A Novel Rhizospheric Bacterium, *Bacillus Velezensis* NKMV-3 as a Biocontrol Agent against *Alternaria* Leaf Blight in Tomato

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Abstract

A novel strain of *Bacillus* isolated from rhizosphere has shown to be excellent biocontrol agents against various plant pathogens. In this study, a first report of a *Bacillus* strain NKMV-3 which effectively controlling *Alternaria solani*, which cause the Early Blight disease in tomato. Based on the cultural and molecular sequencing of 16S rRNA gene sequence, the identity of the strain was confirmed as *Bacillus velezensis* NKMV-3. The presence of the lipopeptide which are antibiotic synthesis genes namely Iturin C, Surfactin A, Fengycin B and D were confirmed through gene amplification. In addition, lipopetides was also confirmed through liquid chromatography. The extract showed inhibitory effect against *A.solani* in-vitro and detached tomato leaf assays. *Bacillus velezensis* strain NKMV-3 based formulations may provide an effective solution in controlling early blight disease in tomato and other crops.

Introduction

Tomato (*Solanum lycopersicum* Linn.), is one of the most important crops cultivated throughout the world in a wide range of climates among solanaceous crops [1, 2]. Tomato is ranked as the second most consumed vegetable after potato due to its rich source of vitamin A, C and K, minerals aminoacids and antioxidants, lycopene[2, 3]. There is a high demand because of its wide use and nutritional values, both in fresh and processed tomato markets. Therefore, higher production is required to fulfil the increasing demandand for value addition. The tomato cultivation is mainly hindered by bacterial, fungal and viral diseases[4]. The early blight caused by *Alternaria solani*, in particular severely affects the tomato production[4, 5]. *A. solani* is an air-borne pathogen inhabiting the soil. It is distributed worldwide, but is highly infective in the tropics and temperate regions[5]. Synthetic fungicides such as mancozeb, pyraclostrobin, azoxystrobin and hexaconazole are utilized for the control of this disease in various crops[6, 7]. However, *A. solani* is gaining resistance to these pesticides and causing serious problems in the yield of tomatoes[8, 9].

Over the past few decades, chemical pesticides have been extensively used by farmers for crop protection, but it posessevere adverseenvironmental and detrimental healtheffects[10, 11]. Unscrupulous usage of fungicides encourage in the development of resistant pest/pathogen strains,destruction of non-target organisms, leads to residual toxicity, and other health and ecological hazards[12–14]. Thus, alternative eco-friendly sustainable agricultural practices like adopting tomicrobial control of pests and diseases is the need of the hour. Bacterial biocontrol agentshaverecently gained worldwide attention for the control of various plant pathogens due to their low cost and ease of production, environment friendliness and non-residual effect[15, 16].Many species of the genus *Bacillus* are potential candidates for development as biocontrol agents against various plant pathogens.[17, 18]. Among Bacillus spp., *B. velezensis* is one of the potential biocontrol agent [19, 20]. Literature reports numerousstrains of *B. velezensis* strains capable of controlling the major plant pathogens (bacteria & fungi) and nematodes[19–23].

In the present study, bacterial strains were isolated from soil samples collected from the roots of tomato plants and screened *in vitro* for antagonistic activity against *A. solani.B. velezensis* was identified by 16srRNA sequencing. Through FTIR and RP-HPLC analyses, the antifungal lipopeptides secreted by *B. velezensis* were characterized and its efficacy against *A. solani* were studied by poison food technique and detached leaf assays.

Materials And Methods

Isolation of Antagonistic bacteria and Alternaria solani causing Early Blight in Tomato

The sampling was conductedduring the months of September to November 2019 in 20 locations of Tamil Nadu known for growing tomato crop in large acreages. Tomato growing fields were selected for sampling soil for the isolation antagonistic bacteria against early blight pathogen, *Alternaria solani*. Healthy tomato plants were uprooted carefully without disturbing the roots. Soil adhering to the roots was shaken and removed into poly Ziplock bags and immediately kept on ice until further processing.

Isolation of putative antagonistic Bacillus isolates

Soil samples collected from various tomato fields were removed from the cold storage and thawed to room temperature. A soil suspension was prepared using 10g of each sample and 90 ml of sterile water. The solution was agitated using a rotary shaker at 180 rev min^{-1} for 30 minutes. The suspensions thus prepared using all the soil samples were heated to 60°C for one hour and cooled immediately by placing in an ice box giving a heat shock reaction for the formation of endospores. The suspensions were serially diluted up to 10^4 and spread plated on nutrient agar medium, followed by incubation at 30°C for 2 days in an incubator. Single colonies isolated were maintained as purified cultures on nutrient agar slants [24]. Purified putative antagonistic *Bacillus* isolates were stored at − 80°C in 60% glycerol stocks.

Screening of putative antagonistic Bacillus isolates against various plant pathogens

Putative antagonistic *Bacillus* isolates were subjected to screening through dual culture technique[25] against major pathogens of crops namely *Fusarium oxysporum f.sp.lycopersici, Alternaria solani, Rhizoctonia solani, Macrophomina phaseolina* and *Pyricularia oryzae*. All the phytopathogens were supplied by the Microbial Type Culture Collection culture (MTCC) bank, Chandigarh, India. Briefly, 5mm mycelial plug was removed from an actively growing plate of each phytopathogen and placed at one edge of the plate. Putative *Bacillus* isolates were streaked on other side of the mycelial plug near the edges of the plate followed by incubation at 28°C in an incubator for 7–10 days. *Bacillus* isolates which showed antagonistic activity through zone of inhibitions were selected. The percentage growth inhibition was calculated as per the below formula adapted from [26].

Inhibition (%) = [(Control-Treatment)/Control] x 100

Where Control is the colony diameter in Control plates and Treatment is the colony diameter in treatment plates. The experiment was performed in triplicates.

Characterization And Identification Of Putative Antagonistic Bacillus Isolates

The putative antagonistic *Bacillus* isolate (NKMV-3) that was shortlisted through dual plate screening was characterized according to Bergey's manual of systematic Bacteriology [27] and using Himedia'sHiBacillus™ identification Kit. In order to identify the putative antagonistic *Bacillus* isolate, a loop full culture of NKMV-3 growing on Nutrient Agar was used for DNA extraction. Quick DNA-Fungal/Bacterial Miniprep Kit ofZymo Research was used for extracting DNA from the putative antagonistic *Bacillus* isolate (NKMV-3). 16S rRNA sequence of the extracted DNA was amplified throughPolymerase Chain Reaction (PCR) with two bacterial universal primers namely 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3')[28]. A 14μLPCR reaction mixture consisting of 8μL Taq Master mix, 2 μL of each forward and reverse primers, 2 μL of DNA template and 2 μL of molecular grade water was used for amplifying the DNA. PCR was performed in an Eppendorf Mastercycler X50s. The PCR conditions were adapted from Zhu et al., 2020 with slight modifications as described:1 minute of Initial denaturation at 95℃, followed by 35 cycles of denaturation at 95℃ for 30 seconds, 1 minute of annealing at 52℃followed by extension at 72℃ for 2 minutes and 30 seconds. A final extension of 72℃ for 10 minutes completed the PCR reaction. The PCR products were purified and sequenced by external sequencing facility.The sequencing results were compared with known bacterial NCBI Genbank sequences using BLAST and the identity was confirmed. The identified bacterial sequence was submitted with NCBI and an accession number was obtained. Mega 11 software was used for constructing a phylogenetic tree using the neighbor-joining method with 100 bootstrap replicates[30].

Effect of crude lipopeptides against Alternaria solani

The effect of the crude lipopeptides obtained from NKMV-3 was tested through a modified poison food technique[31]. The dried crude lipopeptide powder was used for preparing various concentrations starting from 1–5% in potato dextrose agar medium on W/V basis. Media containing various concentrations of the crude lipopeptides was autoclaved and dispensed in petri plates. After solidification, a 5mm disc from an actively growing *A.solani* plate was extracted using a sterile cork borer and placed at the centre of the plate. The PDA plates containing no crude lipopeptides served as control for the trial. The plates were incubated at 28°C for 7 days in an incubator. After 7 days, the plates were observed for the growth of *A.solani*. The inhibition rate of mycelial growth was calculated as follows.

Inhibition rate (%) = [Diameter of colony in Control (mm) – Diameter of colony in treatment (mm)/Diameter of colony in Control (mm)] x 100

The experiment was conducted in triplicates.

Molecular Detection Of Surfactin, Fengycin And Iturin Genes

A loop full of culture from an actively growing slant of NKMV-3 was used for DNA extraction. DNA was extracted as mentioned in the previous section of this article. Each PCR reaction was performed in an Eppendorf Mastercycler X50s thermal cycler using a 14 µL reaction mixture containing Taq Master mix (8µL), forward and reverse primers (2µL each), Molecular grade water (2µL). The primers were chosen from already available literature. The details of primers and expected amplicon sizes are provided in Table 1.The PCR conditions were adapted from [32, 33] with slight modifications. Briefly, *Sfr*A gene was amplified using a 35-cycle reaction consisting of 4 minutes of initial denaturation at 95°C, followed by denaturation for 1 minute at 94°C. Annealing was performed at 52°C for 30 sec, followed by an extension at 70°C for 1 minute. A final extension was performed at 70°C for 5 minutes. *fen* B, *fen* D and *Itu* C genes were amplified using a 40-cycle reaction consisting of 3 minutes of initial denaturation at 94°C, followed by denaturation for 1 minute at 94°C. Annealing was performed at 59°C (*fen* B), 50°C (*fen* D &*Itu* C) for 1 minute, followed by an extension at 70°C for 1 minute. A final extension was performed at 72°C for 10 minutes. The PCR amplicons were analysed through gel electrophoresis on a 1% agarose run at constant voltage of 100V for 40 minutes followed visualization using a gel documentation system (make: Vilber Quantum).

Extraction Of Extracellular Metabolites Produced By Nkmv-3

NKMV-3 was grown in Nutrient brothfor 72 hoursat 37°Cin a rotatory shaker with constant shaking of130 rotations min^{-1}. The cells were harvested after 3 days by centrifugation at 6,000 x*g*for 15 minutes followed by the reduction of pH of the cell free extract to 2.0 by the addition of 3N HCl and left for overnight precipitation at 4°C. The so precipitated crude lipopeptides was separated by centrifugation at 8,000 x*g* for 30 minutes at 4°C. The pellet was dissolved in methanol and extracted thrice and evaporated under vacuum using a rotatory evaporator at 50°C and 65 rpm [34, 35]. The resulting viscous liquid was left for drying at 50°C for 48hours in a hot air oven. The dried crude lipopeptide extract was scrapped and dissolved in Tris HCl pH 7.5 and stored until further use.

Table 1
PCR Details for amplification of AMP genes

S.No	Gene	Primer	Primer sequence	Expected base pairs (bp)
1	*Sfr*A	SRFA-F1/SRFA-R1	5'-AGAGCACATTGAGCGTTACAAA-3’ 5'-CAGCATCTCGTTCAACTTTCAC-3’	670
2	*fen* B	FEN B-F1/ FEN B-R1	5'-CCTGGAGAAAGAATATACCGTACCY-3' 5'-GCTGGTTCAGTTKGATCACAT-3'	670
3	*fen* D	FEN D-F1/ FEN D-R1	5'-GGCCCGTTCTCTAAATCCAT-3' 5'-GTCATGCTGACGAGAGCAAA-3'	670
4	*Itu C*	ITU C - F1/ ITU C - R1	5'-CCCCCTCGGTCAAGTGAATA-3' 5'-TTGGTTAAGCCCTGATGCTC-3'	594

Identification and quantification of Iturin, Surfactin and Fengycin in crude Lipopeptides using Liquid Chromatography

All solvents used were of HPLC grade (Merck) and standards of Iturin, Surfactin and Fengycin were purchased from Sigma, USA. Crude lipopeptides were quantified by Reverse-Phase High performance liquid chromatography using a chromatograph (Waters, USA) equipped with a quartinary pump and diode array detector. Analytical scale Purospher® RP-C18 (250 × 4.6 mm, 5 µm particle size) column was used. 1mg/ml of crude lipopeptide was prepared and filtered and filled into HPLC vials for injection. A volume of 2µl of sample was injected into the column. The mobile phase and chromatographic conditions were adapted from[36]. This method provided a single protocol for the detection and quantification of lipopeptides in a combined method rather than the conventional method of separate chromatographic runs for the identification and quantification of individual lipopeptides. The mobile phase consisted ofMilli-Q Water (Solvent A) and 0.1% HPLC grade trifluoroacetic acid dissolved in Acetonitrile(Solvent B). The elution of lipopeptide homologues was monitored at 210 nm. The conditions of the chromatography are provided in Table 2.

Table 2
Details for HPLC gradient conditions for the isolation and quantification of lipopeptides

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL min ^{1})
0–4	60	40	2
4–11	55	45	2
11–17	40	60	0.8
17–22	30	70	0.4
22–30	15	85	1.5
30–33	5	95	1.0
33–35	60	40	2

Analysis of crude Lipopeptides through Fourier Transformation Infra-Red (FTIR) spectroscopy

The crude methanolic lipopeptide extract was subjected to a FTIR analysis (make:Perkin Elmer) to elucidate the structural groups of the crude lipopeptides.100 mg of KBr and an mg of crude lipopeptide extractof NKMV-3were ground using a pestle and pressed with load for 30 s to obtain translucent pellets.These pellets were subjected to FTIR between a frequency range from 4000 to 400 cm − 1 [37].

Detached leaf bioassays of crude lipopeptides against A.solani in Tomato

Detached leaf bioassays were conducted with slight modifications as described by [38]. Briefly, Tomato (Variety: PKM − 1) leaves from 45 days old potted plants were obtained. The leaves were surface sterilized using 1% sodium hypochlorite solution, followed by two washes with sterile water. The leaves were left for air drying inside the Laminar air flow chamber. Various concentrations of crude lipopeptides were prepared from 1–5% on W/V basis in sterile water and methanol in the ratio of 9:1. Using a handheld atomizer, the leaves were sprayed on both sides with the test solution. Untreated controls were maintained which were sprayed with only sterile water and methanol in the ratios as mentioned above. Control leaves with sterile water spray alone were also maintained. All the leaves were left to air dry inside the laminar air flow chamber. Upon drying, a 5mm disc from an actively growing 7 days old *A.solani* plate was cut and place in the centre of each leaf, except for the untreated control leaves. All the leaves were placed on wet cotton inside petri dishes followed by incubation for 7 days at 28°C in an incubator. After 7 days the leaves were examined for lesions and the extent of lesion formation was measured in centimetres. The inhibition rate was calculated as follows

Inhibition rate (%) = [Diameter of lesion in Control (cm) – Diameter of lesion in treatment (cm)/Diameter of lesion in Control (cm)] x 100

The experiment was conducted in triplicates and statistically analysed.

Statistical analysis

All data was analysed statistically using WASP - Web Agri Stat Package 2.0 and Microsoft excel (2016) to assess statistically significant differences among the various treatments.

Results

Isolation of antagonistic bacteria against A.solani**causing early blight in tomato.**

In this study, a total of 146 morphologically distinct isolates of bacteria were isolated from 20 different locations covering three major districts in Tamil Nadu (Salem, Krishnagiri and Dharmapuri) known for the production of Tomato (Table 3). Of the 146 strains screened by dual culture technique(data not shown), NKMV-3 showed maximum inhibition against *A. solani*.Based on this result we further explored the biocontrol potential of NKMV-3.

Table 3
Locations of soil sample collectionacross major tomato growing districts of Tamil Nadu

S.No	Location	GPS coordinates	District Name
1	Omalur	11.744409995997248,78.04702812571645	Salem
2	ChinnaThirupathi	11.68502179676408,78.16517791092896	Salem
3	Karuvalli	11.853092036241849, 78.02571765381492	Salem
4	Kakapalayam	11.562937807350007, 78.02687110824542	Salem
5	P.N. Patti	11.81249373420619, 77.8163899465155	Salem
6	Mechari	11.832889045711607, 77.94214015355809	Salem
7	Mallikundam	11.879409367196567, 77.90420896048289	Salem
8	Vazhapadi	11.656775354146326, 78.40187057341394	Salem
9	Ayothiapattinam	11.678625602423615, 78.23288918192901	Salem
10	Mallikarai	11.578287994955963, 78.49669332982101	Salem
11	Attur	11.601006780799647, 78.59691957206958	Salem
12	Singipuram	11.625408778112208, 78.41173586328848	Salem
13	Krishnagiri	12.527487369732494, 78.21500036445342	Krishnagiri
14	Rayakottai	12.515828521002238, 78.03191381220594	Krishnagiri
15	Hosur	12.748212609908164, 77.83581406836144	Krishnagiri
16	Gundalapatti	12.178115378796399, 78.17690314167471	Krishnagiri
17	Bommidi	11.985749117328707, 78.25792730721723	Dharamapuri
18	Thoppur	11.944101988529056, 78.05742683411441	Dharamapuri
19	Kadathur	12.095210056909767, 78.29157293530848	Dharamapuri
20	Papparapatti	11.52862749255263, 78.06841315845728	Dharamapuri

Morphological and biochemical characterization of NKMV-3.

The cells of the NKMV-3 strain were Gram positive, motile, short rod-shaped, aerobic bacterium with cell sizes ranging from 1–3 µm. The colony of the strain on nutrient agar and Luria Bertani agar was irregular in form with flat elevation and undulate margins. The colour of the colony was dull white with matte texture. NKMV-3 was positive for gelatin liquefaction, catalase and citrate activity. The isolate was able to utilize different sources of carbon namely glucose and sucrose. The isolate was negative for acetoin production (Voges–Proskauer reaction), utilization of mannitol, arabinose and trehalose (Table 4).

Table 4
Morphological and Biochemical characters of strain NKMV-3

Description	Results	Description	Results
Shape	Short Rod	Catalase activity	+
Cell size (µm)	1–3µm	Nitrate reduction	+
Form of spores	Elliptical	Arginine	+
Mobility	+	Sucrose	+
Aerobic	+	Mannitol	-
Grams Staining	+	Glucose	+
Malonate	+	Arabinose	-
Voges–Proskauer reaction	-	Trehalose	-
Citrate	+		
Gelatin liquefaction	+		

Molecular characterization of 16S rRNA gene of NKMV-3 strain.

The *16S rRNA* gene of strain NKMV-3 was amplified using bacterial universal primers 27F and 1492R. The PCR amplification yielded 1185 nucleotides and sequenced (GenBank accession number: MZ243468). The nucleotide sequence of NKMV-3 *16S rRNA* gene showed high similarity (99.58 %) to the sequence of *B.velezensis* (Fig. 1). 16S rRNA gene sequence from similar *Bacillus* species were used for the construction of the phylogenetic tree. NKMV-3 clustered with *B. velezensis* strainsCBMB205 and FZB42.

Figure 1. **Phylogenetic tree based on** 16S rRNA **gene sequences.** The tree was constructed by UPGMA method. Bootstrap values over 50% (based on 100 replications) are shown at each node. The isolate obtained in the present study is marked with blue symbol.

Antagonistic effects of B. velezensis **strain NKMV-3 against major phytopathogens.**

A dual culture technique was performed to evaluate the efficacy of NKMV-3 strain against major agricultural phytopathogens (Fig. 2). After 7 days of incubation, NKMV-3 strain was most effective in inhibiting the mycelial growth of *A. solani* with an inhibitory percentage of 58.0 ± 0.25 %. NKMV-3 was ineffective against *Macrophomina phaseolina* (Table 5).

Table 5
In vitro antifungal activity of NKMV-3 strain against various phytopathogens on Potato Dextrose Agar Medium

Phytopathogen details	% Inhibition
Alternaria solani	**58.0 ± 0.25**
Fusarium oxysporum f.sp. lycopersici	49.4 ± 0.06
Rhizoctonia solani	41.3 ± 0.25
Pyricularia oryzae	27.9 ± 0.06
Macrophomina phaseolina	0.4 ± 0.12

In vitro effects of crude lipopeptides of B. velezensis **NKMV-3 against** A. solani.

The inhibitory effect of NKMV-3 crude lipopeptide extract was assessed using poison food technique (Fig. 3). It was observed that a 5% crude lipopeptides of NKMV-3 strain significantly (70.8 ± 1.0) inhibited the mycelial growth compared to other tested concentrations (Table 6).

Table 6
In vitro antifungal activity of NKMV-3 *A.solani*

Sample concentration (%)	% Inhibition
1	9.9 ± 1.0
2	13.6 ± 1.0
3	29.0 ± 3.06
4	58.6 ± 1.0
5	70.8 ± 1.0

Amplification of Iturin, Fengycin and Surfactin genes from B. velezensis **NKMV-3.**

Since the crude lipopeptides showed inhibitory effect against *A. solani*. The presence of lipopeptide genes in NKMV-3 namely iturin (*ituC*), surfactin (*sfrA*) and fengycin (*fenB* and *fenD*) were checked by PCR amplification using primers reported in the literature and as describedin the earlier section. Upon PCR amplification, 670 bp corresponding to *sfrA*, *fenB* and *fenD* and 594 bp corresponding to *ituC* were visualized. Thus, all these four lipopeptide genes were present in *B. velezensis* NKMV-3 (Fig. 4).

Identification of Lipopeptides from B. velezensis **NKMV-3 though Reverse Phase – HPLC**

The lipopeptides namely iturin, fengycin and surfactin in crude methanolic extract were separated and identified using a RP-HPLC by comparing its retention time to the specific standards (Sigma, USA).

Compounds similar to iturin, fengycin and surfactins were isolated with retention times ranging from 4 to 7 min, 12 to 15 min and 27 to 30 min respectively (Fig. 5).

Structural Analysis of crude lipopeptides from B. velezensis **NKMV-3 through FTIR.**

The FTIR analysis for crude lipopeptides is exhibited in Fig. 6. A broad peak at 3308 cm^{-1} specifies the existence of –OH or –NH groups. FTIR peaks at 2957.79, 2871.63 and 1466.26 cm^{-1} indicate the existence of the –C-H stretching (-CH3 or CH2) of the aliphatic chain of lipids present in the NKMV-3. The presence of the peptide fraction in sample was revealed by the wavenumbers responsible for amide bond at 603.14, N-H bending of the secondary amides at 1542 and 1649.21 of carbonyl group (C = O). A peak at 1208 cm^{-1} confirms the presence of C-O bending of esters in the NKMV-3.

Effects of crude lipopeptides against A. solani **in detached tomato leaves.**

The inhibitory effect of various concentrations of NKMV-3 crude lipopeptide against *A.solani* was assessed using detached tomato leaf bioassay(Fig. 7). There was an evident reduction of lesion diameter with increase in concentration of the crude lipopeptides. 5% crude lipopeptides showed maximum reduction in lesion diameter (0.4 ± 0.32 %) in comparison to control (Fig. 8).Lesion diameter observed after 7 days after incubation.

Values are averages of three replicates. Bars represent means from replicates, and error bars represent standard errors. Differentletters indicate significant differences between different treatments according to Tukey'stest (α = 0.05)

Discussion

Early Blight of Tomato has become an economically important disease both under field conditions and also under storage of tomato fruits [39, 40], thus garnering interest from both field pathologists and also from post-harvest disease control specialists. Several synthetic fungicides have been used by farmers for the control of early blight of Tomato [42]. But, due to their continuous usage and also non-adherence to prescribed dosages, resistance build-upof resistance has been observed [43] among the pathogens. Hence, a safe and sustainable method of disease control is the need of the hour. Soil samples from 20 different tomato growing regions of the state of Tamil Nadu, India was used for the isolation of 146 bacterial antagonistic bacteria in this study. Among them, NKMV-3 was found inhibit *A. solani* with highest percent of inhibition. Many species of the genus Bacillus are known to exhibit antifungal activity against various plant pathogens especially against *A.solani*[1, 44].

Strain NKMV-3 was identified as *B. velezensis*, based on morphological, biochemical and 16S rRNA gene sequencing. *B. velazensis* is a farmer friendly bacterium reported to promote plant growth [45], control plant diseases [46, 47] and detoxify pollutants [48]. Several *B. velezensis* isolates were found to effectively control various Phyto-pathogens such as *Botrytis cinerea*, *Alternaria solani*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*among others [49]. *B. velezensis* has already been reported to

control R. solanacearum, *F. oxysporum* and *Verticillium dahlia* infecting tomato plant [21, 50]. This research articles is the first report of a *B. velezensis* strain isolated from major tomato growing regions of Tamil Nadu, India which is effective against *A. solani*.

Dual-culture results showed *B. velezensis* NKMV-3 not only inhibit the growth of A. solani, also inhibited other two tomato pathogens, *F. oxysporum*f.sp. *lycopersci*and *R. solani*. These results suggest that *B. velezensis* NKMV-3 could control not only early blight but also vascular wilt and foot rot diseases in tomato. These were tested at the laboratory scale, the efficacy of *B. velezensis* on the field against other tomato diseases will be studied in future.

To further understand the biocontrol mechanism of B. velezensis NKMV-3, *A. solani* was used as an indicator. We confirmed the presence of essential genes for lipopeptides secretion namely, iturins, fengycin and surfactinsin *B. velezensis* NKMV-3 by PCR amplification. The presence of all the three secreted lipopeptides were identified through RP-HPLC and FTIR analyses. To date, lipopeptides secreted by *B. velezensis* have been documented to inhibit the growth of phytopathogens, such as *R. solani, F. oxysporum, A. flavus* and *Ralstonia solanacearum*[19]. In our findings, 5% crude lipopeptide extract effectively inhibited *A. solani* as evidenced by both poison plate technique and detached leaf assay.Chen et al., 2018 showed the lipopeptides secreted by *B. velezensis* LM2303 inhibited *F. graminearum* by damaging the cell membrane permeability. The same mechanism could also work in *A. solani*, this will be tested in future.

Conclusion

This study clearly revealed the biocontrol potential of B. velezensis strain NKMV-3 isolated from the tomato growing regions of Tamil Nadu. *In vitro* investigations through lipopeptide biosynthesis gene detection, RP-HPLC, FTIR analysis and antifungal activities clearly demonstrated its ability to be used as a biocontrol agent for controlling fungal pathogens. The commercialization of the product could be possible with formulations and field trials in future.

Declarations

Conflict of interest

There is no conflict of interest to this work

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

VM: data acquisition, manuscript writing; BNV: data analysis; DM: proofreading; characterization and SRM: project guidance and correspondence.

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Figures

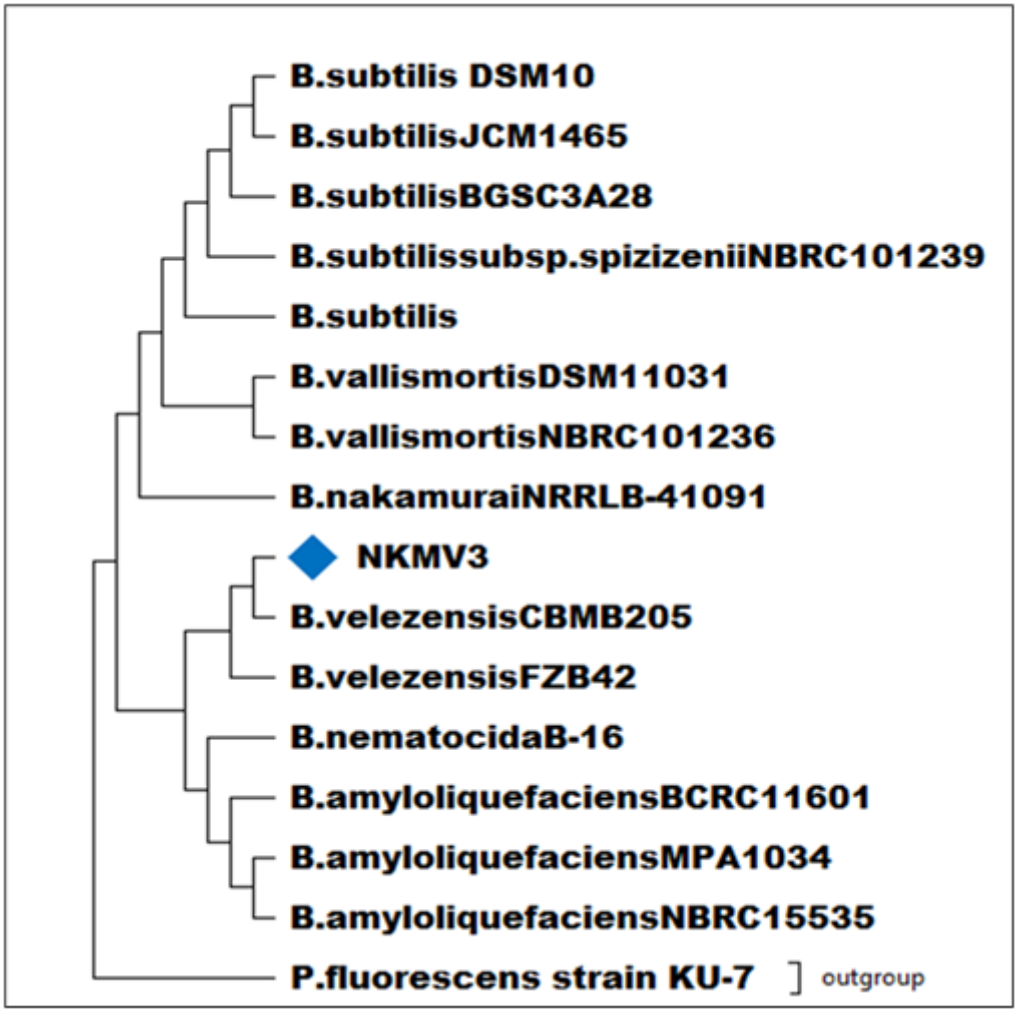

Figure 1

Phylogenetic tree of the B.velezensis NKMV-3 strain based on 16S rRNAsequence analysis constructedusing the neighbor-joiningmethod

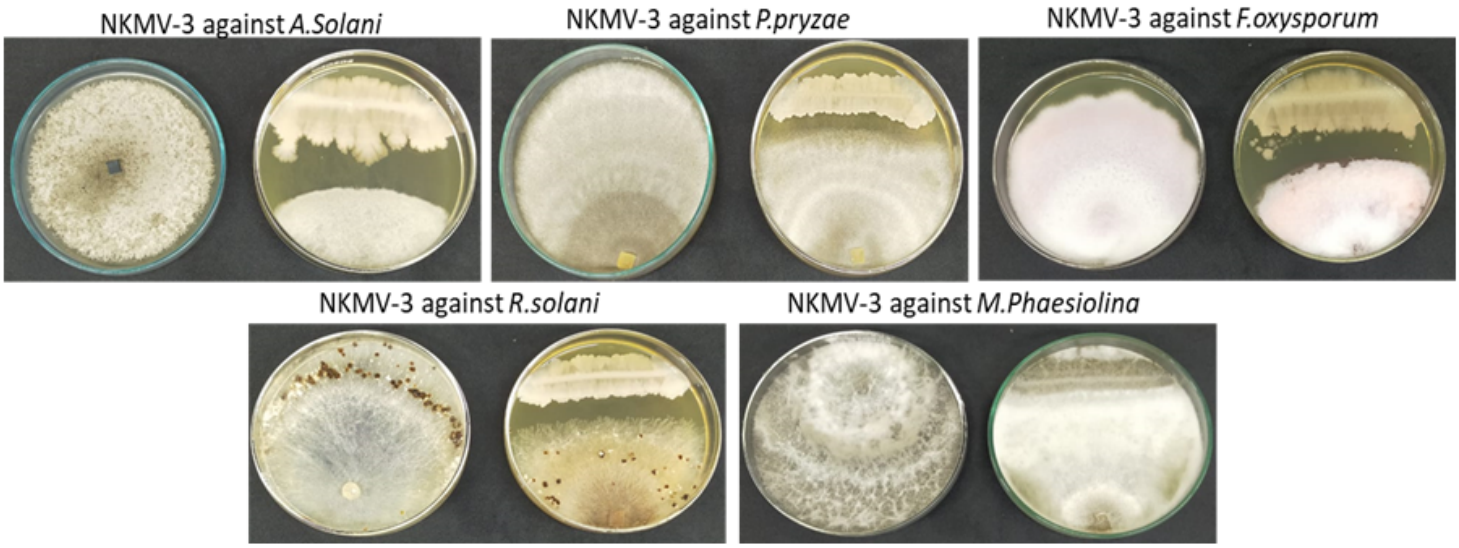

Figure 2

Invitro Antagonistic activity of B.velezensis strain NKMV-3 against various phytopathogens

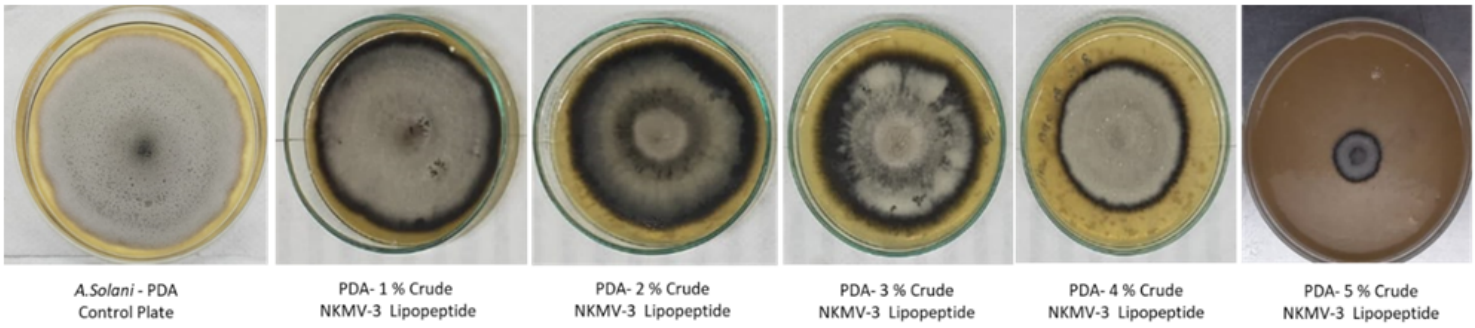

Figure 3

Invitro effect of various concentrations of the crude lipopeptides against A.solani

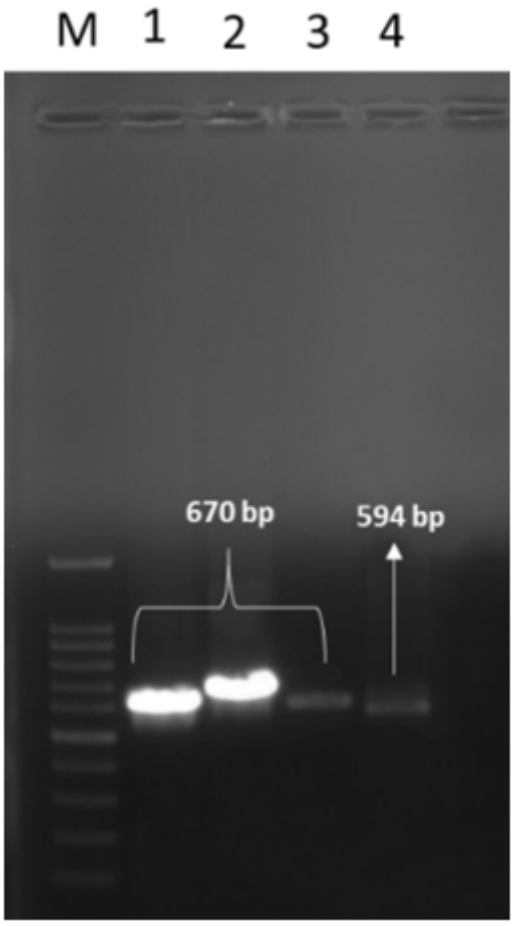

Figure 4

PCR amplification of crude lipopeptide genes from B.velezensis strain NKMV-3. Agarose gel Electrophoresis of PCR products for the genes of (1) Surfactin: Sfr A, (2,3) Fengycin: fen b, fen D and (4) Iturin: ItuC of NKMV-3 strain, M: 1000 bp DNA ladder

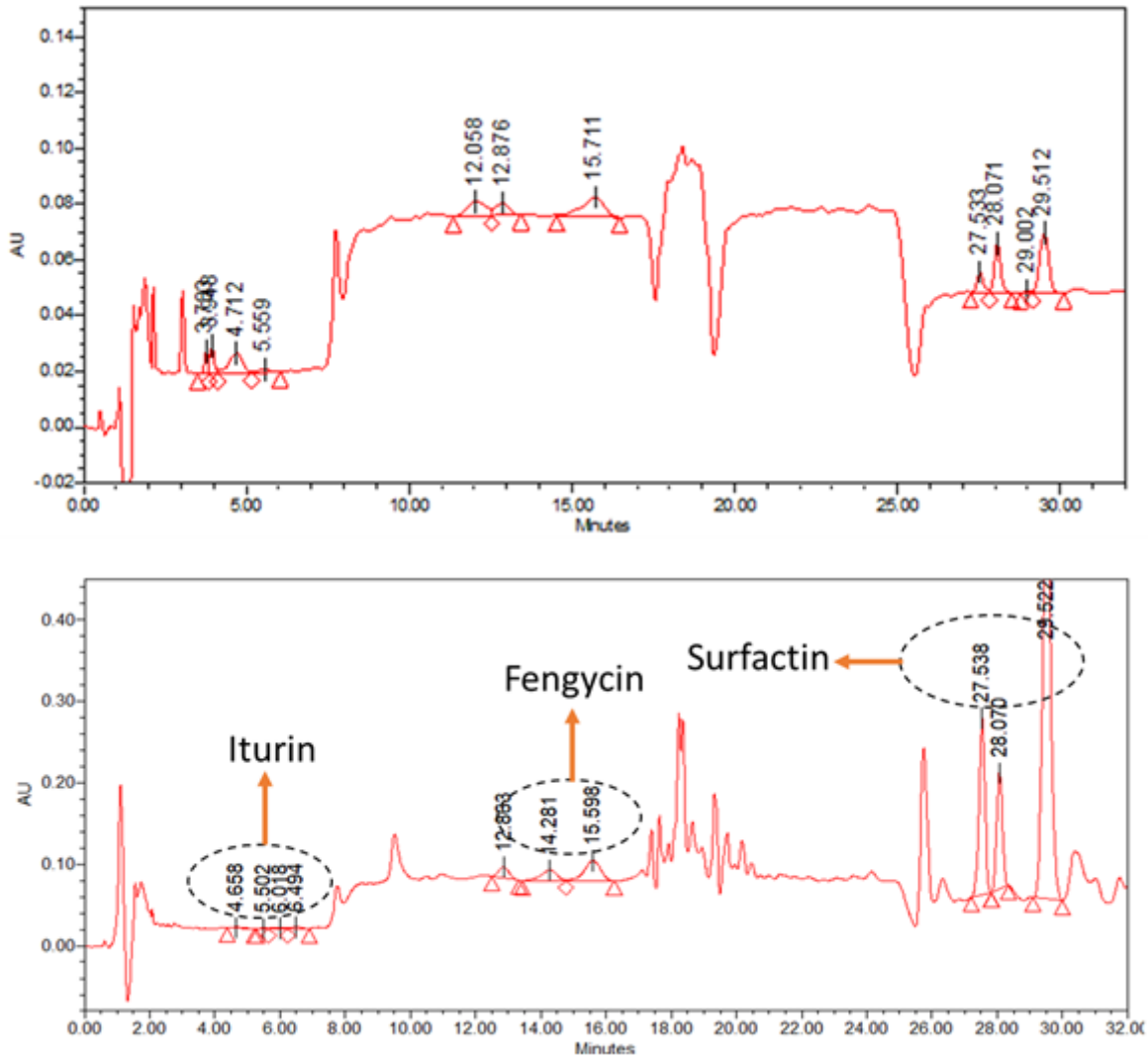

Figure 5

High-performance liquidchromatography (HPLC) profileof crude lipopeptides fromB. velezensis NKMV-3. The crude lipopeptide extract exhibited fractions consisting of Iturin, Fengycin and Surfactin

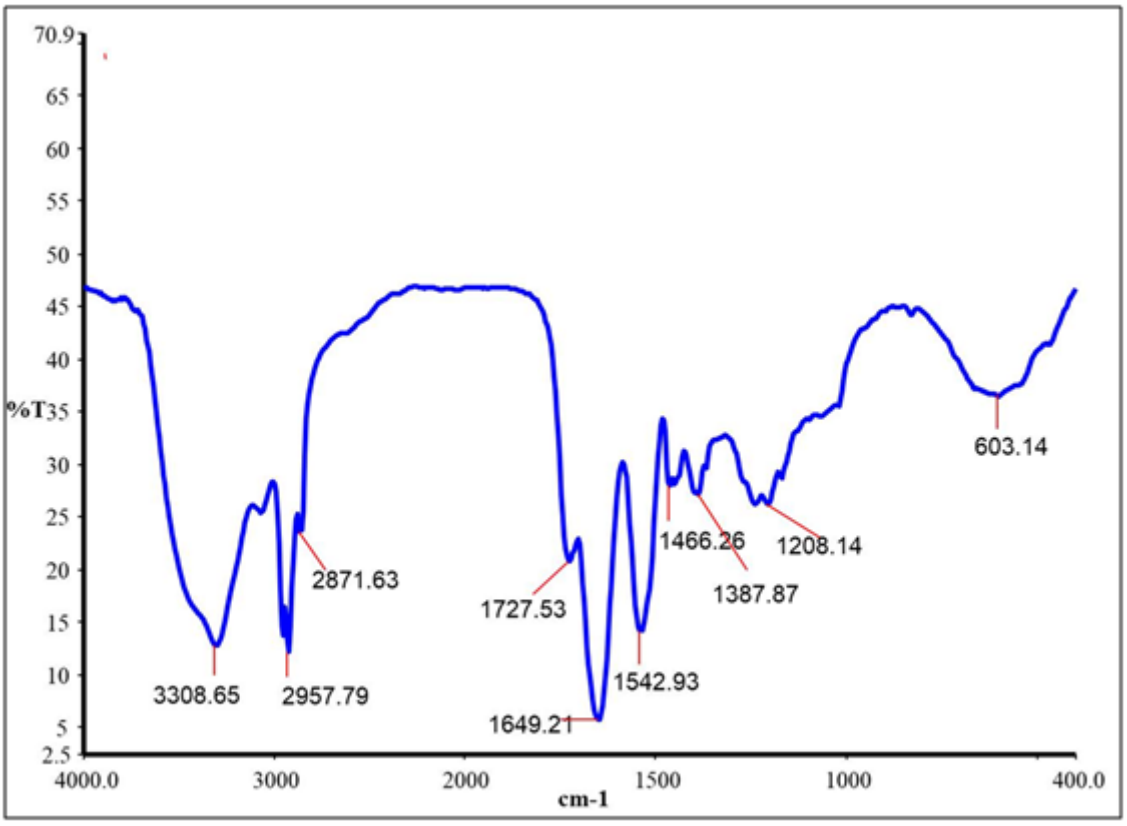

Figure 6

FTIR analysis of Lipopeptide extract from B. velezensisstrain NKMV-3

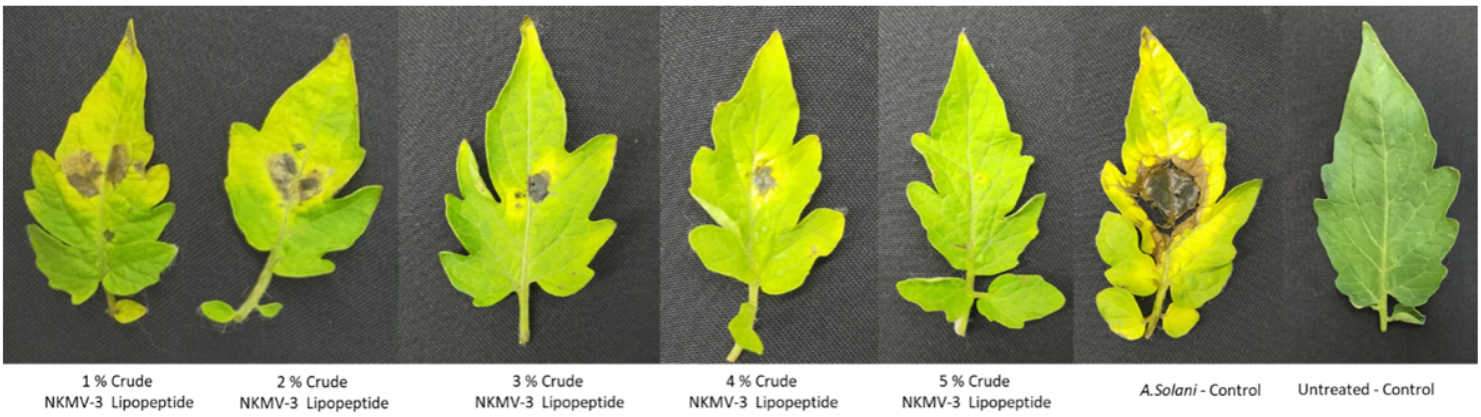

Figure 7

Detached leaf bioassay of crude lipopeptides from B.velezensis strain NKMV-3 against A.solani

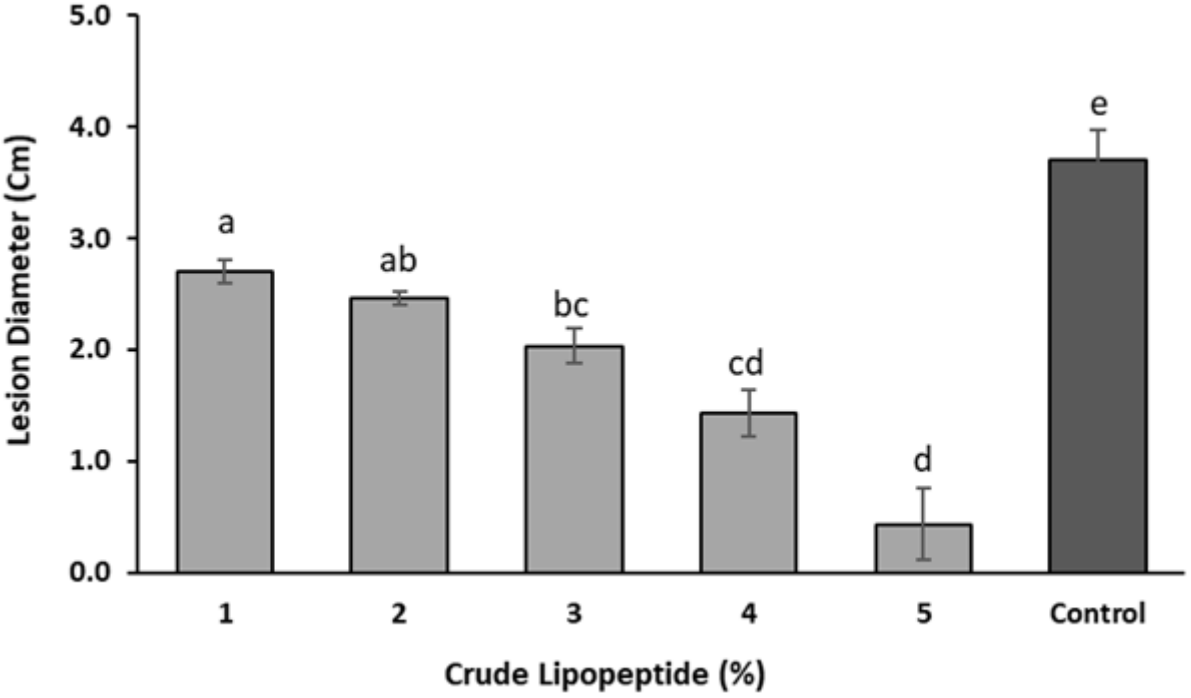

Figure 8

Lesion diameter of detached leaf bioassay of crude lipopeptides from B.velezensis strain NKMV-3 against A.solani