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Identification and Characterization of Lipopeptides from *Bacillus subtilis* B1 Against Sapstain Fungus of Rubberwood Through MALDI-TOF-MS and RT-PCR

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Abstract *Bacillus subtilis* is a potent biocontrol agent producing a wide array of antifungal lipopeptides for the inhibition of fungal growth. *B. subtilis* B1 isolated from market-available compost provided an efficient control of rubberwood sapstain fungus, *Lasiodiplodia theobromae*. The current study is aimed to identify and characterize the lipopeptides responsible for the biocontrol of rubberwood sapstain fungus by *Bacillus subtilis* B1. The bacterial whole-cell surface extract from the dual culture of *B. subtilis* B1 and sapstain fungus (*L. theobromae*) was analysed using MALDI-TOF-MS. The protonated as well as sodium, potassium adducts of homologues of iturin C, surfactin, bacillomycin D and fengycin A and B were identified and expression of the lipopeptide biosynthetic genes could be confirmed through RT-PCR. This is the first report of mycobacillin and trimethylsilyl derivative of bacilysin during antagonism through MALDI-TOF-MS. MALDI-TOF-MS with RT-PCR offered easy platforms to characterize the antifungal lipopeptides. The identification of antifungal lipopeptides can lead to the formulation of prospective biocontrol by-products which have wide-scale utility.

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Introduction

In India, rubberwood (*Hevea brasiliensis*) has emerged as a main source of wood material with immense applications across all sectors of wood industry. Globally, India is the fourth largest natural rubber-producing country and Kerala State accounts for 78 % of the area and 90 % of total rubber produced in the country [13]. Even though an eco-friendly wood material, the non-durability of rubberwood makes it highly susceptible to fungal infection in the warm and humid tropical climate. Sapstain fungi, which cause high economic loss to the timber industry by reducing the aesthetic value of the wood, is also a serious issue in rubberwood industry [8]. *Lasiodiplodia theobromae*, the dominant bluish black sapstain fungus, badly attacks the rubberwood within 1 day of felling and infects the full log in a span of 1 week [12]. Although chemical fungicides are commonly used for rubberwood preservation, environment-friendly biocontrol measures are preferred to reduce the health hazards and environmental pollution. Different microbes like *Trichoderma, Pseudomonas,* and yeast among others were used as biocontrol agents against *L. theobromae* on fruit crops like guava, banana, cashew, annona, etc. [1, 14, 15, 43], but so far no studies had been undertaken on the biological control of *L. theobromae* on rubberwood.

One of the first flourished biocontrol agents used against insects and pathogens was the members of the genus *Bacillus* [36]. *B. subtilis* collectively with other *Bacillus* species represent about half of the industrially available biopesticides in the world market [11]. *B. subtilis* has been regarded as a paradigm of Gram-positive endospore-forming bacteria [29]. Various strains of *B. subtilis* are widely exploited as biocontrol agents because of their safety, extensive distribution in varied habitats and their significant

ability to endure unfavourable conditions by the development of endospores. The biocontrol property of *Bacillus* has been explained by different mechanisms of action including competition for space and nutrients, production of cell wall-degrading enzymes and antibiosis [2]. The potential of *B. subtilis* is based on its ability to produce a broad array of less toxic lipopeptides with powerful antifungal properties, high biodegradability and eco-friendly features as compared to chemical pesticides [6, 28].

Among these, non-ribosomally synthesized lipopeptides like iturin, surfactin and fengycin families are most commonly known for their antifungal and biosurfactant activities [18, 21, 31, 34]. Iturin A and C, bacillomycin D, F, L and LC and mycosubtilin were explained as the seven main members of the iturin family. The fengycin family of lipopeptides includes fengycin A and B, which are also known as plipastatins. Biological control of rubberwood sapstain fungus, *L. theobromae*, had been attempted previously using *Trichoderma* sp. and *Streptomyces* sp., but the results were not promising [41, 48]. The successful and significant inhibition of rubberwood sapstain fungus was observed in the study using *B. subtilis* B1 isolated from market-available compost [42]. Understanding the mechanisms of biological control helps to manipulate the environment to create congenital conditions for better biocontrol [45]. Several studies had been carried out for the identification of antifungal lipopeptides in various strains of *B. subtilis* [4, 21, 34]. Till date, there is no information on the family of antifungal lipopeptides produced by *B. subtilis* during antagonism of sapstain fungus on rubberwood. Matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-TOF-MS) has been regarded as a very fast and reliable tool for the identification of lipopeptides when compared to the conventional methods like culturing and purifying the lipopeptides. The effectiveness of MALDI-TOF-MS for the identification of *Bacillus* cyclic peptides and other lipopeptides has been previously reported [4, 21].

In this context, the objective of the present study was to identify and characterize the antifungal lipopeptides produced by *B. subtilis* B1 during the inhibition of rubberwood sapstain fungus (*L. theobromae*) using MALDI-TOF-MS as well as through reverse transcriptase PCR (RT-PCR).

Materials and Methods

Bacillus subtilis B1 Isolation, Identification and Antagonism

Protocols for the isolation of the bacterial biocontrol agent, morphological/biochemical/molecular identification of *Bacillus subtilis* B1 as well as its antagonistic potential have been standardized [42].

Structural Characterization of the Sapstain Fungus During Antagonism

The dual culture of rubberwood sapstain fungus (*L. theobromae*) as well as the biocontrol agent (*B. subtilis* B1) was performed according to Johnson and Curl [19]. *B. subtilis* B1 was streaked on one side of the potato dextrose agar (PDA) plate and a 6-mm-width disc of actively growing *L. theobromae* was simultaneously inoculated on the opposite side of the plate and incubated at 28 °C. *L. theobromae* culture grown in PDA was used as a control. The mycelial portion from the inhibition zone was viewed under the light microscope as well as subjected to scanning electron microscopic (SEM) study to understand the structural changes in the fungal mycelia during antagonism, induced by the biocontrol agent, *B. subtilis* B1 [5].

MALDI-TOF-MS Analysis of Antifungal Lipopeptides

The antifungal lipopeptide analysis was done from the bacterial whole-cell surface extract obtained during the dual culturing of *B. subtilis* B1 and *L. theobromae*. Preparation of bacterial surface extracts was carried out according to the methodology of Vater et al. [47]. Instead of antibiotic production medium Landy agar, the growth medium, potato dextrose agar, was used in this study. *B. subtilis* B1 was streaked on one side of the plate and *L. theobromae* was placed on the opposite side simultaneously and incubated at 28 °C. On the second day, one to two loops of bacterial cells from the interface of the bacterial–fungal dual culture was suspended in 500 µL of acetonitrile (70 %) with trifluoroacetic acid (0.1 %) for 2 min. This was gently vortexed and the homogenized suspension was pelleted by centrifuging at 5000 rpm for 10 min. Dihydroxy benzoic acid (DHB) was used as the matrix solution in MALDI-TOF-MS analysis. The cell-free supernatant was stored in fresh microcentrifuge tubes at 4 °C for MALDI-TOF-MS analysis. The MALDI-TOF-MS analysis was carried out at Indian Institute of Sciences, Bangalore, India. The mass range used for the analysis was from 200 to 2000 Da. The surface extract of *B. subtilis* B1 grown on potato dextrose agar was used as a control in this study.

Characterization of Antifungal Lipopeptide Synthetic Genes Using Reverse Transcriptase PCR (RT-PCR)

For confirming the antifungal lipopeptide gene expressions during antagonism, the total RNA was isolated from the *B. subtilis* B1 using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The RNA was

isolated from the *B. subtilis* B1, dual cultured with *L. theobromae* having a visible inhibition zone. The concentration and purity of the isolated RNA were determined by measuring the absorbance at 260/280 nm (NanoDrop 1000, Thermo Scientific, USA). The total mRNA was used as the template for the cDNA synthesis using first-strand cDNA synthesis kit (Fermentas, Lifesciences). The synthesized cDNA was used as the template for the RT-PCR. To characterize the genes, the cDNA was amplified using the specific primers for each lipopeptide biosynthetic gene (Table S1). cDNA-PCR amplification was carried out as per standard protocols with a preliminary denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation (94 °C for 1 min), primer annealing (different annealing temperatures). After 35 cycles of PCR amplification, the PCR products were resolved on 1.5 % agarose gel and the eluted products were subjected to Sanger's dideoxy sequencing. The similarity searches for the sequences were carried out using the *BLAST (N)* option in the NCBI genbank (www.ncbi.nlm.nih.gov).

Scale-Up Production of *Bacillus subtilis* B1 in Bioreactor

The batch fermentations were performed in a 3L LS2 bioreactor (Scigenics, India), equipped with an agitator shaft with three impellers and connected to a digital control unit. Temperature and pH were measured using the probes, and the pH was maintained by automatic feeding of 1 N NaOH and 1 N HCl. Compressed air was supplied through the sterile filter. The agitation and aeration could be manually controlled in parallel to the dissolved oxygen. The dissolved oxygen level and pH were controlled and measured. Foaming was eliminated using the antifoam agent silicone oil (Himedia, India).

Based on the preliminary shake flask studies on the standardization of medium and physical factors for the massive growth of *B. subtilis* B1, molasses peptone broth (MPB) with pH 7 at 30 °C was used in the bioreactor for scale-up production. The batch process was started with an initial volume of sterile 2.5 L MPB (Molasses 10 %, peptone 0.25 %) at pH 7 and inoculated with 25-ml shake flask pre-inoculated *B. subtilis* B1 culture. In the bioreactor, the optimum aeration and agitation were standardized using a range from 0.5 to 3 lpm (liquid per minute) and 0 to 350 rpm (rotation per minute), respectively. The dissolved oxygen set at the beginning was 100 % and it was maintained above 20 % manually by controlling the agitation. After every 24 h of growth, the optical density (O.D.) of the samples at 600 nm was measured and viable cell count and spore count were calculated. The plate count method was used to determine the number of viable cells during *B. subtilis* strain B1 growth in the bioreactor [35]. For spore count, the culture was incubated at 80 °C for 10 min in a water bath. It was then serially diluted and plated on nutrient agar and the observation was made after 24 h.

Results

Structural Characterization of the Sapstain Fungus During Antagonism

The antagonistic ability of *B. subtilis* B1 against *L. theobromae* is evident from the observed structural changes in the fungal mycelia during the dual culture (Fig. 1a, b). The fungal mycelia from the zone of inhibition of the dual culture showed swelling and distortions, while the control mycelia were thin, clear and slender under light microscope (Fig. 1c, d). In the scanning electron microscope, the distortion of the fungal mycelia from the inhibition zone was visible and plasmolysis as well as shrinkage of the fungal hyphae leading to the death of the fungus was also observed (Fig. 1e, f).

MALDI-TOF-MS Analysis of Antifungal Lipopeptides

The lipopeptide profiling of *B. subtilis* B1 responsible for the inhibition of sapstain fungus was performed by MALDI-TOF-MS. The whole-cell surface extract MALDI spectrum of *B. subtilis* B1 grown in PDA control plate and the *B. subtilis* B1 obtained from the dual culture clearly distinguished the lipopeptide profiles responsible for antagonism (Fig. S1a, b). The mass spectra obtained from the whole-cell surface extract of *B. subtilis* B1 from the dual culture showed clear peak clusters of the members of iturin, surfactin and fengycin families (Figs. 2a, b), while these peak clusters were almost absent in the spectrum from the control plate. The peaks at *m/z* 994.44, 1008.45, 1022.47 and 1036.49 (Fig. 2a) observed in the mass spectra were the protonated forms of C12 to C15 surfactin homologues [30]. The peaks at *m/z* 1030.43, 1044.45, 1058.47 and 1072.47 (Fig. 2a) in the mass spectra were the protonated forms of C13 to C16 iturin C homologues, while the peaks at *m/z* 1017.4, 1031.4, 1045.4 and 1059.4 (Fig. 2a) were the protonated forms of C13 to C16 homologues of bacillomycin D, respectively [38, 44]. In addition to these four antifungal lipopeptides, two other antifungal lipopeptides which are not commonly mentioned, mycobacillin (protonated forms of C14 to C16 homologues) and trimethyl silyl derivative of protonated, sodium and potassium adducts of bacilysin were also detected in the MALDI-TOF-MS analysis based on their molecular weight and observed peaks (Figs. 2b, c; Table S2).

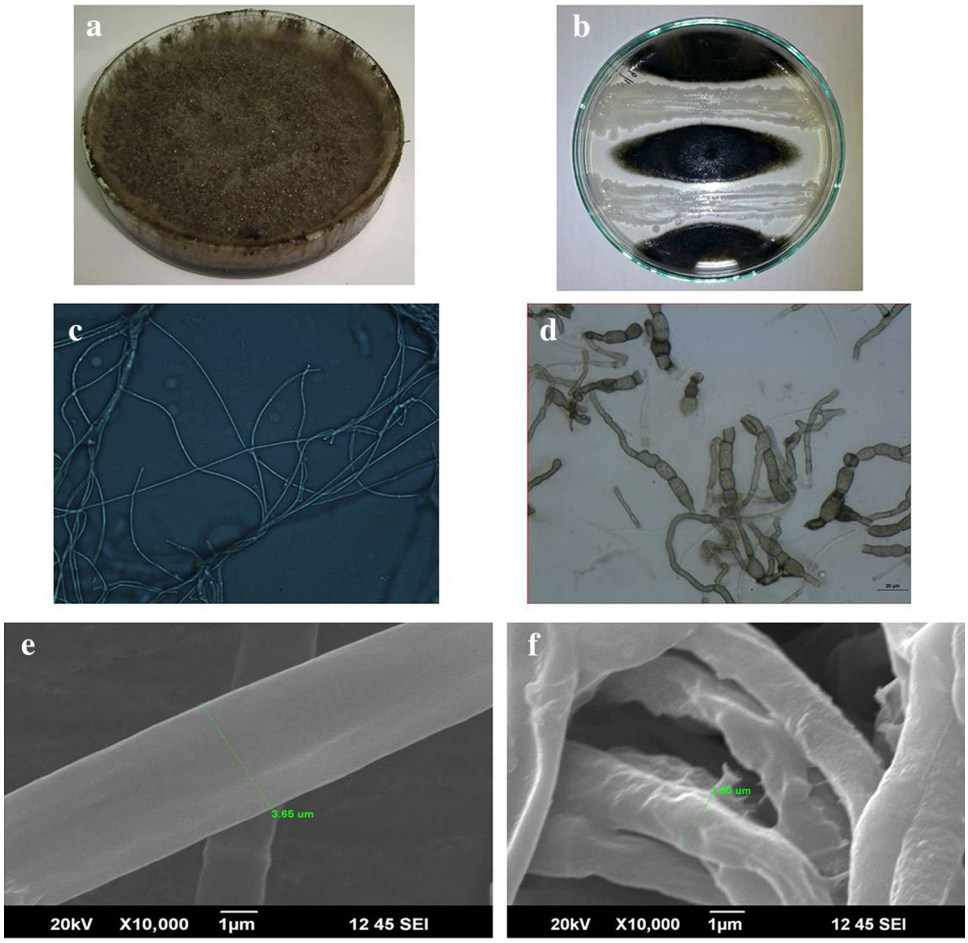

Fig. 1 Pure and dual culture of *Lasiodiplodia theobromae* under light microscope and SEM. **a** Pure culture of *Lasiodiplodia theobromae* isolated from infected rubberwood pieces. **b** Dual culture showing the effective inhibition of *L. theobromae* by *B. subtilis*. **c** Thin slender hyphae of normal *L. theobromae* under light microscope. **d** Distorted fungal hyphae from the zone of inhibition under light microscope. **e** Clear normal mycelia under scanning electron microscope. **f** Plasmolysed and shrunken fungal mycelia from the zone of inhibition under scanning electron microscope

Characterization of Antifungal Lipopeptide Biosynthetic Genes Using RT-PCR

RT-PCR carried out to confirm the gene expressions of antifungal lipopeptides (viz. bacilysin, fengycin, bacillomycin, mycosubtilin, surfactin and iturin) during antagonism gave rise to specific amplified products of 500, 964, 875, 550, 675 and 650 bp for these lipopeptide genes, respectively (Fig. S2). NCBI-BLAST homology searches were carried out to confirm the coding sequences of these respective lipopeptide genes.

Scale-Up Production of *Bacillus subtilis* B1 in Bioreactor

The batch cultivation of *B. subtilis* B1 in the 3 L bioreactor with controlled optimal conditions of 1 lpm aeration, 250 rpm agitation and 20 % dissolved oxygen could scale up the production in 24 h and the viable cell count was 1.13×10^{11} CFU ml^{-1} after 24 h (Table S3). The optical density value at 600 nm and viable cell and spore counts after each 24 h up to 168th hour (7th day) are given in Table S4.

Discussion

The biocontrol potential of *B. subtilis* has been mainly attributed to the ability to produce non-ribosomal antifungal lipopeptides with synergistic effects [3, 45]. The inhibition of the fungal growth by an antagonistic bacterium is generally accomplished through plasmolysis, shrinkage and lysis of the fungal mycelium [45]. Lytic antifungal lipopeptides alter the structure of cell membrane and lead to increased permeability with leakage of metabolites and fungal cell death [7]. The plasmolysis, shrinkage and lysis of the fungal hyphae observed in the present study have been reported previously against *Curvularia lunata*, *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* [3, 5, 15]. RT-PCR analysis performed from the *B. subtilis* B1 colonies at the time of dual culture confirmed the expression of lipopeptide biosynthetic genes, coding for surfactin, fengycin, iturin, bacillomycin, mycosubtilin and bacilysin.

Antifungal lipopeptides from the whole-cell surface extracts of *B. subtilis* have been reported previously using MALDI-TOF-MS [4, 46]. This is the first instance of employing MALDI-TOF-MS for the precise identification

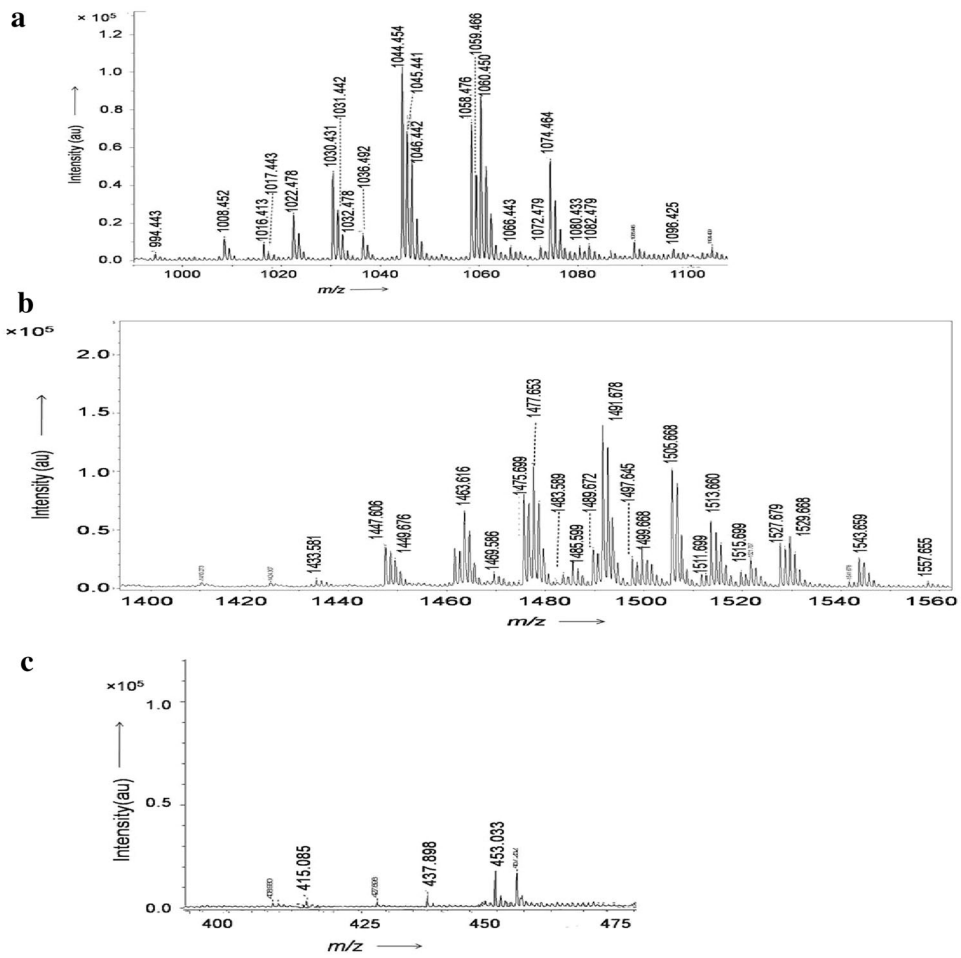

Fig. 2 MALDI-TOF mass spectra of *Bacillus subtilis* B1 cell surface extracts during the inhibition of sapstain fungus. **a** Peaks of protonated as well as sodium and potassium adducts of bacillomycin D (*m/z* 1017–1059), iturin C (*m/z* 1030–1096) and surfactin (*m/z* 994–1046). **b** Peaks of protonated as well as sodium and potassium adducts of fengycin A and B (*m/z* 1433–1515) and mycobacillin (*m/z* 1529–1557). **c** Peaks of protonated as well as sodium and potassium adducts of trimethyl silyl derivative of bacilysin (*m/z* 415–453)

of the antifungal lipopeptides by *B. subtilis* B1 against rubberwood sapstain fungus (*L. theobromae*). Generally, bacterial antibiotics are best produced in the antibiotic production medium, Landy agar. But in the presence of the fungus, the bacteria are capable of producing the essential antifungal lipopeptides even in the growth medium (PDA) to antagonize the fungus. High antibiotic production was also reported in the inhibition zone of dual culture in PDA medium [24]. In the present study, the MALDI-TOF-MS spectrum (Figs S1a, b) of the cell surface extract, for control as well as dual cultured *B. subtilis* B1 with sapstain fungus on PDA medium, clearly substantiated the presence of antifungal lipopeptides on bacterial cell surface extract.

The inhibitory effects of the iturin family lipopeptides are reported to be based on the osmotic perturbation due to the formation of ion-conducting pores on cell membrane [25]. The permeability of yeast plasma membrane is reported to be altered by mycosubtilin and bacillomycin with tyrosine residue, which resulted in the release of nucleotides, proteins and lipids [23, 39, 49]. Iturin A is the commonly produced antifungal antibiotic of the iturin family produced by *B. subtilis* [21]. But in the present study, instead of iturin A (*m/z* 1043.5, 1057.5, 1071.5) another antibiotic iturin C (*m/z* 1044.45, 1058.48, 1072.48), with the *m/z* value varying by 1 Da due to asparagine (Asn) to aspartic acid (Asp) replacement at amino acid position 1, was identified [33]. This is the first report on the identification of iturin C from *B. subtilis* whole cell during sapstain fungal inhibition using MALDI-TOF-MS analysis. Similarly, LC–ESI–MS analysis of the lipopeptides of banyan endophyte *B. subtilis* could reveal the presence of iturin C [34]. Another member of iturin family, bacillomycin D, is also identified during the sapstain fungal inhibition. C13 isoform of bacillomycin D has been detected in the present study, whereas only C14–C17 isoforms were previously reported [38]. The long-chain iturins are more hydrophobic, readily form oligomers and therefore may interact more effectively with ergosterol-containing membranes of fungi and yeast [27]. In this study, C16 homologue of iturin C and C17 homologue of bacillomycin have been detected by the MALDI assay which can also contribute strongly for the fungal inhibition. Even though RT-PCR could detect the expressions of mycosubtilin gene and itu D gene (coding for iturin A) during

inhibition, its presence in the form of *m/z* peaks was not detected in MALDI-TOF-MS.

Iturin with its amphiphilic nature can readily associate and tightly anchor into lipid layers and can form irreversible pores by the insertion of surfactin-rich clusters in the membrane and causes synergistic effect through membrane solubilization or disruption [21, 26, 27, 31, 37]. Surfactin-producing *B. subtilis* strains have high swarming motility and biofilm formation potential which in turn results in the bacterial cell motion [22]. This is evidenced by the even distribution of *B. subtilis* cells on the wood blocks after 1 week of inoculation. The lipopeptides, iturin and surfactin are surface-active substances which promote adhesion of *B. subtilis* onto diverse solid substrates by changing the hydrophobicity of the cells [2].

Among the three antifungal lipopeptide families, fengycins are reported as the most effective with high fungicidal, bactericidal and insecticidal properties [16, 20]. Even in low concentrations, fengycin acts as detergents which solubilize the membranes by interacting with the lipid layers and forms pores on them [10]. The production of all the five reported homologues of fengycin A and fengycin B during the fungal inhibition was clearly observed in the present MALDI-TOF-MS analysis [46]. The synergistic action of surfactin and iturin, surfactin and fengycin as well as iturin and fengycin had been reported previously for the biological control of fungal pathogens [26, 32, 40].

The cyclic tri-decapeptide mycobacillin, which is antifungal in nature, lacks the hydrocarbon tail but it binds to fungal cell membrane by its tyrosine residue and solubilizes it [30]. MALDI spectrum of mycobacillin has not been reported previously. Based on the reported molecular weight of mycobacillin (1528 Da), three homologues of mycobacillin (1529.6, 1543.6 and 1557.6) could be identified in the present study [50]. The presence of protonated as well as the sodium and potassium adducts of trimethyl silyl derivative of bacilysin in this study clearly indicates the role of bacilysin also in the sapstain fungal inhibition [50]. Chung et al. [9] had confirmed the trimethyl silyl derivative of bacilysin peak (*m/z* 415) in the GC–MS analysis of the *B. subtilis* culture filtrate.

Earlier studies reported molasses as one of the ingredients in the bacterial growth medium along with other nitrogen sources [52]. The viable cells obtained in the present batch cultivation after 24 h was high as compared to the mass production of *B. subtilis* in batch reaction with low-cost medium containing soyflour and molasses [51].

The present study could identify specific antifungal lipopeptides viz. iturin C, surfactin, fengycin A and B, bacillomycin D, bacilysin and mycobacillin produced by *B. subtilis* B1 during the inhibition of sapstain fungus, *L. theobromae*, using MALDI-TOF-MS as well as could confirm the expression of the lipopeptide biosynthetic genes in *B. subtilis* B1 through RT-PCR. More studies using real-time PCR are warranted to further quantify the expression levels of antibiotic genes during the sapstain fungal antagonism. Mass production of the biocontrol agent, *B. subtilis* B1, against the sapstain fungus on rubberwood using an economically viable medium offers scope for its commercialization. Further experiments are progressing in our lab for the formulation and field efficacy testing of the biocontrol agent as well as for the real-time quantification of lipopeptide biosynthetic gene expression levels during antagonism.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

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