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MFAP9: Characterization of an extracellular thermostable antibacterial peptide from marine fungus with biofilm eradication potential

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ABSTRACT

An extracellular thermostable antibacterial peptide designated as MFAP9 was purified from marine *Aspergillus fumigatus* BTMF9 by ammonium sulfate precipitation followed by ion exchange chromatography on a DEAE-sepharose column. The molecular weight of MFAP9 was found to be~3 kDa in SDS-PAGE gel corresponding a single intensity peak in MALDI-TOF. The distinct peak with a retention time of 32.5 min appeared in high performance liquid chromatography (HPLC), further confirming the purity. Isoelectric focusing, two-dimensional gel electrophoresis and peptide mass fingerprinting were performed for the characterization of MFAP9. Functional analysis of purified MFAP9 exhibited strong antibacterial activity against *Bacillus circulans* (NCIM 2107) with MIC and MBC values of 0.525 μg/mL and 4.2 μg/mL, respectively. The *in vitro* antibiofilm effect of MFAP9 was analyzed against bacteria which have strong biofilm forming potential. The antibiofilm effect of MFAP9 treatment on *Bacillus pumilus* was examined using scanning electron microscopy. MFAP9 was found to be active at high temperatures and a wide range of pH (28). In addition, it showed varied sensitivity towards proteolytic enzymes. The peptide was nontoxic to human RBCs at higher concentrations. These results indicate that MFAP9 is an antibacterial peptide, suitable for the development of novel anti-infective agent with strong antibiofilm potential.

1. Introduction

The global increase in the emergence of multiple drug-resistant pathogens and the shortage of effective antibiotics persuade to the development of effective alternatives. Bacterial biofilms are cellular aggregates or surface-attached microorganisms which are often resistant to antimicrobial agents [1]. Bacteria embedded in biofilms can be 1000-fold or more resistant to antibiotic treatment in comparison with planktonic bacteria. These resistant strains on biomedical devices are the leading cause of death worldwide [2].

Antimicrobial peptides (AMPs) are important contributors in this context as they exhibit a wide spectrum of activity against various Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells [3]. AMPs facilitate the development of therapeutic agents against emerging infectious diseases. Defensins are cysteine-rich peptides with a cationic and amphipathic nature, one of the largest groups of AMPs distributed across the eukaryotic kingdom. Defensins produced by invertebrates, plants and fungi are composed of a α-helix linked to an antiparallel β-sheet by disulfide bridges. This structure is called cysteine-stabilized α-helical and β-sheet (CSaβ) motif, which is a common motif in peptides with antimicrobial activity [4]. Most AMPs selectively interact with the negatively charged surfaces of microbial membranes due to their cationic nature. The most common mechanism is the membranolytic effect, where the peptide translocation towards an intracellular target leads to membrane lysis. In contrast to that of conventional antibiotics, this unique mechanism enables AMPs to avert the common resistance mechanisms [5].

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Marine filamentous fungi are significant sources for the discovery of drug leads owing to their chemical diversity and rich biological activity. In comparison with the bioactive compounds from on-land sources, marine peptides showed diverse sequences, unique structures and prominent bioactivities. Among the bioactive peptides, antibacterial cyclic peptides of marine origin fijimycin A–C, cyclomarin A and salinamide A and B are less explored classes with pharmaceutical applications [6].

Fungi represent an attractive source of antimicrobial compounds. However, only a few fungal CSaβ defensins have been characterized to date such as plectasin, copsin and eurosin, which are synthesized by *Pseudoplectania nigrella*, *Coprinopsis cinerea* and *Eurotium amstelodami*, respectively [7,8]. The filamentous fungal strain *Aspergillus fumigatus* has been reported to be the producer of metabolites with a broad range of biological activities apart from being an opportunistic pathogen affecting immuno compromised patients [11]. A series of novel natural compounds such as fumiquinazoline K, nordammarane triterpenoid, diketopiperazines and tryptoquivaline F have been isolated from the marine fungus *A. fumigatus* KMM 4631 [9]. A recent study with a marine *A. fumigatus* strain CUGBMF170049 described the characterization of two new spiroheterocyclic lactam derivatives, cephalimysins M and N, pseurotin A, and FD-838, along with already reported helvolic acid derivatives [10].

In view of this, the present study is an attempt to purify, characterize and functionally evaluate the antibacterial and antibiofilm activity of an extracellular peptide, MFAP9 from a marine sediment derived fungus, *A. fumigatus* BTMF9. To the best of our knowledge, this is the first report of the characterization of a novel defensin like peptide other than various compounds like terpenoids and cyclic peptides, which have already been identified from marine *A. fumigatus* strains.

2. Materials and methods

2.1. Strains and growth conditions

Humus rich soil samples from terrestrial environment were collected from central region of Kerala, India - Ernakulam and Alappuzha districts and marine sediment samples from onboard the research vessel FORV Sagar Sampada (Cruise No.271) were used for screening. The active strain BTMF9 obtained from marine sediment sample was grown on Mycological agar (MA) medium (HiMedia, Mumbai, India) and was identified by morphological characteristics as well as genotypic identification based on the sequencing the amplified regions of 18S rRNA gene using ITS1 and ITS4 primer pair followed by BLAST analysis. The cultures were maintained on MA slants at 4 °C and stored in glycerol at −20 °C. The indicator bacteria were procured from National Centre for Industrial Microorganisms (NCIM, Pune, India). The microorganisms used for the activity screening were propagated in media appropriate for species used.

2.2. Production of MFAP9

A. fumigatus BTMF9 isolate (2.4×10^6 spores/mL) inoculated to 100 mL of sterile Czapek-Dox (CD) minimal media taken in 250 mL Erlenmeyer flasks. The inoculated broth was incubated at 28 °C in a shaking incubator (Orbitek, Scigenics, Chennai, India) for five days at 150 rpm. Culture supernatant was obtained by filtration through four layers of cheese cloth followed by centrifugation at 13,000 *g* at 4 °C (Sigma, Osterode, Germany) for 15 min. This extracellular supernatant was used as the crude extract to evaluate antibacterial activity. The fungal strain that produced -MFAP9 after secondary screening was identified by morphology and molecular characterization.

2.3. Bacterial susceptibility assay

The disc diffusion assay was performed to screen the antibacterial activity of fungal crude extracts and ammonium sulfate precipitated fractions. Critical dilution assay was employed to identify the titer of antibacterial activity [11]. Two- fold serial dilutions of peptide sample was prepared and from each dilution, 5 μL was spotted on the surface of MH agar plates previously swab inoculated with suspension of log-phase cells of test organism (OD at 600 nm ~1). Plates were incubated at 37 °C for 18 h and zone of growth inhibition was observed. One activity unit (AU) is defined as 5 μL of the highest dilution of peptide yielding a definite zone of inhibition on the lawn of the test organism. The highest dilution was multiplied by 200 to obtain the activity units per mL (AU/mL).

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of MFAP9 were determined by using the slightly modified resazurin based broth microdilution method [12]. Two-fold dilution of MFAP9 was prepared in deionized water, at a volume of 30 μL per well in 96-well U-bottom microtiter plates. Further, 100 μL nutrient broth followed by 10 μL standardized inoculum (OD at 600 nm ~1) of *Bacillus circulans* were added to each well and incubated at 37 °C for 24 h. After incubation, 1 μL resazurin (1% stock) was added to each well and observed for colour change by incubating at 37 °C for 30 min. Blue coloured resazurin turns pink/colourless in wells which contain living cells. The highest dilution at which blue colour retained was noted and considered as the MIC value. To determine the MBC, 10 μL of broth from wells that showed no apparent growth in MIC assay was inoculated on MHA plates and incubated at 37 °C for 24 h. MBC was defined as the lowest peptide concentration that killed all or >99.9 % of the total bacteria. Ampicillin (0.1 mg/mL) was taken as the positive control.

In addition, the effect of MFAP9 on most sensitive indicator strain *B.circulans* was evaluated by scanning electron microscopy (SEM) [13]. Briefly, bacterial cells treated with partially purified MFAP9 fraction (1 μg/1 μL) and untreated control cells were kept for 1 h. Following incubation, treated and control cells were centrifuged and washed. The samples were then fixed with 3 % gluteraldehyde in phosphate buffer (pH 7.4) and dehydrated through a graded series of ethanol (20, 40, 60, 80, and 100 %). The images of treated and control samples were captured using SEM (JEOL Model JSM - 6390 L, Peabody, MA, USA).

2.4. Peptide purification

Cell-free culture filtrate was subjected to ammonium sulfate precipitation (0–30 % and 30–90 %) at 4 °C. The precipitate obtained after centrifugation was dissolved in minimum volume of 10 mM phosphate buffer (pH 7.5), and dialyzed for 24 h at 4 °C with regular changes of same buffer using benzoylated dialysis tube with cut off value of 2 kDa (Sigma-Aldrich, Mumbai, India) and kept at −20 °C until use. Dialyzed sample was lyophilized (Yamato, Neocool, Japan) and resuspended in 10 mM phosphate buffer(pH 7.5) and further applied to 25 × 1.5 cm econo column of DEAE sepharose (Bio-Rad, Hercules, CA, USA) pre-equilibrated with 10 mM phosphate buffer (pH 7.5). The elution was done initially with 10 mM phosphate buffer (pH 7.5) followed by elution of bound peptides with a linear gradient of 10−50 mM NaCl in the same buffer. Fractions were collected at a flow rate of 1 mL/min and absorbance was detected at 280 nm. Peak fractions were assayed for antibacterial activity. The fraction harboring antibacterial activity was collected and molecular weight was determined by Glycine SDS-

PAGE (18 %) using protocol as described by Laemmli [14], followed by Coomassie Brilliant Blue staining and silver staining.

2.5. Reversed-phase high-performance liquid chromatography

The active fraction got from gel filtration ion exchange chromatography (20 μL, 0.1 mg/mL) was subjected to reverse phase high-performance liquid chromatography (RP-HPLC) on Schimadzu LC 2010 using Phenomenex C18 HPLC column (22.5 mm ID × 250 mm length) (Phenomenex India Pvt Ltd, Hyderabad, Telangana, India). The solvent reservoirs contained the following eluents: (A) 0.1 % (*v/v*) trifluoroacetic acid (TFA) (B) 0.1 % (*v/v*) TFA in 80 % (*v/v*) acetonitrile. The elution program consisted of a gradient system (0–100 % solvent B in 45 min) with a flow-rate of 1.0 mL/min. The elution pattern was monitored by measuring the absorbance at 214 nm.

2.6. Electrophoretic analysis for molecular mass and bioactivity assay on gel by Agar overlay method

The active fraction obtained after ammonium sulfate precipitation was run in two lanes along with protein molecular weight marker (GeNei, Bengaluru, India) in 18 % SDS-PAGE gel. After electrophoresis, the gel was divided into two parts and one half was assayed for detecting MFAP9 band with antibacterial activity. The gel slice was immersed thrice in 50 mL Tween 80 (0.1 %) for 30 min each so as to remove SDS, followed by washing with deionized water. The gel was then placed on MH agar base plate and overlaid by soft MH agar (0.8 % agar) seeded with 50 μL ($OD_{600} \sim 1$) of test organism *B. circulans* [15]. Zone of clearance at the corresponding active band was observed after overnight incubation at 37 °C. The other half containing the sample and molecular weight marker was stained with Coomassie Brilliant Blue in order to find exact position of active MFAP9.

2.7. Isoelectric focusing (IEF) and 2-D gel electrophoresis

Isoelectric point (pI) of the purified MFAP9 was determined by isoelectric focusing (Bio-Rad, Hercules, CA, USA). Two Immobilized pH gradient (IPG) strips with range of pH 3–10 were taken for both IEF followed by 2D gel electrophoresis. After electrophoresis, IPG strips were removed from focusing tray. Then, one of the strips was stained with Coomassie brilliant blue and the other strip was placed on the precast 16 % SDS-PAGE gel for further electrophoresis followed by silver staining.

2.8. Intact mass determination by MALDI-TOF MS and peptide mass fingerprinting

The MFAP9 band with antibacterial activity was excised and electroeluted in a 2-kDa benzoylated dialysis tubing (Sigma Aldrich, Mumbai, India). For this, a voltage of 30 V was applied to the gel pieces in dialysis tubing immersed in the buffer for overnight at 4 °C, after which the electrodes were reversed and voltage of 30 V was applied for 30 min. Then, Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was employed to determine the intact molecular mass of the electro eluted peptide (Bruker Daltonics, Bermen, Germany) at Indian Institute of Science (IISc), Bengaluru, India. Additionally, the resultant peptides extracted after treatment of MFAP9 with iodoacetamide and trypsin for alkylation and digestion respectively were analyzed by MALDI-TOF-TOF mass spectrometer using a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Spectra acquired were analyzed using Mascot sequence matching software (http://www.matrixscience.com) to identify peptide of interest with Swiss-Prot and NCBInr database [16]

2.9. Effect of pH, temperature, and enzymes on the activity of MFAP9

To evaluate the effect of temperature and pH, MFAP9 was exposed to temperatures ranging from 4 °C–100 °C for 1 h and incubated with equal amount of buffers with pH ranging from 2–13 and kept for 18 h at 4 °C. The buffer systems used included hydrochloric acid- potassium chloride buffer (pH 2), citrate buffer (pH 3–6), phosphate buffer (pH 7), Tris (hydroxy methyl amino methane) buffer (pH 8 and 9), carbonate- bicarbonate buffer (pH 10 and 11), sodium hydroxide- potassium chloride buffer (pH 12 and 13) [17]. The samples after the treatment were tested for the antibacterial activity.

To understand the sensitivity to proteases, the purified sample was subjected to treatment with varying concentrations of proteinase K, pepsin and trypsin (from Bovine pancreas, SRL, Mumbai, India) ranging from 20−100 μg for 1 h at 37 °C. The treatment effect was analyzed by observing the residual antibacterial activity of MFAP9, where MFAP9 without treatment with the proteases was used as positive control. The proteases and starting buffer were used as blank controls. All assays were conducted in triplicate.

2.10. Antibiofilm assay

Indicator bacteria used for biofilm inhibition assay were taken from the laboratory collection of Department of Biotechnology, CUSAT. They are categorized as strong biofilm producers (unpublished data) based on the standard microtiter plate assay [18]. In this study, a microtiter plate assay was used to grow and quantify *in vitro* biofilms of the bacteria treated with or without MFAP9. For biofilm inhibition assay, 220 μL of tryptone soya broth (TSB) was placed in each well of 96-well polystyrene microplates. Then, fresh bacterial cell suspensions were prepared in sterile saline solution (0.9 % NaCl) to obtain cell count 10^8 CFU/mL (OD_{600} ~1) and inoculated into plates at 20 μL/well in 96-well plates. The biofilms were allowed grown to stationary phase when plates kept for 24 h at 37 °C. After incubation, the experimental wells with preformed biofilms were added with 30 μL of MFAP9 solution in TSB (5x MIC) and wells with only TSB was maintained as negative control. The microtiter plates were further incubated for 24 h at 37 °C to study the effect of MFAP9 on preformed biofilm. After 24 h incubation, the media and planktonic bacteria cultures were decanted, washed 3 times with 300 μL of phosphate buffer (0.01 M, pH 7.5). The remaining attached cells were fixed with methanol per well for 15 min and stained with 1 % (*w/v*) crystal violet (CV) for 5 min. After staining, the plates were washed and dried, and CV was solubilized with 33 % (*v/v*) glacial acetic acid and the absorbance was measured at 570 nm. Three independent experiments were performed in triplicate.

2.11. Scanning electron microscope analysis of biofilm eradication

The effect of MFAP9 on 24 h pre-established *in vitro* biofilm on cover slip by the most sensitive *Bacillus pumilus* BT3 was done and visualized using SEM [13]. Cover slip was kept in culture broth for biofilm formation for 24 h. Peptide sample was added to the pre-established biofilm and incubated at 37 °C for 24 h and the control biofilms were treated with PBS. After incubation, the coverslips were washed and fixed by immersion in 2.5 % gluteraldehyde for 1 h at room temperature. The cover slips were rinsed three times using 0.1 M sodium phosphate buffer (pH 7.3) and then treated with a graded series of alcohol (25–90 %) for 5 min each followed by 100

Table 1
Antibacterial activity of ammonium sulfate precipitated protein fraction by Critical dilution method.

Test Organism	Antibacterial activity (AU/mL)
B. circulans	3200
B. coagulans	1600
S. aureus	3200
B. pumilus	1600
B. cereus	800

% ethanol two times for 10 min each. The images were captured under SEM (JEOL Model JSM - 6390 L, Peabody, MA, USA).

2.12. Hemolytic assay

Hemolytic activity of the MFAP9 was evaluated by measuring the hemoglobin release from fresh human erythrocytes. Fresh hRBCs were washed three times with PBS (pH 7.5) and 8 % cell suspension was prepared with the same buffer. For this assay, MFAP9 solution was two fold serially diluted with deionized water. Then, aliquots of an 8 % suspension of red blood cells were transferred to each tube and incubated for 30 min at 37 °C. The reaction mixtures were centrifuged at 1000 × *g* for 10 min to remove intact erythrocytes and then supernatant containing released hemoglobin was measured at 414 nm. No hemolysis (0 %) and full hemolysis (100 %) were determined in the presence of PBS and 0.1 % Triton X-100, respectively. Hemolytic activity was expressed as a percentage hemolysis using the following equation:

$$\%\text{hemolysis} = \frac{(\text{A414nm with peptide solution-A414nm in PBS})}{(\text{A414nm with 0.1\% Triton-X-A414nm in PBS})} \times 100$$

2.13. Statistical analysis

Graph pad prism software (version 8.4.3) was used for statistical analysis. Results were analyz ed using ANOVA, followed by Bonferroni's test for multiple comparisons. The minimum value set as $P \leq 0.05$ was considered as significant. All the assays were conducted in triplicates and the results were expressed as mean ± SD.

3. Results and discussion

3.1. Production and purification

Therapeutically efficient fungal defensins are more advantageous than mammalian AMPs owing to their high antibiotic activity, low toxicity and serum stability [19]. In the present study, preliminary screening of 120 terrestrial and 10 marine fungal strains resulted in the segregation of fifteen fungal isolates with antibacterial activity. The crude extract of the shortlisted fungi were partially purified using ammonium sulfate precipitation, where the fungal strain BTMF9 exhibited maximum antibacterial activity in 30–90 % fraction (Table 1). The strain was identified as *A. fumigatus* BTMF9 (NCBI GenBank accession No.HQ285882) by observing colony morphology, microscopic observation as well as molecular analysis of fungal rDNA. Sequence analysis with BLAST software showed 100 % identity with already available sequences of *A. fumigatus* in the GenBank [20]. MFAP9 was purified to homogeneity by ion exchange chromatography using DEAE sepharose column. A step by step increase in the specific activity was observed in each stage of purification. Ammonium sulfate precipitation showed a four fold increase in the antibacterial activity and a further purification by anion exchange column containing 0.3 M NaCl in phosphate buffer (pH 7) resulted a six fold increase (Table. 2). Similar procedure has been employed for purifying Pc-Arctin from Arctic *P. chrysogenum* [21]. The purity of active ion exchange fraction was further confirmed by analytical RP-HPLC with a retention time of 32.5 min (Fig. 2.A). Majority of the antimicrobial peptides have been purified employing reversed phase chromatography and the purity of peptide 5812-A/C isolated from *Streptomyces* sp. INA-5812 preparation to be 93.2 % [22].

3.2. Bacterial susceptibility

The purified MFAP9 exhibited notable antagonistic effect towards Gram-positive bacteria which includes *Bacillus cereus* (NCIM 2155), *Bacillus circulans* (NCIM 2107), *Bacillus coagulans* (NCIM 2030), *Bacillus pumilus* (NCIM 2189) and *Staphylococcus aureus* (NCIM 2127) and no activity was observed against the Gram-negative test bacteria. A fungal defensin eurocin exhibited similar pattern in the activity against Gram-positive bacteria rather than Gram-negative bacteria [8]. AfusinC, a putative peptide produced in *Escherichia coli* is the only report available on anionic fungal defensin from *Aspergillus fumigatus* [23]. The quantitative estimation of antibacterial activity of the ammonium sulfate fraction of BTMF9 was determined by the critical dilution assay against the sensitive test bacteria. Among the test organisms used, *Bacillus circulans* (NCIM 2107) showed consistent sensitivity towards MFAP9 and hence, selected as the indicator organism for further studies (Fig. 1.A). The MIC and MBC of MFAP9 was determined as 0.525 μg/mL and 4.2 μg/mL respectively compared to the standard antibiotic ampicillin which showed the values of 0.937 μg/mL and 3.75 μg/mL. In contrast to the MIC which provides inhibitory activity information, MBC gives bactericidal activity of the test at a specific time point. The obtained MBC value of MFAP9 showed only a slight variation when compared to MBC value of ampicillin. The mode of action of AMPs is different from that of conventional antibiotics. The interactions of AMPs with target cell membranes can result membrane depolarization, membrane permeabilization or lysis, and leakage of intracellular components, eventually leading to cell death. Such a characteristic diverse mechanism of action enables AMPs in order to avoid the common resistance mechanisms observed towards conventional antibiotics.In the case of eurocin [8], the MIC for *Staphylococcus* sp. was found to be in the range 0.5–128 μg/mL and for *Enterococcus* 0.25–128 μg/mL. Similarly, antimicrobial activity of AfusinC [23], a putative anionic fungal defensin from *A. fumigatus* against Gram-positive bacteria showed MIC ranging from 4 to 64 μg/mL. Taking this in to account, the purified MFAP9 has better MIC values compared to ampicillin and also towards the previously reported AMCs eurocin and AfusinC. In addition to this, the antibacterial activity of fungal peptide was also visualized by electron microscopy. The SEM images (Fig. 1.B) of the control and the treated *B. circulans* cells (Fig. 1C) showed considerable variations in the morphology. The cells were clumped together in the treated samples and cell surface damage was visible in the electron micrograph, which may be attributed to the effectiveness of the AMP.

3.3. Bioactivity assay on SDS-PAGE gel, IEF and 2-D gel electrophoresis

The 3 kDa MFAP9 fraction obtained after ion exchange chromatography was visualized as a single band on the SDS-PAGE gel confirming its purity and homogeneity (Fig. 2 B). The antibacterial activity of the purified MFAP9 band was confirmed on the gel using zymography indicating bacterial growth inhibition as a clear zone (Fig. 2C). Purified AfusinC revealed the molecular weight of ~4 kDa in Tricine-SDS-PAGE [23]. The isoelectric point (pI) of the MFAP9 was calculated to be six. In contrast, the pI value of PgAFP

Table 2
Summary of the purification steps of MFAP9 from the culture supernatant.

Purification step	Protein conc. mg/mL	Activity AU/mL	Specific activity AU/mg	Fold of purification
Crude	0.1	1600	16000	1*
Ammonium sulfate	0.29	6400	22068.96	1.38
Ion exchange	0.13	12800	45176.47	6.15

* Value taken arbitrarily.

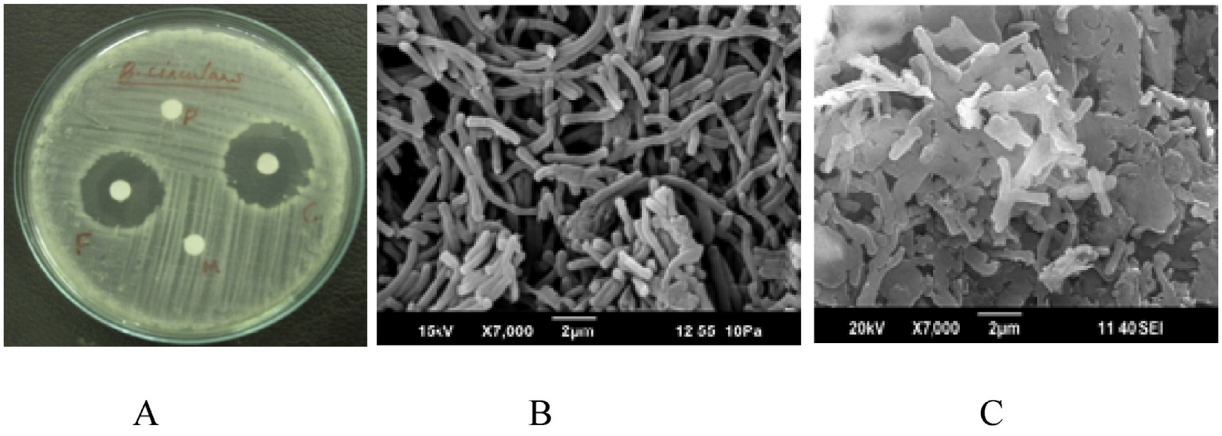

Fig. 1. A Antimicrobial activity of 30-90 % ammonium sulfate fraction against *Bacillus circulans* (NCIM 2107) by disc diffusion assay. 1.B SEM image of untreated *B. circulans* strain.1.C *B. circulans* treated with MFAP9.

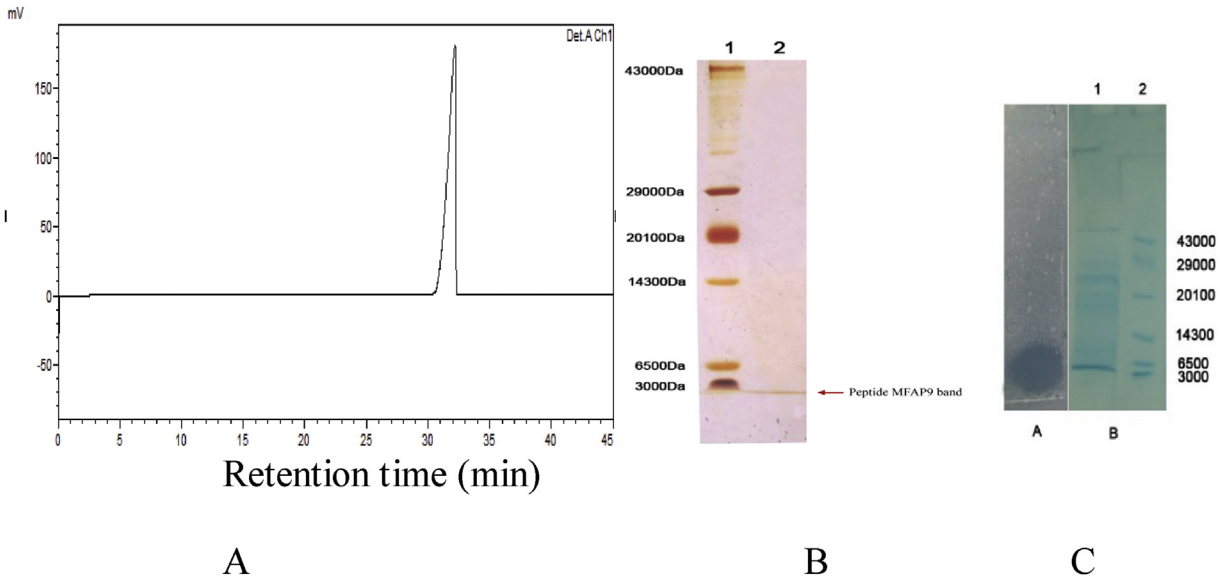

Fig. 2. A Reverse phase chromatography profile of purified MFAP9 on C-8 column on HPLC system. Absorbance was recorded at 214 nm. 2.B Glycine SDS-PAGE profile of the MFAP9 after ion exchange chromatography. Lane 1 – GeNei protein marker; Lane 2- Purified MFAP9. 2.C Glycine SDS-PAGE profile of partially purified (30-90 %) protein fraction: (A) Zone of growth inhibition corresponding to the position of MFAP9 band in gel overlaid with viable cells of *B. circulans* (~10^6 CFU/mL) seeded in MH soft agar; (B) Lane 1 partially purified active sample, and lane 2 molecular weight marker.

purified from *Penicillium chrysogenum* was estimated as 9.22 corresponding to the electrophoretic mobility of marker proteins [24]. Besides, the theoretical pI values of most of the defensin-like antifungal proteins secreted by filamentous fungi ranges from 7.14 to 9.27 [25]. Thus the obtained pI of six in the present study highlights the unique anionic characteristic feature of MFAP9. In contrast to *E. coli* membranes, the highly anionic AP1 binds strongly to the positively charged lipid lysyl phosphatidyl glycerol (LPG) of *S. aureus* present in the membrane head group region [26]. The net charge of AMPs are not responsible for the antimicrobial activity in certain cases. Instead, a single positive amino acid of the loop 3 of CSαβ defensins like AfusinC (Lys-32) is determining the antimicrobial activity [23]. 2D gel electrophoresis of MFAP9 resolved as a single spot further confirmed the purity and the absence of multiple bands with same pI value.

3.4. Intact mass determination by MALDI-TOF MS and peptide mass fingerprinting

The intact molecular mass determination by MALDI-TOF MS showed a single prominent peak with 3.055 kDa confirming a clear-cut agreement with the relative mass resulted in SDS- PAGE (Fig. 3A). The molecular mass of the extracellular, defensin-like antifungal proteins secreted by filamentous fungi ranges from

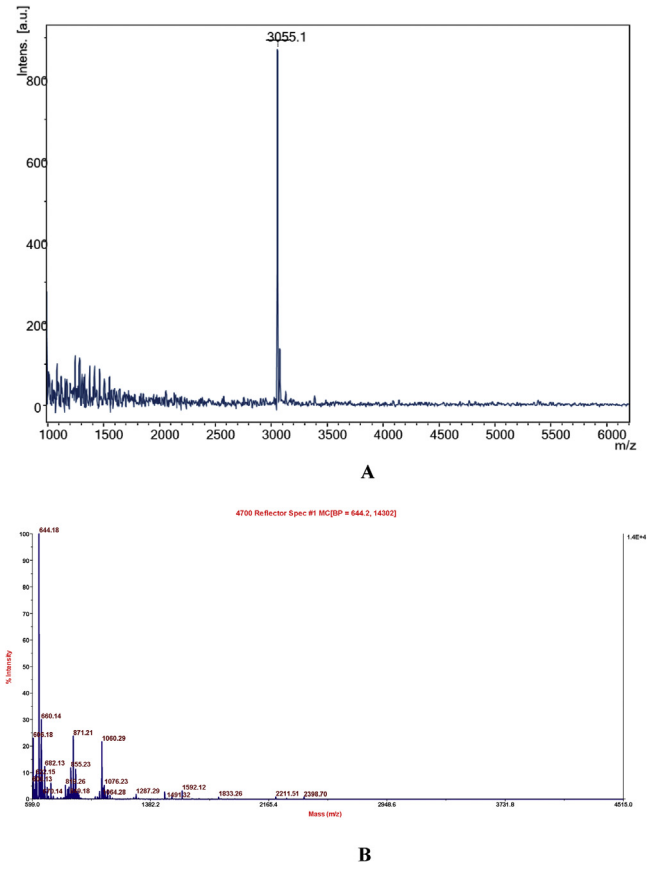

Fig. 3. A Molecular weight analysis of MFAP9 by MALDI-TOF MS.3.B MALDI MS/MS spectra of the tryptic digest of MFAP9 from gel. The peptide mass peaks marked with numbers are those subjected to MASCOT search.

5.8 kDa to 6.6 kDa, the mass of AfusinC was 3.96 kDa and eurocin from fungus has a molecular mass of 4.3 kDa [8,25]. Peptide mass fingerprint of MFAP9 was analyzed with the MASCOT database search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) showed matching with NADH-ubiquinone oxidoreductase chain 4 L (EC 1.6.5.3) of *A. niger* (Fig. 3B). However, MFAP9 showed limited homology with existing peptides in the database and therefore it can be a novel peptide having distinct structure and sequence homology. In an earlier report, MASCOT search tool analysis of the mass spectra obtained after peptide mass fingerprinting of an inhibitor isolated from *Solanum tuberosum* did not show resemblance to any of the plants inhibitors [27].

3.5. Effect of temperature, pH and proteases on the stability of MFAP9

The peptide MFAP9 was found to be stable over a wide range of temperature from 4 °C to 90 °C with optimum activity exhibited at 50 °C. It was noticed that the complete loss of antibacterial activity was not occurred even after 1 h incubation at 100 °C (Fig. 4A). From the results presented in Fig. 4B, the MFAP9 was found to be stable at both acidic and alkaline pH with a maximal activity recorded at pH 4 and complete loss of activity at pH 13. The thermostable and pH tolerance nature of MFAP9 in the presence of physical denaturants such as temperature, pH suggests that the intramolecular disulfide bridges are presumably responsible for that particular functional stability [28].

The effect of proteases on the stability of MFAP9 was studied using proteinase K, pepsin and trypsin (Fig. 4C). It was observed that the treatment with trypsin and pepsin on MFAP9 resulted in the reduction of antibacterial activity rather than complete loss of activity. Complete inactivation of MFAP9 was observed at a higher concentration of proteinase K. The variations in the sensitivity of MFAP9 towards proteases may be due to the specificity of these proteases; for example, activity of plectasin against *S. aureus* was not affected by pepsin and papain [29].

3.6. Effect of MFAP9 on preformed biofilm

Bacteria within biofilms are resistant to antibiotics, and high concentrations of antibiotics are required to eradicate the established biofilms. The preformed bacterial biofilm eradication potential of MFAP9 revealed significant inhibitory effect with an average biofilm inhibition percentage of > 85 % against all test bacteria (Fig. 5A). MFAP9 exhibited the highest biofilm removal activity against *Bacillus pumilus BT3* (99 % *v/s* control) and SEM analysis substantiated the potential of MFAP9 as a strong antibiofilm agent. SEM analysis of control bacteria revealed smooth-walled bodies, elongated, entangled in a thick biofilm mass forming three-dimensional biofilms (Fig. 5B). However, the treatment on preformed biofilm of *B. pumilus* BT3 with MFAP9 resulted a significant loss of biofilm organization. In the treated plates, only a few cells were visible scattered on the bottom shall be due to the disruption of cell-to-cell connections.The aggregated cells entirely disintegrated and bacterial cells lost their original shape demonstrating a distorted, irregular topograpic features with grooves and pore-like lesions on the surface (Fig. 5C). Furthermore, complete destruction of biofilm architecture made of extracellular polymeric substances was evident in the MFAP9 treated cells. The biofilm eradication potential of the MFAP9 is highly promising against *B. pumilus*, a human pathogen involved in the sepsis of newborns, immune-compromised individuals, in central venous catheters and causing cutaneous infections [30].

3.7. Hemolytic activity of MFAP9

Hemolysis associated toxicity is a limiting factor for the development of therapeutic compounds. Hemolytic or hemagglutination tests remains a fundamental strategy for determining safety and selectivity of proteins for potential applications. Hence, the hemolytic activity of MFAP9 was studied against hRBCs and proved to be non hemolytic even at a higher concentration of 100 μg/mL.The hemolysis of MFAP on hRBCs was only 0.6 % at 100 μg/mL. In comparison, treatment of AfusinC sheep erythrocytes caused 3.0 ± 0.8 % and 55 ± 8% of hemolysis at 100 μg/mL and 200 μg/mL respectively [23].

4. Conclusion

Antimicrobial resistance is multifaceted, multidimensional, and is the second largest cause of death in the world. Both the Gram-positive and Gram-negative bacteria are getting refractory to current armamentarium of antimicrobial drugs. In this respect, AMPs are considered as promising antimicrobial agents for producing new generation antimicrobials. Though, there are several obstacles to be considered for therapeutic applications, natural and synthetic AMPs are still attractive sources to the pharmaceutical companies. In order to facilitate commercial development of peptide antibiotics, it is reasonable to focus on small peptides. In this juncture, this study highlights the potential of an anionic fungal antibacterial peptide MFAP9 to control the bacterial infections. Purified MFAP9 showed an effective antibacterial and biofilm destruction activity against the test organisms, well thought-out to be the major pathogens in food borne illnesses and other infections. This low molecular weight MFAP9 is highly efficient, active

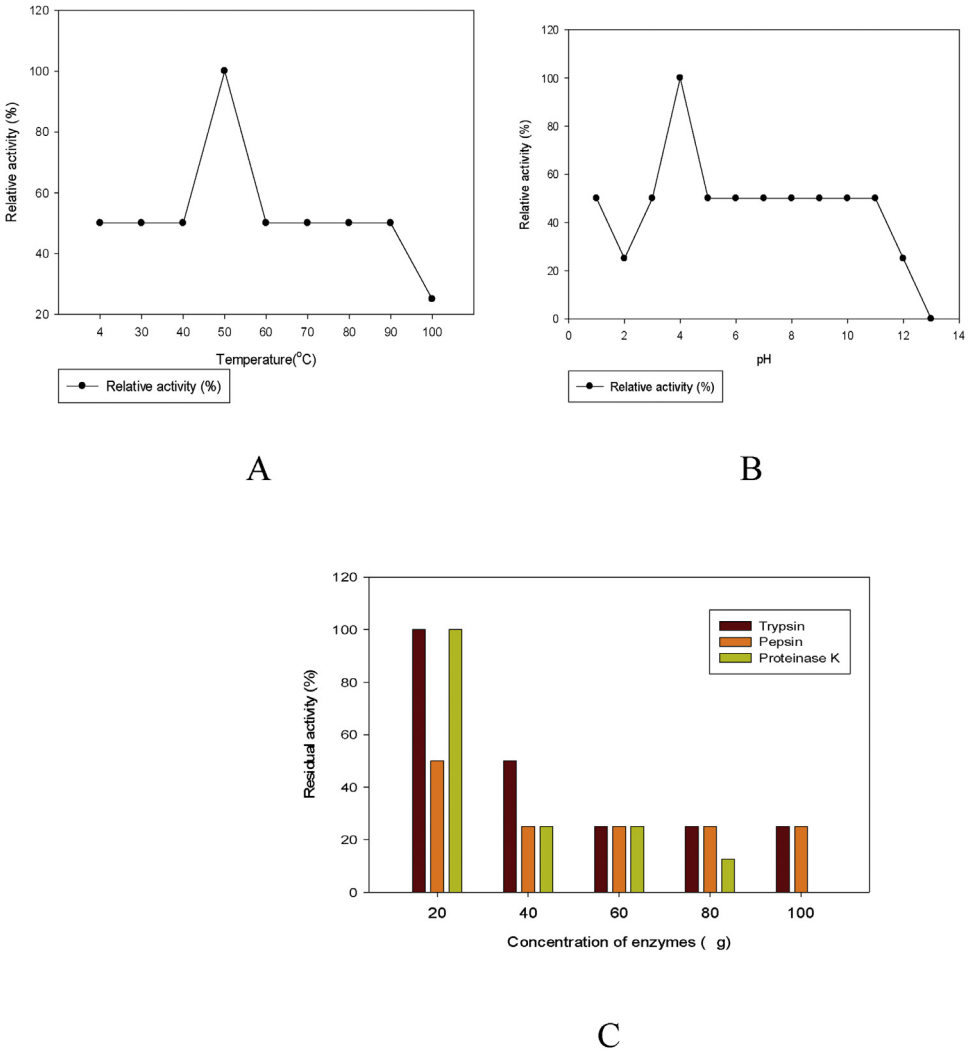

Fig. 4. A Effect of temperature on MFAP9. 4.B Effect of pH on MFAP9 activity. 4.C Effect of proteases on MFAP9.

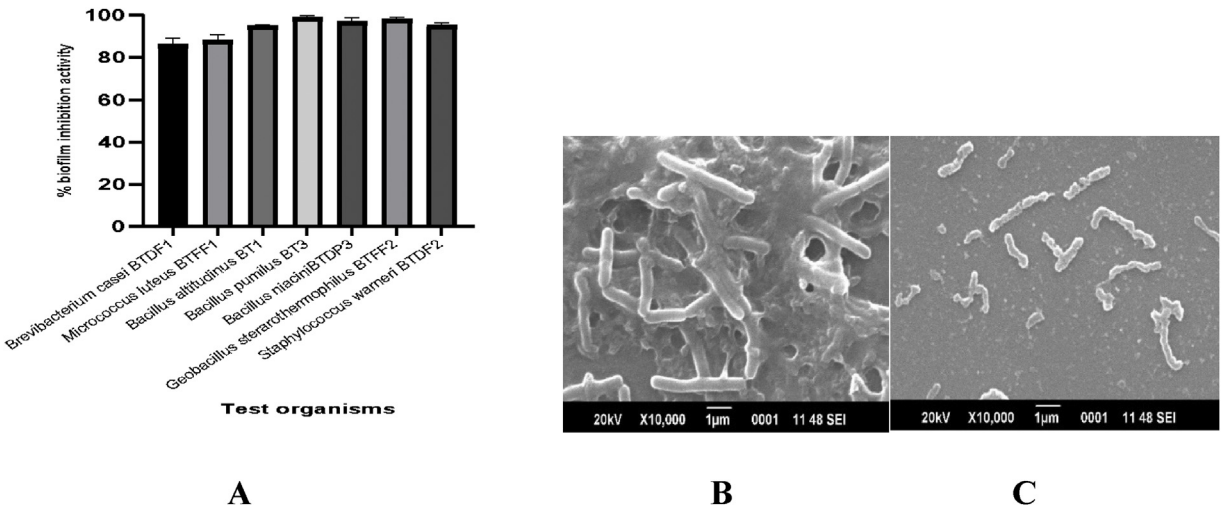

Fig. 5. A Antibiofilm assay: Effect of peptide MFAP9 on strong biofilm producers. The results are expressed as a percentage of the biofilm inhibition, with respect to untreated control taken as 100 %. Data are the mean ± SD of experiments performed in triplicate. *P < 0.05 *vs* control, ANOVA test followed by Bonferroni's post-test. 5.B SEM images showing the untreated *Bacillus pumilus BT3* biofilm. 5.C SEM images showing the effect of MFAP9 on *Bacillus pumilus BT3* biofilm.

even at very low (in μg) concentrations, inhibiting an array of Gram-positive pathogenic organisms which are concerned either in gastroenteritis and/or food poisoning. Likewise, stability and reproducibility in strong antibacterial activity over a wide range of pH and temperature may be advantageous for scaled-up and devising into deliverable products. Hopefully, with the utilization of bioinformatics and modification strategies, the fungal peptide MFAP9 could be a promising peptide for various clinical applications.

CRediT authorship contribution statement

Rekha Mol K.R: Investigation, Methodology, designed the experiments, Data curation, Writing - original draft, Writing & editing. **Manzur Ali P.P:** Conceptualization, Methodology, Writing - review & editing. **Sapna Kesav:** Visualization, Methodology, Writing - review & editing. **Abraham Mathew:** Resources, Methodology. **Sarita G Bhat:** Investigation, Methodology, Project administration. **Mohamed Hatha AA:** Conceptualization, Methodology. **Elyas KK:** Project administration, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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