate and incubated at 27± 2 °C. PDA plates without lipopeptides were maintained as control. Observations on radial mycelial growth of *S. sclerotiorum* were measured by using formula $(R_1-R_2)/R_1$, where R_1 is radial growth of control, R_2 is radial growth of treatment (Jeyaseelan *et al*., 2012) and per cent inhibition was calculated and statistically analyzed (Leelasuphakul *et al*., 2008).

RESULTS

Isolation and purification of *B. subtilis* isolated from Western Ghats of India

A total of 25 soil samples were collected from different regions of the Western Ghats of India. Based on morphological, Gram stain and microscopic observations, *Bacillus* spp. were identified from 15 soil samples. Colonies showed rough, non-glassy surfaces with flat margins and creamish white in colour. The isolates were short rods, positive for Gram's reaction with endospore forming ability when viewed in a microscope. The isolates were initially assigned as NBAIR BSWG1, NBAIR BSWG2 etc. indicating Western Ghats *B. subtilis* isolates.

Molecular characterization of *B. subtilis* isolates by 16S rRNA analysis

The purified Gram-positive bacteria were identified by 16S rRNA sequence analysis. Amplified products were resolved at 1400 bp (Figure 1). Blast analysis indicated that ten belonged to *B. subtilis* and the remaining five could be designated only as *Bacillus* spp. The NCBI accession numbers were depicted in Table 1.

Extraction and quantification of lipopeptides

The lipopeptides produced by different *B. subtilis* isolates were extracted and quantified. The results indicated that the

Table 1. Details of *B. subtilis* isolates with NCBI accession number

Sl. No.	Name of *B. subtilis* isolate	Locations	NCBI Accession No.
1	*B. subtilis* NBAIR BSWG1	WG_Melkoppa 1	OP318064
2	*B. subtilis* NBAIR BSWG2	WG_Meega 1	OP326110
3	*B. subtilis* NBAIR BSWG3	WG_V pura 1	OP351364
4	*B. subtilis* NBAIR BSWG4	WG_Meega 2	OP363347
5	*B. subtilis* NBAIR BSWG5	WG_Belandur 1	OP363348
6	*Bacillus* sp. NBAIR BSWG6	WG_Asamballi	OP326225
7	*Bacillus* sp. NBAIR BSWG10	WG_V pura 2	OP800101
8	*B. subtilis* NBAIR BSWG11	WG_Asardalli 1	OP800105
9	*B. subtilis* NBAIR BSWG16	WG_Honnavalli 1	OP800113
10	*Bacillus* sp. NBAIR BSWG13	WG_Nalbur 1	OP800107
11	*Bacillus* sp. NBAIR BSWG14	WG_Meega 4	OP800108
12	*B. subtilis* NBAIR BSWG15	WG_V pura 3	OP800112
13	*Bacillus* sp. NBAIR BSWG17	WG_Melkoppa 3	OP800115
14	*B. subtilis* NBAIR BSWG18	WG_Meega 3	OP800116
15	*B. subtilis* NBAIR BSWG19	WG_Melkoppa 2	OP800117

Table 2.Quantification of lipopeptides produced by five *B. subtilis* isolates

***Bacillus subtilis* isolates**	**Quantity of lipopetides mL/L of culture media**
NBAIR BSWG1	29.50(44.84)[a]
NBAIR BSWG2	24.10(37.78)[b]
NBAIR BSWG3	21.46(34.70)[bc]
NBAIR BSWG4	22.00(35.86)[bc]
NBAIR BSWG5	19.26(34.12)[c]
C.D.	1.261

Values in parentheses are arcsine-transformed values. Means in a column followed by same letters are not significantly different according to DMRT at 5% level.

Table 3. Antagonistic activity of *B. subtilis* isolates against *S. sclerotiorum* using dual culture inhibition assay

Treatment	***S. sclerotiorum* at 5 DAI***	
	****Average radial growth of mycelial (cm)**	**Per cent inhibition**
NBAIR BSWG1	4.23[c]	29.50
NBAIR BSWG2	4.46[d]	25.67
NBAIR BSWG3	4.67[c]	22.17
NBAIR BSWG4	4.80[bc]	20.00
NBAIR BSWG5	4.90[b]	18.33
Control	6.00[a]	0.00
CD (0.05)	0.16	-

*DAI - Days after inoculation. ** Average radial growth of three replications. Values in the same column followed by same letter do not differ significantly and different letter indicates significant difference accordingly to Duncan's Multiple Range Test ($P \leq 0.05$).

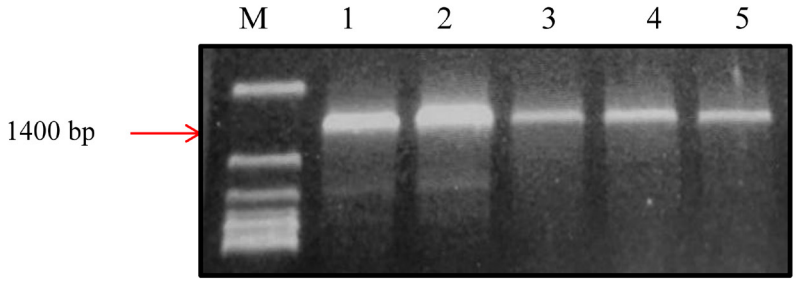

Figure 1: Gel electrophoresis pictures showing amplification of 16S rRNA for five *B. subtilis* isolates M: 1kb ladder, 1: NBAIR NBAIR BSWG1, 2: NBAIR BSWG2, 3: NBAIR BSWG3, 4. NBAIR BSWG4, 5: NBAIR BSWG5

highest quantity of lipopeptides was produced by NBAIR BSWG1 (29.50 mL/L), followed by NBAIR BSWG2 (24.10 mL/L), NBAIR BSWG3 (21.46 mL/L), NBAIR BSWG4 (22.00 mL/L), and the lowest was observed with NBAIR BSWG5 (19.26 mL/L) (Table 2).

In vitro bioassay of *B. subtilis* isolates against *S. sclerotiorum*

Bio-assay of the five selected *B. subtilis* against *S. sclerotiorum* was studied using the dual culture assay (Li *et al.,* 2012). Data recorded are mentioned in the Table 3. Results indicated that NBAIR BSWG1 showed maximum inhibition of 29.5 % followed by NBAIR BSWG2 (25.67 %), NBAIR BSWG3 (22.17 %), NBAIR BSWG4 (20.00 %) and NBAIR BSWG5 (18.33 %) (Figure 2).

5. Poison food technique

The antimicrobial activity of lipopeptides isolated from five different strains of *B. subtilis* isolates was tested against *S. sclerotiorum* using the poison food technique (Table 4, Figure 3). Highest percent inhibition of 88.89 % was obtained with a lipopeptide concentration of 100 µl/ml that was purified from NBAIR BSWG1. The lowest of 21.56 % was observed with 12.50 µl/ml concentration. No inhibition was observed in the control.

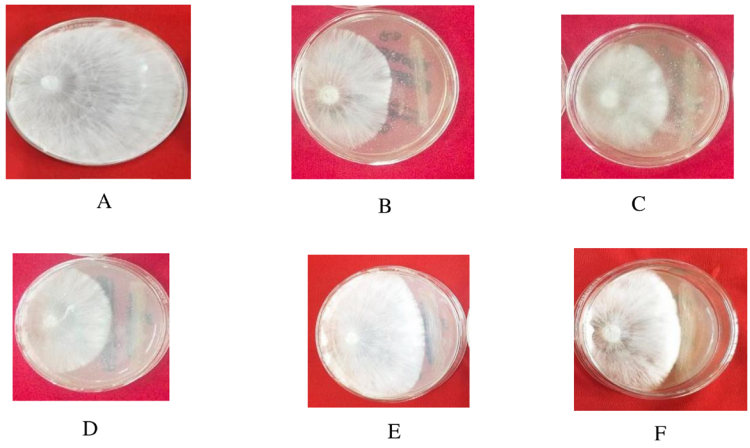

Figure 2: Dual culture bioassay of *B. subtilis* isolates against *Sclerotinia sclerotiorum,* A. control, B. NBAIR BSWG 1, C. NBAIR BSWG 2, D. NBAIR BSWG 3, E. NBAIR BSWG4, F. NBAIR BSWG5

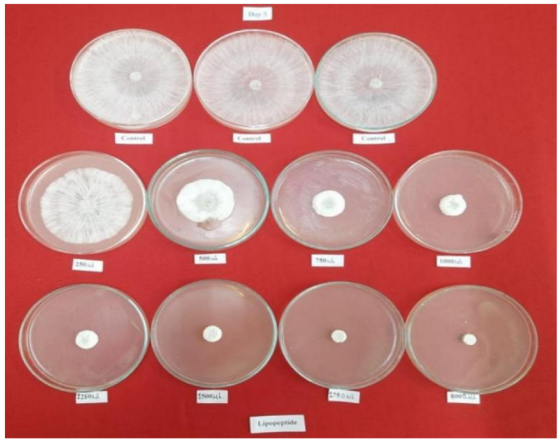

Figure 3: Poison food technique using lipopeptides from *B. subtilis* isolate NBAIR BSWG1 against *S. sclerotiorum*

Table 4. Antagonistic activity of lipopeptides produced by *B. subtilis* isolates (NBAIR BSWG1) against *S. sclerotiorum* using Poison food assay

Concentration µL/mL	***S. sclerotiorum* at 5 DAI***	
	****Average radial growth of mycelial (cm)**	**Per cent inhibition**
12.5	3.53[b]	21.56
25.0	2.20[c]	51.11
37.5	1.36[d]	69.78
50	1.00[e]	77.78
67.5	0.80[f]	82.22
75.0	0.66[g]	85.33
87.5	0.50[h]	88.89
100	0.50[h]	88.89
Control	4.50[a]	0.00
C.D.	0.12	-

*DAI - Days after inoculation. **Average radial growth of three replications. Values in the same column followed by same letter do not differ significantly and different letter indicates significant difference accordingly to Duncan's Multiple Range Test ($P \leq 0.05$)

DISCUSSION

The present study was focused on isolation and testing the biocontrol ability of lipopeptides secreted by *B*. *subtilis* isolates extracted from soil samples of Western Ghats of India. Zaccardelli *et al*. (2020) could identify 133 different spore-forming *B. subtilis* colonies from 300 bacterial isolates present in composts. The identity of *B. subtilis* was further confirmed by 16S rRNA sequence analysis. High-throughput sequencing of the full gene has only recently become a realistic prospect (Johnson *et al*., 2019). It is suggested that sequences of > 95 % identity represent the same genus, whereas sequences of > 97 % identity represent the same species (Schloss and Handelsman, 2005). In our studies, the amplified products were resolved at 1400 bp which is almost the full gene length with >97 % similarity.

Lipopeptides produced by *B. subtilis* mostly pertain to surfactins, iturins, and fengycins (Zhang and Sun, 2018). In our dual culture studies > % inhibition of *S. sclerotiorum* was obtained with *B. subtilis* NBAIR BSWG1 which produces fengycin, surfactin and bacillomycin. When we tested the lipopeptide extract there was 88.89 % inhibition of the pathogen. Lipopeptides have an amphiphilic nature, interfering with biological membrane structures. Their antimicrobial properties include activity against bacteria, fungi, oomycetes, and viruses Penha *et al*., 2020). Recent studies also highlight the ability of these compounds to stimulate defense mechanisms of plants and biofilm formation, which is a key factor for the successful colonization of biocontrol organisms (Penha *et al*., 2020; Sicuia *et al*., 2008). Mardanova *et al*., *(*2016) tested the antagonistic activity of lipopeptide from *B. subtilis* GM5 against Doratomyces sp. and observed 79 % inhibition*. B*. *subtilis* GM5 had a greater ability to inhibit the growth of all micromycetes viz. *Alternaria alternata*, *Fusarium avenaceum* and *F*. *redolens* by 72 %, 66 % and 65 % respectively.

In this study, we could isolate and characterize five lipopeptide producing *B*. *subtilis* isolated from Western Ghats of India. The isolates harbored lipopeptide expressing genes like surfactin, fengycin and bacillomycin. Lipopeptide extract of *B. subtilis* NBAIR BSWG1 showed 88.89 % inhibition of *S. sclerotiorum* at 100 µl/ml. Hence lipopeptide producing *B. subtilis* isolates can have huge potential in biological control. Further research is needed in the formulation and field application of lipopeptides.

CONCLUSION

Bacillus subtilis strain NBAIR BSWG1 produced three types of lipopeptides under *in vitro* conditions, which significantly suppressed *Sclerotinia sclerotiorum* as compared to control. The present study demonstrates the significance of lipopeptides-producing *Bacillus subtilis* isolates obtained from soils of the Western Ghats of India in the suppression of *S. sclerotiorum* under *in vitro* conditions. Further studies are being carried out in formulating the application of lipopeptides under field conditions.

ABBREVIATIONS

BLAST - Basic Local Alignment Search Tool
BSWG - *Bacillus subtilis* Western Ghats
HCL - Hydrochloric Acid
LB broth - Luria Bertani broth
LPs - Lipopeptides
NBAIR - National Bureau of Agricultural Insect Resources
NCBI - National Center for Biotechnology Information
PCR - Polymerase Chain Reaction
PDA - Potato Dextrose Agar

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