Multifarious allelochemicals exhibiting antifungal activity from *Bacillus subtilis* MBCU5

Urja Pandya¹ · Sunita Prakash² · Kishor Shende³ · Umesh Dhuldhaj⁴ · Meenu Saraf¹

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Abstract A potential antagonist, designated strain *Bacillus subtilis* MBCU5 was previously isolated from vermicompost-amended soils of Gandhinagar, Gujarat, India. Crude allelochemicals from strain MBCU5 displayed strong antifungal activity against *Macrophomina phaseolina* as well as *Rhizoctonia solani*. These crude allelochemicals were tentatively identified as iturin, fengycin and surfactin through TLC and HPTLC analysis. Lipopeptides produced by MBCU5 were identified by MALDI-TOF-MS and LC-ESI-MS/MS analysis showed that iturin homologues (m/z 1020–1120), surfactin (m/z 1008.7 and m/z 1022.7), fengycin A and fengycin B (m/z 1400–1550) types of allelochemicals which are responsible for antifungal activity against pathogens. PCR analysis showed presence of genes (i.e. Iturin A synthetase KJ531680 and Surfactin synthetase KJ601726) involved in the biosynthesis of allelochemicals. Many reports showed lipopeptides from *Bacillus* species; this is the first report executed of multifarious

allelochemicals from vermicompost-amended soil due to the presence of predominant *Bacillus* species.

Keywords Allelochemicals · Antifungal · *Bacillus* · Lipopeptides · Mass spectrometry

Introduction

Soil amendments such as inorganic and organic types have important role in improving the soil fertility in terms of biological as well as chemical constituents. Among different types of amendments, vermicompost is proving to be highly nutritive fertilizers over the conventional composts (Sinha et al. 2009). Vermicompost provided efficient nutrients to the soil and this would increase microbial population in the soil. Earthworm excreted mucus from its digestive system and it can trigger antagonism between microbial populations and which resulted in the production of allelochemicals which are biologically synthesized molecules (Pandya et al. 2014).

Use of various chemical-based fertilizers resulted in imbalance of ecological diversity of microbes inhabiting soil, ground water contamination, and resistant strains of various pathogens, which lead to health risks in humans and animals. The alternative approach is to use microbial-derived products based on their antifungal activity for eco-friendly disease management (Mnif et al. 2016). Allelochemicals are very important part of integrated disease management. These compounds worked as natural pesticides and solved the problems due to application of chemical fertilizers (Barazani and Friedman 2001; Farooq et al. 2001; Saraf et al. 2014). The employment of Plant growth promoting rhizobacteria (PGPR) specifically as biocontrol agent is known as an alternative strategy for control of all types of soil borne pathogens such as bacteria, fungi, etc.

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✉ Meenu Saraf
sarafmeenu@gmail.com

¹ Department of Microbiology and Biotechnology, School of Sciences, Gujarat University, Ahmedabad 380 009, India

² Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

³ Department of Biotechnology and Bioinformatics Center, Barkatullah University, Bhopal, Madhya Pradesh 462026, India

⁴ Department of Biotechnology, School of Life Science, North Maharashtra University, Jalgaon 425001, India

which are harmful to sustainable agriculture (Berg and Smalla 2009). A wide range of cyclic lipopeptides has been reported by many species of *Bacillus* which can inhibit growth of fungal pathogens (Okigbo 2005).

These types of allelochemicals can inhibit fungal pathogens directly and indirectly. Therefore, these attributes make *Bacillus* species effective biocontrol agent. Thus, the present work was aimed to study *Bacillus subtilis* MBCU5 isolated from vermicompost area of Gujarat for their allelochemicals production. Crude lipopeptides were checked for their antifungal activity. Fourier transform infrared spectroscopy analysis was studied for determination of functional groups of allelochemicals. Further, detection of allelochemicals was carried out by different techniques.

Materials and methods

Bacterial strain

The bacterial strain MBCU5 isolated in a previous study from ecological diversity of rhizobacteria from vermicompost-amended soils in Gujarat (Pandya et al. 2014). Preliminary characteristics such as morphological, physiological and biochemical were studied for its identification at our laboratory (Krieg and Holt 1984). Bacterial identification (16S rRNA sequencing) was carried out at Xcelris Labs, Ahmedabad, India. Sequence homology analysis was carried out with the aid of Nucleotide BLAST against 16S rRNA database, NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and phylogeny was derived with closely (>90% homology) related *Bacillus* species using MEGA 6 software tool (Tamura et al. 2013).

Production and extraction of allelochemicals

The MBCU5 strain was activated on nutrient agar medium (g/l; peptone 5, beef extract 2, yeast extract 3, sodium chloride 5 and agar 18, pH 7.0) and then inoculated in 500 ml of Tryptic soy broth under shaking conditions for 72 h at 37 °C. The broth was centrifuged at 10,000g at 4 °C for 20 min and supernatant was collected. Briefly, allelochemicals were concentrated by acid precipitation and then acid precipitates were solubilized in anhydrous methanol. The yellow gummy material was remained after evaporation of excess methanol was collected and further used for analysis (Pathak et al. 2012).

Fungal strains and antifungal activity of crude allelochemicals

Two fungal strains viz. *M. phaseolina* and *R. solani* were obtained from the Cultural Collection of Gujarat

University, Ahmedabad. A 10^8 cfu/ml of spore suspension was prepared from both fungus and then incorporated in PDA medium at 45 °C in Petri dishes. Different concentrations of crude allelochemicals such as 0, 2, 4, 6, 8 and 10 µl were taken and prepared disc (Whatman Filter No. 1) and placed on the agar medium and incubated at 25 °C for 24–48 h. Antifungal activity was observed by the formation of inhibition halos around the discs (Calderia et al. 2011).

TLC and high performance thin layer chromatography (HPTLC) analysis

The crude extract was fractionated by TLC on silica gel plates, developed chloroform: methanol: water (65:25:4, v/v/v) as solvent system. Free amino acids were detected with ninhydrin and iodine vapor. Similarly crude extract further studied for HPTLC analysis as method reported by Al-Wahaibi et al. (2014).

Fourier transform infrared spectroscopy (FT-IR) analysis

Structural groups from allelochemicals were identified through FT-IR analysis. In brief, 1 mg of crude allelochemicals was taken and mixed with 100 mg of KBr. It resulted in translucent pellets. FTIR spectra were recorded between 400 and 4000 cm^{-1} (Kumar et al. 2009).

Identification of allelochemicals from *B. subtilis* MBCU5

MALDI-TOFF analysis from crude allelochemicals was carried out as per method of Pathak et al. (2012). For LC–ESI–MS/MS analysis, crude allelochemicals were analyzed as per method of Pathak and Keharia (2013).

Amplification of allelochemicals genes

MBUC5 strain processed for genomic DNA and electrophoretic analysis according to standard protocols. For surfactin, forward primer SFP-F (5'-ATG AAG ATT TAC GGA ATT TA-3') and reverse SFP-R (5'-TTA TAA AAG CTC TTC GTA CG-3') were used for amplifications (Velho et al. 2011). In case of iturin, forward primer ITUA-F (5'-TGC CAG ACA GTA TGA GGC AG-3') and reverse ITUA-R (5'-CAT GCC GTA TCC ACT GTG AC-3') were used for amplification (Cao et al. 2012). Amplification was performed with a PCR system DNA thermal cycler (Applied Biosystem Veriti Model) program as per protocol of Cao et al. (2012). The obtained PCR fragments were sequenced at Xcelris Labs (Ahmedabad, India).

Results and discussion

Bacterial identification by phylogenetic analysis

The identification of the bacterial strain MBCU5 was confirmed with partial 16S rRNA sequencing (Fig. 1). Set of 16S rRNA sequences were aligned by ClustalW program and phylogeny was derived using MEGA6 software. Clustering was done by maximum likelihood method using substitution model Kimura 2 parameter and test of phylogeny was done by Bootstrap method with 1000 replicates. *B. subtilis* of strain MBCU5's 16S RNA sequence is partial of length 1348 bps, so ML method was selected to derive the phylogeny. Based on all informative sites, the sequence is clustered with sequences of other *B. subtilis* strains as an external link and closely related to all other *Bacillus subtilis* group species as, *B. tequilensis*, *B. malacitensis*, *B. mojavensis*, *B. axarquinensis*, *B. siamensis*, *B. amyloliquefaciens* and *B. methylotrophicus*, *B. nematocida* and *B. vallismortis*. *B. sonorensis*, *B. licheniformis* and *B. aerius*, whereas *B. atrophaeus* is externally linked. All these species have shown similarity more than

99%. A cluster of *B. safensis* and *B. pumilus* is linked externally with similarity in between 98 and 99%. Though the 16S rRNA sequence of *B. subtilis* strain MBCU5 is partial but still due to similar sequence patterns associated with the *B. subtilis* group, it has been clustered in this group and therefore the strain is of *B. subtilis*. The partial sequence of *B. subtilis* MBCU5 has KF758454 genebank accession number.

Antifungal activity of crude allelochemicals

At higher concentration of crude allelochemicals showed maximum fungal growth inhibition. The methanolic extract from strain MBCU5 exhibited concentration-dependent inhibition of growth of *R. solani* and *M. phaseolina*. The maximum growth inhibition of *M. phaseolina* (2.07 mm) and *R. solani* (1.54 mm) was recorded at 10 µl concentration of MBCU5 crude allelochemicals (Table 1). Arrebola et al. (2010) also reported higher concentration of lipopeptides from *B. subtilis* and *B. amyloliquefaciens* extract showed higher growth inhibition of fungal pathogens. Similar

Fig. 1 Phylogenetic analysis based on 16S rRNA gene sequences of strain MBCU5 (*Bacillus subtilis*). Clustering method: maximum likelihood, substitution model: Kimura 2 and test of phylogeny: bootstrap with 1000 replicates. Tree scale shows 1 nucleotide change per 100 nucleotides

Fig. 2 Analysis of crude methanolic extract of the strain MBCU5 (*B. subtilis*) through (a) TLC and (b) HPTLC

Fig. 3 Fourier transform infrared (FT-IR) spectrum analysis of crude allelochemicals produced by the strain MBCU5 (*B. subtilis*)

observations have been documented by Pandya and Saraf (2014) in their in vitro antifungal assay of *B. sonorensis* MBCU2 against *M. phaseolina*. Results are similar to

Mnif et al. (2016) studied efficiency of *B. subtilis* SPB1 lipopeptides for growth inhibition of *R. bataticola* and *R. solani* in vitro conditions.

Fig. 4 MS/MS spectra of the extract of strain MBCU5 (*B. subtilis*) for molecular mass ions at m/z 1449.9 (a) and m/z 1477.9 (b)

Fig. 5 MS/MS spectra of the extract of strain MBCU5 (*B. subtilis*) precursor ions at m/z 1463.9 and m/z 1505.9

TLC and HPTLC analysis

Partially purified methanolic fractions from strain MBCU5 showed the presence of major four spots with R_f values of 0.71, 0.68, 0.30 and 0.09 (Fig. 2). Romero et al. (2007)

reported standard R_f values of three lipopeptides. Results showed iturin A (0.3 R_f), surfactin (0.7 R_f) and fengycin (0.09 R_f). Kumar et al. (2009) reported TLC analysis with chloroform: methanol: acetic acid (40:4:1) as solvent system and detected one spot of R_f 0.67 value.

Fig. 6 MS/MS spectrum of the extract of strain MBCU5 (*B. subtilis*) at m/z 1008.7

Fig. 7 MS/MS spectrum of the extract of strain MBCU5 (*B. subtilis*) at m/z 1022.7

FTIR (Fourier transform infrared spectroscopy) analysis

The IR spectrum of methanolic extract showed different groups through FT-IR analysis. The IR spectrum in KBr showed N-H group at 1658.78 cm^{-1} and between 3300 and 3500 cm^{-1} , C=O at 1114.80 cm^{-1} and most important

is C-H (alkane) group at 1463.97 cm^{-1} . All these groups indicated the presence of peptide compounds (Fig. 3). *B. subtilis* extract showed similar presence of functional groups that indicated lipopeptide nature of compounds (Romero et al. 2007). The FTIR spectrum of *B. subtilis* ATCC 6633 showed strong absorption bands of peptides at 3312 , 1647 and 1539 cm^{-1} resulted due to the stretching

Fig. 8 MALDI TOFF analysis of iturin from the extract of strain *B. subtilis* MBCU5

mode of N–H, stretching mode of the C=O bond, and the deformation mode (combined C–N stretching mode) of the NH bond, respectively (Dehghan-noudeh et al. 2005).

Mass spectrometry-based characterization of *B. subtilis* MBCU5 allelochemicals

MALDI-TOF MS was used in positive ion mode for detection of allelochemicals from methanolic extract of strain *B. subtilis* MBCU5. For MS analysis, iturin, surfactin and fengycin families were detected using two clusters of molecular mass ions, i.e., (1) m/z 900–1150 and 2) m/z 1400–1550 respectively.

Molecular mass ions for Fengycins were recorded in the range from m/z 1400–1550 in the LC–ESI–MS spectrum (Suppl. File 1). Many researchers were reported these molecular mass ions as fengycins (Vanittanakom et al. 1986; Vater et al. 2002). Fengycins are cyclic lipopeptides with decadepsipeptide with β -hydroxy fatty acid moiety. Fengycins are microheterogeneous lipopeptides varying cyclic peptide ring as well as in the chain length of β -hydroxy fatty acid. Pathak et al. (2012) reported five different classes of fengycins due to variation in peptide ring at amino acid positions 6, 10. To characterize the fengycin cluster, each of the putatively assigned fengycin ions was subjected to auto MS/MS analysis. Figure 4 showed molecular mass ions at m/z 1449.9 and m/z 1477.9 from MS/MS spectra analysis. Both the MS/MS spectra at precursor ions, m/z 1449.9 and m/z 1477.9 shows similar types of characteristics of product ions at m/z 966.5 and 1080 (Madonna et al. 2003; Pathak et al. 2012). These ions could be assigned as $-y_8$ and $-y_9$ ions generated due to neutral

Table 1 Statistic analysis of growth inhibition radius (mm) of fungal pathogens from lipopeptide extract from *Bacillus subtilis* (MBCU5) after 48 h of incubation

Lipopeptide extract concentration (μ l)	Fungal growth inhibition (mm)	
	<i>M. phaseolina</i>	<i>R. solani</i>
0	0	0
2	0.03 $\pm$ 0.99	0.11 $\pm$ 0.04
4	0.19 $\pm$ 0.09	0.19 $\pm$ 0.07
6	1.35 $\pm$ 0.01	1.11 $\pm$ 0.10
8	1.49 $\pm$ 0.02	1.26 $\pm$ 0.09
10	2.07 $\pm$ 0.04	1.54 $\pm$ 0.10

Data are presented as the mean $\pm$ SE of three different experiments with three replicates

loss of Orn-Glu- β -hydroxy fatty acid moiety and Glu-hydroxy fatty acid, respectively. On the basis of characteristic ions, ions at m/z 1449.9 and m/z 1477.9 could be identified as Fengycin A homologues with C14 and C16 hydroxy fatty acid (Pathak et al. 2012). Fengycin A homologues are varying from Fengycin B homologues with variation of Ala⁶ (Fengycin A) to Val⁶ (Fengycin B) (Vanittanakom et al. 1986; Madonna et al. 2003). Similarly, present data indicated MS/MS spectra of precursor ions at m/z 1463.9 and m/z 1505.9 characteristic product ions at m/z 994.5 and m/z 1008.6 as shown in Fig. 5. These ions are also assigned as $-y_8$ and $-y_9$ ions generated due to neutral loss of Orn-Glu- β -hydroxy fatty acid moiety and Glu-hydroxy fatty acid, respectively. The precursor ions at m/z 1463.9 and m/z 1505.9 could be identified as C14 and C16 homologues of Fengycin B with Val at position 6 (Pathak et al. 2012).

Table 2 PCR amplification and sequence analysis from biosynthetic genes

Primer name	Match with proper genes by BlastN with accession number	Identity of corresponding gene by BlastN (%)	Match with probable proteins obtained by BlastX with accession number	Identity of corresponding gene by BlastX (%)	Present study deposited accession number
<i>ItuA1F/ituA1R</i>	<i>ituA</i> JN093024.1	100	Mycosubtilin synthase subunit A JN093024.1	82	Iturin A synthetase KJ531680
<i>sfp1F/sfp1R</i>	<i>sfp</i> X63158.1	100	4'-phosphopantetheinyl transferase <i>sfp</i> P39135.2	99	Surfactin synthetase KJ601726

The signals varying from m/z 994 to 1120 were putatively assigned to members of surfactin family based on literature as reported by researchers (Kowall et al. 1998; Grangemard et al. 2001; Vater et al. 2002) (Suppl. File 2). The precursor ions at m/z 1008.7 and m/z 1022.7 were subjected to MS/MS analysis. The MS/MS spectrum at m/z 1008.7 shows series of $-b_1$ to $-b_6$ and $-y_3$ to $-y_5$ ions facilitating identification of surfactin homologue as C_{13} surfactin (L/I^2 , L/I^4 , L/I^7) (Pathak et al. 2014a, b) (Fig. 6). Similarly, peptide sequence assignment from the MS/MS spectrum of protonated precursor ion at m/z 1022.7 can be seen in Fig. 7. On the basis of literature molecular mass ion at m/z 1022.7 can be assigned as C_{14} Surfactin (L/I^2 , L/I^4 and L/I^7) surfactin homologue (Pathak et al. 2014a, b). Surfactins are cyclic lipodepsipeptide with β -hydroxy fatty acid. Surfactins variants differ from each other at amino acid positions 2, 4, 6 and/or 7 as well as in the chain length β -hydroxy fatty acid (Kowall et al. 1998; Vater et al. 2002). The common peak at m/z 685.5 observed in all two spectra of protonated precursor ions of surfactin homologues in CID-MS/MS of m/z 1022 and m/z 1036 (Hue et al. 2001). It is to be observed that the peak at m/z 685 is observed in all spectra; hence, it could be the characteristic marker ion for the surfactin homologues. The peak at m/z 686.5 indicates the presence of internal protonated fragment ion $[(H) \text{Leu}^2\text{-Leu}^3\text{-Val}^4\text{-Asp}^5\text{-Leu}^6\text{-Leu}^7 (OH)+H]^+$ of intact protonated precursor ions of surfactin homologues (Pathak et al. 2014a, b).

Iturin data showed mass range for m/z 1020–1120 from methanolic extract of MBCU5. Iturin A homologue confirmed at m/z 1043.6 and 1057.6 and iturin D confirmed at m/z 1073.5 (Fig. 8) (Pathak and Keharia 2013; Pathak et al. 2014b). Sodium and potassium adducts were appeared with their protonated species in mass analysis (Williams and Brodbelt 2004; Sabareesh and Balaram 2006). It has been reported that *Bacillus* spp. produce mixture of iturin and based on the variation of C-length of β AA as well as based on the isomers of β AA (normal, *iso* and *anteiso*), iturin A homologues (iturin A1–A8) (Isogai et al. 1982; Tilvi and Naik 2007).

Identification of biosynthetic genes from MBCU5

PCR analysis results indicated that the presence of both genes were responsible for iturin and surfactin production. Both

sequences were deposited with accession number at NCBI. BlastN and blastp results showed the maximum homology (Table 2). The sequence of the fragment by primers *ituA-F/ituA-R* showed 82% homology with the Mycosubtilin synthetase subunit A (JN093024.1). The sequence of the fragment by primers *sfp-F/sfp-R* showed 99% homology with the 4'-phosphopantetheinyl transferase *sfp* (P39135.2). Surfactin synthetase and iturin synthetase proteins belong to the families phosphopantetheinyl transferase and polyketide synthase. These two families indicated non-ribosomal peptides (NRPs) which are multidomain enzymes that catalyze the formation of various types of lipopeptides (Ongena and Jacques 2008).

Conclusion

Bacillus subtilis MBCU5 isolated from vermicompost-amended soil produced antifungal allelochemicals in vitro conditions. Preliminary analysis by FT-IR shows the presence of lipopeptides. Secondary analysis by MALDI-TOF-MS indicated iturin, surfactin and fengycin types of homologues from strain *B. subtilis* MBCU5. The presence of NRPs genes were deposited in Genbank with accession numbers. In the light of all these results, strain *B. subtilis* MBCU5 isolated from vermicompost-amended soil could be excellent candidate for sources of lipopeptides production with antifungal activities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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