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ORIGINAL ARTICLE

Streptomyces sp. MNP32, a forest-dwelling *Actinomycetia* endowed with potent antibacterial metabolites

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Abstract

The *Actinomycetia* isolate, MNP32 was isolated from the Manas National Park of Assam, India, located in the Indo-Burma biodiversity hotspot region of Northeast India. Morphological observations and molecular characterization revealed its identity to be *Streptomyces* sp. with a 99.86% similar to *Streptomyces camponoticapitis* strain I4-30 through 16S rRNA gene sequencing. The strain exhibited broad-spectrum antimicrobial activity against a wide range of bacterial human pathogens including WHO-listed critical priority pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii.* The ethyl acetate extract was found to disrupt the membrane of the test pathogens which was evidenced through scanning electron microscopy, membrane disruption assay and confocal microscopy. Cytotoxicity studies against CC1 hepatocytes demonstrated that EA-MNP32 had a negligible effect on cell viability. Chemical analysis of the bioactive fraction using gas chromatography-mass spectrometry (GC–MS) showed the presence of 2 major chemical compounds that include Phenol, 3,5-bis(1,1-dimethylethyl)- and [1,1′-Biphenyl]-2,3′-diol, 3,4′,5,6′-tetrakis(1,1-dimethylethyl)- which have been reported to possess antimicrobial activity. The phenolic hydroxyl groups of these compounds were proposed to interact with the carbonyl group of the cytoplasmic proteins and lipids leading to destabilization and rupture of the cell membrane. These findings highlight the potential of exploring culturable actinobacteria from the microbiologically under-explored forest ecosystem of Northeast India and bioactive compounds from MNP32 which can be beneficial for future antibacterial drug development.

Keywords Antibacterial activity · Drug-resistance · Bacterial pathogens · Secondary metabolites · Membrane disruption

Introduction

Antibiotics have played an instrumental role in the field of medicine, but the emergence of antibiotic resistance has created unforeseen problems in the treatment of life-threatening diseases. Researchers are therefore seeking novel classes of antibiotics whose mode of action is different from the traditional antibiotics (Konwar et al. 2022). Despite the discovery of many antibiotics, including the last resort antibiotics, resistant strains has emerged, prompting the search for novel classes of compounds that have minimal chances of developing resistance and can target intricate bacterial metabolic pathways (Sekyere 2016; Jochum et al. 2021; Mohapatra et al. 2021).

Natural products from microorganism have been a valuable source during the golden age of antibiotics and it possess the potential in discovering novel compounds since there is a vast majority of non-culturable species in different natural environments. Whole genome sequencing and metagenomics have further augmented the potential of finding novel secondary metabolites from non-culturable microorganisms. The quality of an ecosystem greatly influences the microflora of a region and the microorganism have more affinity to strive in undisturbed and unperturbed natural environments where they are exposed to a plethora of external stimulus for an extended period of time. This influences their genetic makeup and their secondary metabolite production

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capability. Therefore, probing for novel strains of microbes from unexplored ecosystems has been the focus of study in recent years. The ecosystems ranging from forest soil to vaginal microflora have been investigated in the search for novel antimicrobials with the help of new techniques and instruments that greatly enhance the probability of finding important species of microbes producing potent antimicrobials (Ma et al. 2014; Donia et al. 2014; Ling et al. 2015).

The forest ecosystems of Northeast India represent a largely unexplored region in terms of microflora since there have been very few reports on the diversity of microorganisms present in the soil systems of the region. The class *Actinomycetia* that includes important microorganisms such as *Streptomyces* has been studied extensively for their high potential of producing important secondary metabolites (Mazumdar et al. 2023). Studies have been carried out where the forest regions of Northeast India have been explored in the search for species of *Actinomycetia* species that can produce potent antimicrobials (Thakur et al. 2007; Sharma et al. 2016; Das et al. 2018; Sharma and Thakur 2020). Moreover, other important *Streptomyces* species have also been found in other natural environments that possess biologically active secondary metabolites (Wahaab and Subramaniam 2018; Dan et al. 2018; Paderog et al. 2020; Chakraborty et al. 2022). Therefore, screening for important *Actinomycetia* species in the unexplored forest ecosystems of Northeast India provides hope for finding novel antimicrobials.

Antimicrobial compounds that falls under the class of peptide antibiotics have an intrinsic capability of membrane disruption. Antibiotics such as colistin and polymyxin B are examples of such antimicrobial peptides. The resistance in bacteria against such membrane-active compounds are hard to develop because of the mechanical mode of action that targets the bacterial cell membrane. But this also generates a problem of cytotoxicity in animal cells as well which impedes its development as a potential drug. Therefore, the search of compounds having membrane disruption capability is of considerable importance since these compounds will have low chances of resistance development. Cytotoxicity values against animal cells should also be considered in the screening process for the development of a proper antimicrobial drug.

In this work, we isolated a *Streptomyces* sp. designated as MNP32 from the soil samples of Manas National Park of Assam, India, and investigated its antimicrobial potential against a variety of bacterial human pathogens, particularly Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* (*A. baumannii).* The chemical constituents in the active fraction of the ethyl acetate extract were identified through GC–MS. The outcome of this study demonstrates the importance of searching for compounds in the genus *Streptomyces* from the forest ecosystems of Northeast India and encourages further investigations into the mechanism of actions of various compounds present in the strain.

Materials and methods

Sample collection and sampling site

Soil samples were collected in the month of February 2021 from varied locations in Manas National Park of Assam, India. The national park experiences a semi-dry hot climate in summers with maximum temperatures ranging from 31 to 35 °C and in winters it experiences cold with minimum temperatures ranging from 6 to 8 °C. The annual average rainfall is around 333 cm. The national park is located at the foothills of the eastern Himalayan region encompassing the manas river and consists of grasslands and dense hilly forests. Soil samples were collected from 4 different types of environmental niches such as forest topsoil, grass rhizosphere soil, tree rhizosphere soil and river sediment soil. Approximately 50 g of samples were collected from each site and transferred aseptically to the laboratory.

Isolation of the *Actinomycetia* strain

The strain MNP32 was isolated from the soil of Manas National Park through a serial dilution technique using Starch Casein Nitrate Agar as the isolation media. The soil samples from each site were homogenized with 0.9% NaCl and then serially diluted. Optimal dilutions were plated in replicate in the isolation plates and incubated at 28 °C for 7–10 days. The isolation media was supplemented with rifampicin (2.5 µg/mL) and amphotericin B (75 µg/mL) for limiting the bacterial and fungal contamination on the isolation plates (Sharma et al.2016). The pure culture of the isolate was sub-cultured on modified Glucose Malt agar medium (GLM) (Yeast extract, 3 g; malt extract, 3 g; peptone, 5 g; starch, 10 g; agar, 20 g; distilled water, 1000 mL; pH 7.4) and was preserved in glycerol stocks using 50% glycerol at -80 °C for future use.

Characterization of the *Actinomycetia* strain

Morphological and cultural characterization

The *Actinomycetia* strain was identified and isolated after observing its peculiar morphological characteristics on GLM agar. The colony morphology, micromorphology and spore chain morphology of the isolate were observed by stereo zoom microscope (Zeiss Stemi 508, Carl Zeiss Microscopy, USA) and compound microscope (Zeiss Primo Star, Carl Zeiss Microscopy, USA) respectively. The morphology of the mycelia was observed by Field Emission Scanning

Electron Microscopy (SEM) (Zeiss Sigma VP, Carl Zeiss Microscopy, USA). GLM medium was used to determine the temperature range (15–45 °C), the pH range (4–12) and NaCl tolerance (1–5% NaCl w/v) for optimal growth of the isolate. Sensitivity and resistance of the isolate to commonly used antibiotics were determined using the disc diffusion method.

Identification of the isolate through 16S rDNA sequencing

For genomic DNA extraction, the isolate was cultured in GLM broth for 7 days and the cell pellet was obtained after centrifugation at 7000 rpm for 10 min. Nucleo-pore gDNA Fungal Bacterial Mini Kit (50) (Catalogue number NP-7006D, Genetix Biotech Asia Pvt. Ltd.) was used for the genomic DNA extraction following the manufacturer's protocol.

Universal primer set 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Weisburg et al. 1991; Sharma et al. 2016) was used for the amplification of 16 s rRNA gene. PCR reactions were performed in a T100 Thermal cycler (Bio-Rad Laboratories, USA) in a total volume of 50 µL. A total of 50 ng template DNA, 1 X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Taq DNA polymerase and 0.2 µM of each primer. The PCR program was as followed: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 7 min. The amplified product was visualized in 1% (w/v) agarose gel electrophoresis. The amplified product was then sent for sanger sequencing to 1st Base (Apical Scientific, Malaysia). The raw sequence was analyzed and assembled using UGENE open-source software and a similarity search was carried out using BLAST N. Before submission of the sequence to GenBank, the probability of chimeras in the sequence was checked using DECIPHER Chimera package (DECHIPHER version 2.24.1).

Antimicrobial screening of MNP32

For assessing the in vitro antimicrobial activity of MNP32, the following test organisms were used: *Staphylococcus aureus (S. aureus)* (MTCC 96), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC–BAA 43300), *Escherichia coli* (*E. coli*) (ATCC 25922), *Escherichia coli* (ATCC-BAA 2469), *Streptococcus epidermidis* (*S. epidermidis*) (MTCC 435), *Micrococcus luteus* (*M. luteus*) (MTCC 11948), *Proteus vulgaris* (*P. vulgaris*) (MTCC 426), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC-BAA 1705), *Pseudomonas aeruginosa* (*P. aeruginosa*) (MTCC 741), *Acinetobacter baumannii* (*A. baumannii*) (MTCC 1425), *Bacillus cereus* (*B. cereus*) (MTCC 1272). The strains were cultured and maintained on Nutrient Agar and Nutrient broth medium and were made into glycerol stocks with 25% v/v glycerol and stored in − 80 °C for future experiments.

MNP32 strain was selected after two rounds of screening including a primary screening with a candidate gram-positive bacterium (*S. aureus* MTCC 96) and a candidate gram-negative bacterium (*E. coli* ATCC 25922) and a secondary screening using a panel of bacteria that includes drug-resistant strains (Methicillin-resistant *Staphylococcus aureus* ATCC–BAA 43300 (MRSA), Carbapenem-resistant *K. pneumoniae* ATCC-BAA 1705 and multi-drug resistant *E. coli* ATCC-BAA 2469). The screening was carried out using the spot inoculation method (Sharma et al. 2016; Sharma and Thakur 2020).

Evaluation of the antimicrobial activity of MNP32

Preparation of crude extract and optimization of the antimicrobial activity of MNP32

Before extraction, the activity of MNP32 in broth was optimized using different media and variable pH. Six different media were used for culturing MNP32 in broth conditions that include Thornton's media (TM), Norris media (NM), Czapex dox yeast extract media (CD), GLM media (GLM), Nutrient broth (NB), and Starch casein nitrate media (SCNA) (Thakur et al. 2009). Zone of inhibition of the culture broth was determined by the well-diffusion method on Mueller Hilton Agar (Nathan et al. 1978). The dry weight of the culture was also determined after 7 days of growth in the different media. A range of pH of GLM broth (4–11) was used to check the optimal zone of inhibition. *S. aureus* MTCC 96 was used as the test organism in the experiments.

For extraction of metabolites from MNP32, it was cultured in GLM broth in shaking condition (180 rpm) at 28 °C, pH 9 for 8 days. The crude extract was obtained after solvent extraction of the culture broth with ethyl acetate mixed in 1:1 ratio. The ethyl acetate extract of MNP32 (EA-MNP32) was recovered by evaporation in a rotary evaporator (Buchi, Switzerland) and was dissolved in 10% DMSO at a concentration of 1 mg/mL for antimicrobial bioassay.

Antimicrobial activity and determination of minimum inhibitory concentration (MIC) of EA-MNP32

Antimicrobial activity of the EA-MNP32 was assessed against 5 bacterial pathogens through disc diffusion method according to guidelines from the Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org/standards/products/microbiology/documents/m02/). Appropriate controls were used during the assay where gentamycin was used as a positive control against *A. baumannii* MTCC 1425 and Co-trimoxazole was used against MRSA ATCC-BAA 43300

because MRSA was found to be resistant to gentamycin. 10% DMSO was taken as a negative control.

MIC was determined according to the guidelines from CLSI, 11th edition (https://clsi.org/media/1928/m07ed11_sample.pdf), with slight modification using the microbroth dilution technique in 96 well plate. Stock solution of 1 mg/mL of EA-MNP32 was serially diluted (single fold) to prepare decreasing concentrations of the extract (500–0.975 µg/mL) in the wells. Overnight grown bacterial cultures were diluted to 0.5 MacFarland turbidity standard (1.5×10^8 CFU/mL) and 10 µL was used to inoculate each well of the 96 well plate containing different concentrations of EA-MNP32 in 100 µL of Mueller Hilton Broth. MIC was determined after 24 h of incubation at 37 °C. MIC was recorded as the lowest concentration of the compound that inhibits the visible growth of inoculated test organism after 24 h. Controls were prepared with 10% DMSO without an antimicrobial agent (negative control) and with standard antibiotics rifampicin, gentamycin, and amphotericin B (positive control).

SEM analysis of the antimicrobial activity of EA-MNP32

EA-MNP32 was further evaluated for its antimicrobial activity against test pathogens MRSA ATCC 43300 and *A. baumannii* MTCC 1425. Sample preparation was carried out according to Sharma et al. (2016) with slight modification. Test organisms were grown initially for 4 h and then treated with 1 X MIC of EA-MNP32 and then further grown for 8 h. Cells were harvested after centrifugation (7000 rpm for 10 min) and washed with 1 X Phosphate Buffer Saline (PBS) (Sigma Aldrich, USA). 2.5% glutaraldehyde solution was used for fixing the cells and incubated for 4 h at 4 °C. After fixation, the fixative was removed and washed with 1 X PBS and then serially dehydrated by gradually increasing the concentration of ethanol from 30 to 100%. Finally, the dehydrated cells were spread on a glass substrate and mounted onto a steel stub. Sputter coating with gold–palladium alloy under vacuum was used for coating the samples and scanned using Zeiss Sigma VP SEM.

Dose-dependent membrane leakage assay of EA-MNP32

Membrane active potential of EA-MNP32 was assessed through membrane leakage assay against MRSA ATCC 43300 and *A. baumannii* MTCC 1425 according to Paderog et al. (2020) with slight modifications. Overnight grown cells of test organisms were diluted to 0.5 MacFarland turbidity standard and then were exposed to varying concentrations of EA-MNP32 which includes, 1 X MIC, 1/2 X MIC, 1/4 X MIC, and 1/8 X MIC. 10% DMSO and 5% Tween 80 was taken as negative and positive control respectively. Measurements of the nucleic acid and protein content that leaked out of the cells were measured after pelleting down the cells at 7000 rpm for 10 min and then measuring the optical density of the cell-free supernatant using a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA). Optical density was measured at 260 nm and 280 nm which corresponds to DNA and protein peak absorbance respectively.

Confocal laser scanning microscopy (CLSM) analysis of the antimicrobial activity of EA-MNP32

CLSM was performed to further evaluate the membrane disruption efficacy of EA-MNP32. MRSA ATCC 43300 and *A. baumannii* MTCC 1425 were selected as test pathogens. For assessment of the bacterial viability as a function of its membrane integrity, LIVE/DEAD BacLight Bacterial Viability kit was used (Thermo Fisher Scientific, USA) where SYTO9 and Propidium Iodide (PI) fluorescent dyes are used. SYTO9 enters the live cells and makes them fluoresce green whereas PI can enter only membrane-compromised cells and fluoresce them red. Samples were prepared according to the manufacturer's protocol and according to Robertson et al. (2019) with slight modifications. Overnight grown test organisms were diluted in 0.9% NaCl to 0.5 MacFarland turbidity standard and were grown in Nutrient broth in 1 mL volume for 4 h and then exposed to 1 X MIC of EA-MNP32. 10% DMSO and 5% Tween 80 were taken as positive and negative control respectively. It was then allowed to grow for 6 h and then cells were obtained after centrifugation at $7000 \times g$ for 7 min. The cells were resuspended in 200 µL of 0.9% NaCl. Then, 100 µL of cell suspension were added to 2 mL of 0.9% NaCl and incubated at room temperature for 1 h with regular mixing at 15 min interval. The cells were then washed twice with 0.9% NaCl and resuspended in 1 mL of 0.9% NaCl. To the 1 mL of bacterial suspension, 3 µL of an equal mixture of SYTO9 and propidium iodide was added and incubated at room temperature in the dark for 15 min. 5 µL of stained bacterial suspensions were then spread onto a glass slide and viewed under confocal laser scanning microscope (Leica, Mannheim, Germany) equipped with a visible light laser, inverted light microscope, and an 63X/1.40 Oil immersion objective. The images were viewed using Leica Application Suite X software.

Cytotoxicity assay

CC1 hepatocytes (primary hepatic cell line from normal Wistar rat liver) were cultured in 96 well plate in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution and incubated in 5% CO_2 at 37° C. Cells were treated with EA-MNP32 for 12 h with doses ranging from 500 µg to 0.24 µg/mL dissolved in 0.1% DMSO in 1X PBS as vehicle control. A normal control group (treated only with vehicle control), a positive control

(treated with 5% Tween80) and a negative control (treated with 10 µg/mL of gentamycin) was also included in the assay. Cell viability assay was conducted using Alamar blue dye at the end of 12 h incubation and colorimetric optical density (OD) was measured using a multimode scan at the wavelength of 570 nm and 600 nm.

GC–MS analysis of secondary metabolites

For analysis of secondary metabolites, EA-MNP32 was separated through column chromatography using a silica column (mesh size 100–200) with methanol and chloroform as the solvent system, yielding six enriched fractions (10%, 20%, 40%, 60%, 80% and 100% methanol in chloroform). The antibacterial activity of the fractions was then analyzed through the disc diffusion method. MRSA ATCC-BAA 43300 was used as the test organism for the assay. The active fraction obtained from the column chromatography was then subjected to GC–MS analysis for identification of the chemical compounds. The analysis was performed according to Sharma et al., (Sharma and Thakur 2020) with slight modifications. EB-5 MS coloumn of length 30 m and thickness of 0.25 µm was used for sample analysis using the Shimadzu GC 2010 plus with triple quadrupole MS (TP-8030). 1 mg/mL of enFr2 was prepared with HPLC grade methanol and filtered through 0.2 µm syringe filter. The method for GC–MS was developed as follows: the initial oven temperature started at 40 °C which was held for 5 min and then increased at 10 °C/min to a temperature of 280 °C which was again held for 10 min. It was raised again to 285 °C at a rate of 5 °C/min and finally held for 10 min. Helium was used as carrier gas and the sample was injected at a temperature of 300 °C in split-less mode. The mass spectrophotometer was operated at electron ionization mode at 70 eV with a scanning range from 45 to 600 m/z. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST, USA) library.

Results

Characterization of the *Actinomycetia* isolate

Morphological characterization of the MNP32 strain was carried out using light microscopy and Scanning electron microscopy. The strain was found to be gram positive and highly filamentous in nature (Fig. 1C). The colony morphology was wrinkled and rough with a moderate amount of

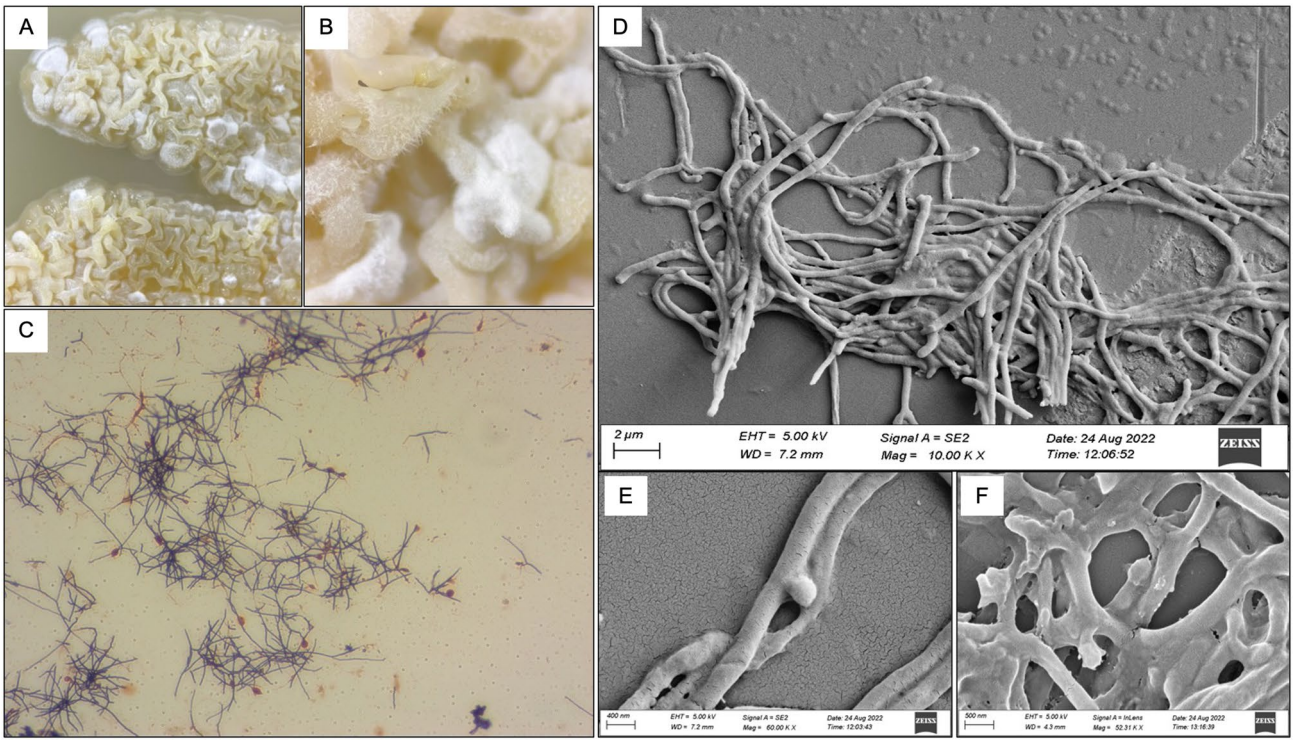


Fig. 1 Morphological characterization of the MNP32 strain using light microscopy and Scanning electron microscopy. **A** and **B** Shows the colony morphology of the strain where colony morphology was found to be wrinkled and rough with moderate amount of white coloured spores borne on the aerial mycelium. **C** Shows the light microscopy image of MNP32 where the mycelial structure with globose spores borne on the mycelium can be observed. **D**–**F** Shows the SEM images of MNP32 where the long filamentous structure of the mycelium was observed. The smooth surface and globose shape of the spores can be observed in **E**

white-coloured spores borne on the aerial mycelium (Fig. 1A and B). The branched hyphae was found to be straight and flexible. SEM revealed the spore shape to be globose and the spore surface morphology to be smooth (Fig. 1D–F). The physiological characteristics and antibiotic sensitivity are shown in Table 1. The strain was found to grow in the temperature range of 20–37 °C and the optimal temperature was found to be at 28 °C. The pH growth range was from 5 to 11 where the maximum mycelial dry weight was found to be at pH of 9. The mycelial dry weight which represents the amount of growth, was found to be highest at a pH of 9 (0.646 ± 0.5 g) and the lowest was found to be at a pH of 4 (0.247 ± 0.25 g) (Fig. 2C).

Table 1 Morphological and physiological properties and antibiotic sensitivity of MNP32

Sl. no	Characteristics	Result
Morphological		
1	Aerial mycelium colour	White to light brown
2	Substrate mycelium colour	Yellow to brown
3	Diffusable pigment	Faint brown
4	Melalnin pigment	–
5	Spore chain morhology	Straight and flexible
6	Spore shape	Globose
7	Spore surface morphology	Smooth
Physiological		
1	Temperature range for growth	20–37 °C
2	Optimum temperature	28 °C
3	pH range for growth	5–11
4	Optimum pH	9
Antibiotic sensitivity (µg/disc)		
1	Ampicillin (10)	R
2	Cefotaxime (30)	R
3	Cephalothin (30)	R
4	Co-trimoxazole (25)	R
5	Gentamycin (10)	S
6	Nalidixic acid (30)	R
7	Nitrofurantoin (300)	R
8	Norfloxacin (10)	S
9	Pefloxacin (10)	S
10	Levofloxacin (5)	S
11	Gatifloxacin (10)	S
12	Chloramphenicol (30)	S
13	Imipenem (10)	S
14	Meropenem (10)	S
15	Polymyxin B (300 units)	S
16	Furazolidone (100)	S
17	Aztreonam (30)	S
18	Cloxacillin (1)	R

'S' for sensitivity and 'R' for resistance

Partial 16S rRNA gene sequence (1383 nucleotides) was analyzed and assembled using the Ugene bioinformatics tool. Accession number OP278937 was obtained against the submission of the assembled sequence to the NCBI GenBank database. The strain showed the highest similarity (99.49%) to *Streptomyces camponoticapitis* strain I4-30 16S ribosomal RNA gene, partial sequence (MW164960.1). Since the genomic data indicated that the strain represented a strain of the genus *Streptomyces*, the strain was referred to as *Streptomyces* sp. strain MNP32.

Assessment of the antimicrobial activity of MNP32

During the screening of MNP32 for assessing the capability of producing antimicrobial secondary metabolites, the spot inoculation method in GLM agar revealed that the strain MNP32 showed antimicrobial activity against gram-positive and gram-negative bacterial pathogens. The average colony size was observed to be (9 ± 2) mm in diameter after 8 days of incubation at 28 °C on GLM agar. Inhibition zone (mm ± SD) was found to be highest against *S. aureus* MTCC 96 and *MRSA* ATCC 43300 (40 ± 1.5 mm) and the lowest was found against *P. aeruginosa* MTCC 741 (15 ± 1.5 mm). The values of a zone of inhibition against all 9 pathogens are listed in Table 2.

Capability of producing bioactive secondary metabolites in submerged broth culture varies considerably from solid agar culture. Therefore, MNP32 was optimized to produce bioactive secondary metabolites in broth culture under shaking conditions using various media for various number of days and a range of pH. The most activity was found when the strain is cultured in GLM broth for 9 days and at a pH of 9 (Fig. 2).

Antimicrobial activity of EA-MNP32

After optimization of the activity, the cultured broth was extracted using 1:1 v/v ethyl acetate and was termed as EA-MNP32. Antimicrobial activity of the extract using the disc diffusion method revealed its antagonistic effect against *S. aureus* MTCC 96, MRSA ATCC-BAA 43300 and *A. baumannii* MTCC 1425 (Fig. 3). The interpretation of the zone of inhibition was in accordance with the Performance Standards for Antimicrobial Disc Susceptibility Tests, CLSI and EUCAST where gentamicin sensitivity is considered when the zone of inhibition is > 15 mm. And for co-trimoxazole, sensitivity is considered when the zone is > 17 mm. EA-MNP32 showed a zone of inhibitions greater than the standard zone size which is considered as sensitive according to CLSI and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (18 ± 0.5 mm for *S. aureus*, 17 ± 0.4 mm for *MRSA*, and 16 ± 0.6 mm for *A. baumannii*).

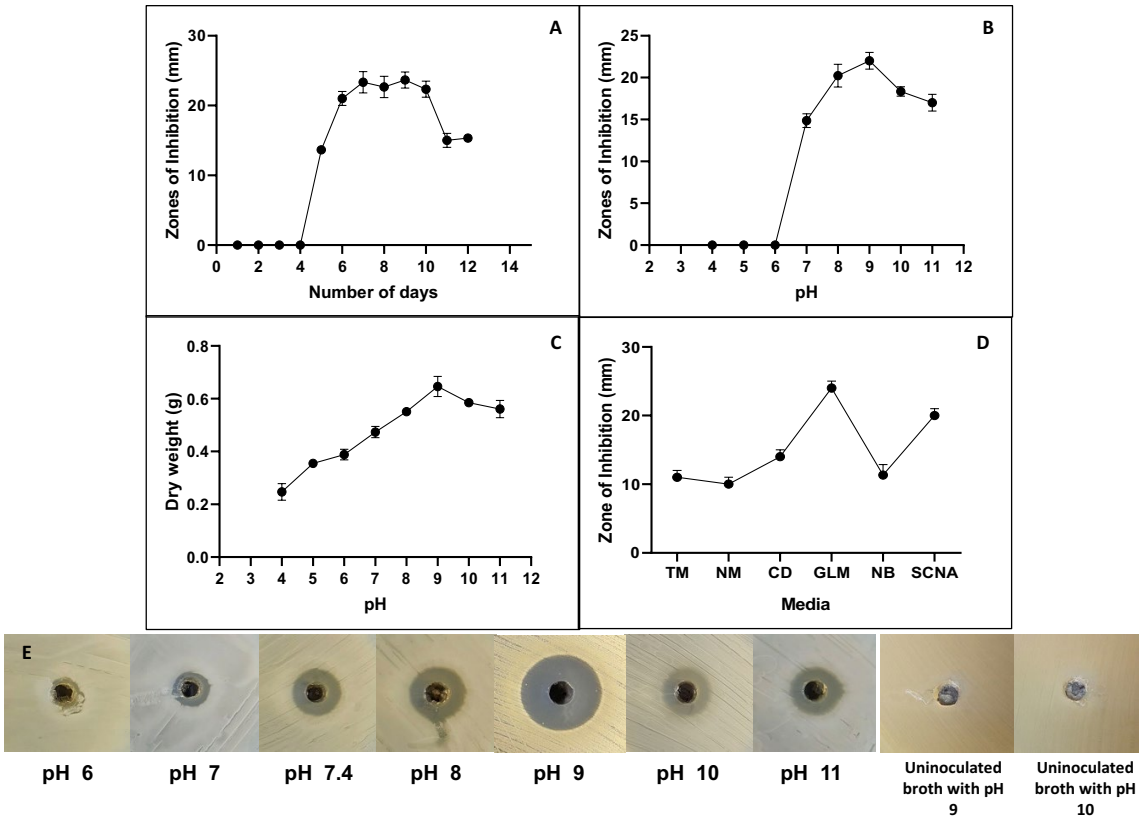


Fig. 2 Optimization of the antibacterial activity of MNP32 under shaking conditions (broth culture). **A** Represents the optimization of the fermentation media for achieving the highest activity according to the number of days. It can be observed that at 9th day, the culture showed the highest activity. **B** Represents the optimization of the fermentation broth according to the pH in GLM broth. A pH of 9 corresponds to the highest activity. **C** Represents the optimization of the pH for obtaining the highest growth of the culture, determined through the dry weight of the culture. It was observed that at pH of 9, the culture showed the highest growth. **D** Represents the optimization of the culture in different media. GLM broth showed the highest activity. **E** Shows the zone of inhibition of the cell free fermentation broth of different pH and appropriate controls of high pH media without inoculation

Table 2 Antimicrobial activity assessment of MNP32 using spot inoculation method

Sl. no	Test organism	Zone of inhibition (mm ± SD)[a]
1	*S. aureus* MTCC 96	40 ± 1.5
2	*E. coli* ATCC 25922	38 ± 1.3
3	MRSA ATCC-BAA 43300	40 ± 1.0
4	*A. baumannii* MTCC 1425	34 ± 0.7
5	*E. coli* ATCC 2469	24 ± 0.5
6	*K. pneumoniae* ATCC-BAA 1705	26 ± 1.5
7	*P. aeruginosa* MTCC 741	15 ± 1.5
8	*S. epidermidis* MTCC 435	18 ± 1.2
9	*P. vulgaris* MTCC 426	22 ± 1.5

[a]Zone of inhibition by spot inoculation method on GLM agar medium. Average size of colony of MNP32 in GLM agar was (9 ± 1.5) mm diameter after 8 days of incubation. Zone of inhibition values are given as mean ± SD ($n = 3$)

MIC of EA-MNP32

MIC of EA-MNP32 was determined against MRSA and *A. baumannii* since EA-MNP32 showed prominent activity against these organisms and these two organisms falls under the list of priority pathogens by WHO. MIC values against MRSA and *A. baumannii* was found to be 12 µg/mL and 25 µg/mL. According to the MIC values, MRSA and *A. baumannii* was found to be intermediately susceptible to EA-MNP32 since CLSI guidelines states that MIC values ≤ 8 µg/mL should be taken as susceptible, ≤ 16 µg/mL should be taken as intermediate and ≤ 32 µg/mL should be taken as resistant.

Visualization of the antimicrobial effect of EA-MNP32 on MRSA and *A. baumannii*

The antibacterial effect of EA-MNP32 against MRSA and *A. baumannii* was visualized through SEM (Fig. 4A). The MRSA cells treated with 1 X MIC of EA-MNP32 had a

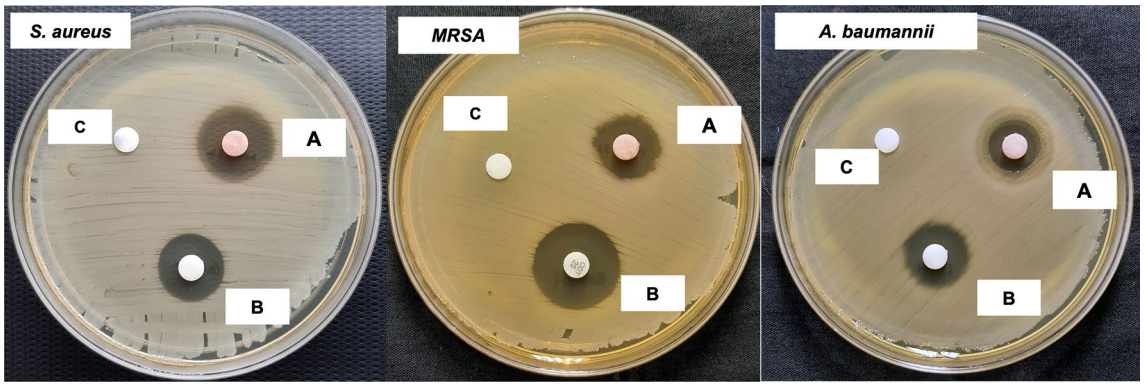


Fig. 3 Assessment of the antimicrobial activity of EA-MNP32 against bacterial pathogens through disc diffusion method. The zone of inhibitions for EA-MNP32 against the test pathogens are as follows: 18 ± 0.5 mm for *S. aureus*, 17 ± 0.4 mm for *MRSA*, and 16 ± 0.6 mm for *A. baumannii*. **A** Disc contains EA-MNP32 in the concentration of 50 µg. **B** Disc contains Gentamicin as positive control, concentration in the disc is 10 µg. **B#** Co-Trimoxazole as positive control for MRSA, concentration in the disc is 25 µg. **C** 10% DMSO as negative control

prominent effect which was characterized as cell leakage since there was considerable cell constituents leaked out of the contorted and deformed cells. In the case of *A. baumannii* cells, the treated cells were mostly deformed, and the outer layer seems to be morphologically different from the smooth physical appearance in the control (negative control) cells. 5% Tween 80 (a known membrane-disrupting detergent) which was taken as a positive control was found to have a profound membrane-disrupting effect on the cells. 10% DMSO control had no effect on the cells (negative control).

Dose-dependent membrane leakage assay

The membrane-disrupting capability of EA-MNP32 was assessed using membrane leakage assay against MRSA and *A. baumannii*. It was observed that most of the cell constituents were leaked out of the cell at the MIC concentration (Fig. 4B). The disruption capability gradually decreased as the concentration of EA-MNP32 decreased, indicating a dose-dependent effect of EA-MNP32 on the test organisms. The cell constituents were characterized by measuring the nucleic acid contents at 260 nm and the protein contents at 280 nm. 5% Tween 80 was taken as positive control where it was seen that the leakage of the cell constituents were comparable to MIC concentration. 10% DMSO was taken as a negative control.

Confocal laser scanning microscopy

CLSM was performed to further evaluate the membrane disruption capability of EA-MNP32 against MRSA and *A. baumannii* (Fig. 5). It was observed that in the cells treated with 1 X MIC of EA-MNP32, more PI florescence was observed compared to the negligible SYTO9 florescence. Similarly, PI florescence increased in the membrane-compromised cells treated with 5% Tween 80 (positive control). Whereas, in the cells treated with 10% DMSO, there was maximum SYTO9 florescence. This provides evidence that cells treated with EA-MNP32 has compromised cell membrane.

Cytotoxicity of EA-MNP32

Since membrane-active compounds have high cytotoxic activity against animal cells, EA-MNP32 was checked for its cytotoxicity in the CC1 hepatocyte cell line. Concentrations ranging from 500 to 0.240 µg/mL of EA-MNP32 were used for the assessment of cytotoxicity. It was observed that only 500 µg/mL was able to decrease the cell viability which is characterized by the inability of the compound to reduce resazurin into resorufin. Whereas 0.1% DMSO control and other lower concentrations of EA-MNP32 had no effect on the cell viability. Concentration nearing the MIC of EA-MNP32 (i.e. 15.6 µg/ml) and concentrations both above and below the MIC (125 µg/mL and 0.24 µg/mL) had no effect on the cell viability and hence were considered as non-cytotoxic (Fig. 6A). This can also be seen in the microscopic images of the treatments where the CC1 cells are seen to be morphologically affected by the treatment of 500 µg/mL of EA-MNP32 and 5% Tween 80. Whereas the control cells and the lower concentrations of EA-MNP32 had no effect on the cellular morphology (Fig. 6B).

GC–MS analysis of secondary metabolites

Coloumn chromatography of the EA-MNP32 resulted in 6 fractions which were 10%, 20%, 40%, 60%, 80% and 100% methanol in chloroform. The fraction, 20% methanol in

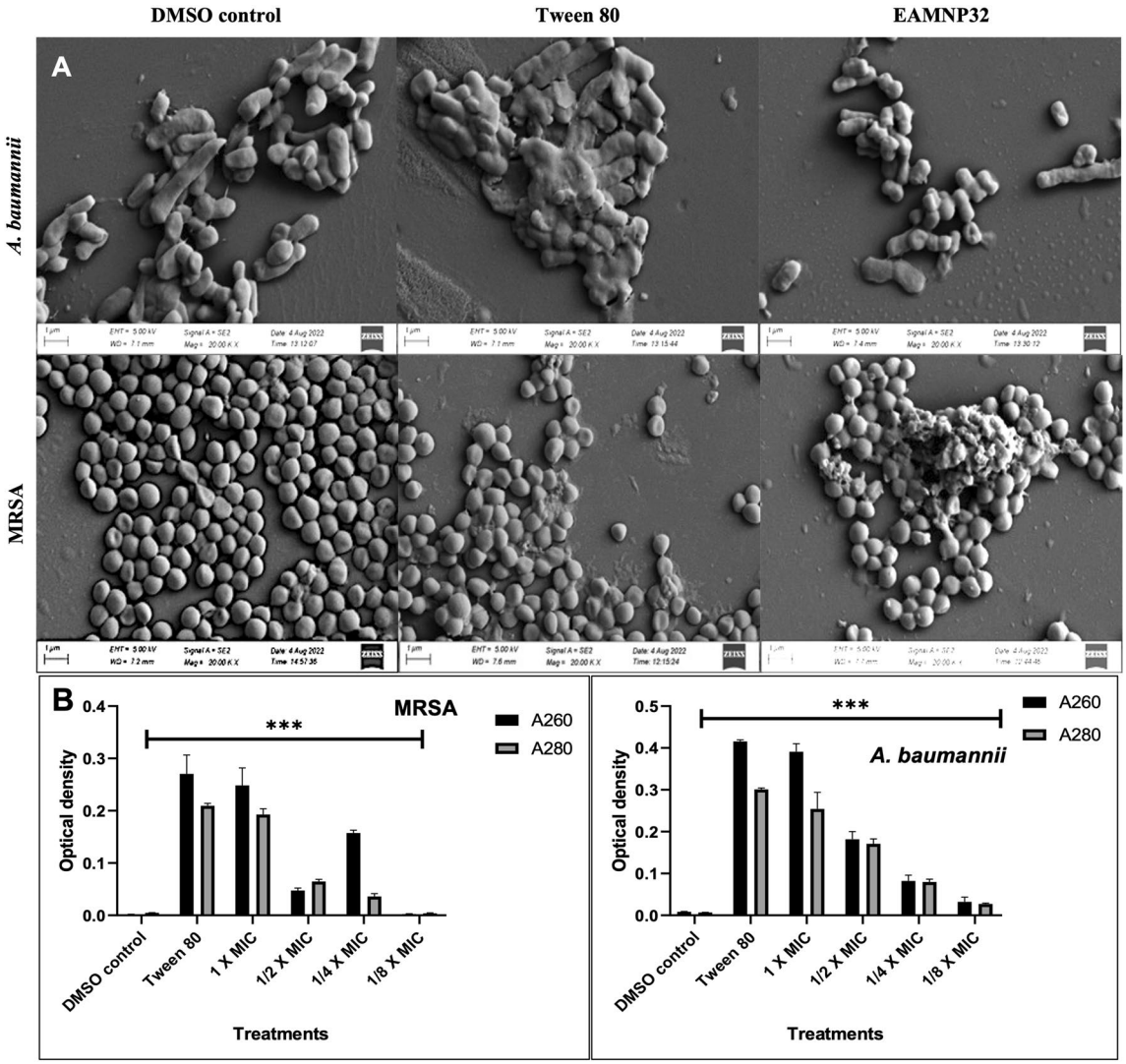


Fig. 4 SEM visualization of the antimicrobial activity of EA-MNP32 and membrane leakage assay. **A** SEM images of the effect of EA-MNP32 (1 X MIC) of the cells of MRSA and *A. baumannii*. The morphology of the treated cells were found to be significantly distinct from the control cells. Moreover, in the EA-MNP32 treated MRSA and *A. baumannii* cells, the cell constituents were seen to have been leaked out of the cells. **B** The graphs represents the findings of membrane leakage assay using EA-MNP32 in various concentrations against MRSA and *A. baumannii*. In 1 X MIC concentration, most of the cellular contents was observed to be leaked out which was determined by the absorbance of DNA and protein at 260 nm and 280 nm respectively. 10% DMSO and 5% Tween 80 were taken as negative and positive controls respectively for both the experiments. Data was analysed using Two-Way ANOVA in GraphPad Prism version 8.0.1 and expressed as mean ± SE where *** represents $p \leq 0.001$

chloroform (enFr2) was found to be the active fraction and therefore was further subjected to GC–MS analysis.

The enriched fraction (enFr2) was evaluated for its chemical composition through GC–MS analysis. In the enriched fraction, 2 major compounds were detected and were identified by using the NIST library after a comparison of their mass spectra along with the retention time, area percentage, height percentage and base m/z peak. The compounds are depicted in Table 3, and the total ion chromatogram along with their corresponding illustrated figures are shown in Fig. 7.

Discussion

Actinomycetia are known for their ability to produce bioactive secondary metabolites and other small molecules of biological importance. *Actinomycetia* from the unexplored ecosystem hold great potential in producing new biologically important secondary metabolites that would be helpful in combating drug-resistant pathogens. Previous reports by Sharma and Thakur (2020), Das et al. (2018) and Thakur et al. (2007) have highlighted the importance of isolation of *Actinomycetia* from the protected forest ecosystems of

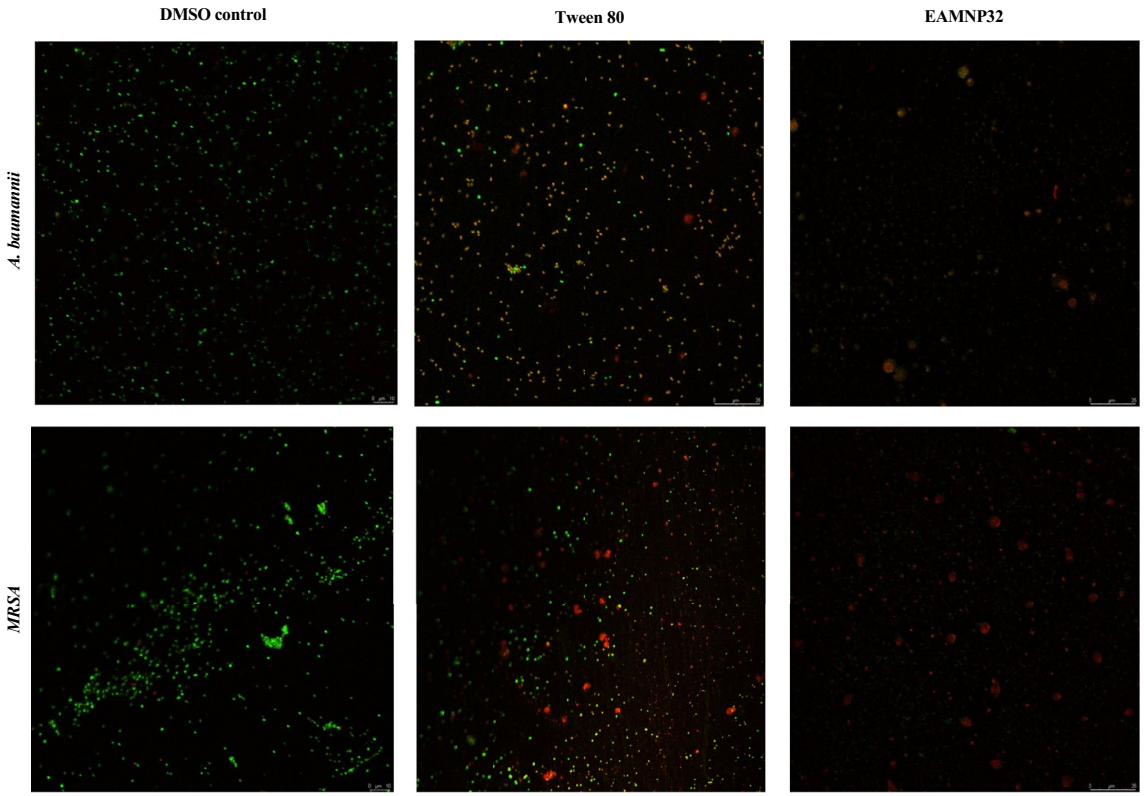


Fig. 5 Confocal Laser Scanning Microscopy of the membrane disrupting capability of EA-MNP32 on cells of MRSA and *A. baumannii*. The EA-MNP32 treated cells of MRSA and *A. baumannii* had increased red fluorescence since Propidium Iodide (PI) was able to enter the cells because of compromised cell membrane integrity. Concentration of EA-MNP32 was 1 X MIC and 10% DMSO and 5% Tween 80 was taken as negative and positive controls respectively

Northeast India for the production of bioactive secondary metabolites. However, Manas National Park, situated in the Baksa district of Assam remain unexplored, except for a single report from P Das et al. (2022), where they isolated *Streptomyces* sp. PSAA01 from the forest ecosystem of Manas National Park having antibacterial properties. Thus, the isolation of *Actinomycetia* strains from Manas National Park was undertaken in this study to further explore the potential of this unexplored ecosystem.

Based on the 16S rRNA identification, the isolate MNP32 was observed to be of the *Streptomyces* sp. since the 16S rRNA region (query length—1383 nucleotides), showed the highest similarity with *Streptomyces camponicapitis* strain I4-30 16S ribosomal RNA gene, partial sequence (MW164960.1) (99.86%) in the non-redundant nucleotide database in the BLASTN search. Whereas matches in the reference database (ribosomal RNA gene database) also showed the highest similarity to *Streptomyces camponoticapitis* strain 2H-TWYE14 16S ribosomal RNA, partial sequence (NR_152020.1) (99.49%). Both had a query cover of 100%.

During the screening of the isolate to assess its capability of producing antibacterial secondary metabolites, it was observed that MNP32 demonstrated remarkable activity against all the test pathogens using the spot inoculation method on agar plates. The highest activity was shown against gram-positive bacteria (*S. aureus* and MRSA) which was characterized by the zone of inhibition. MNP32 also showed activity against important gram-negative bacteria such as *A. baumannii, E. coli, K. pneumoniae, P. aeruginosa, and P. vulgaris* which are major drug-resistant bacteria causing life-threatening infections. Since *Actinomycetia* display varied capability of producing bioactive metabolites in submerged broth culture (Thakur et al. 2009), MNP32 was further analyzed for this purpose in submerged broth culture after confirming its activity in agar plates. To this end, various media were selected at different pH and cultured for the varied duration. Our results indicate that the highest activity (determined through the zone of inhibition against the test pathogen MRSA ATCC 43300) was observed in MNP32 when cultured in GLM broth at pH 9 for 9 days. These results are consistent with the findings of El-Naggar et al. (2016), and Dan et al. (2018), where an optimal growth period of more than 8 days was reported for producing bioactive molecules in the isolated *Streptomyces* sp. Also, the strain showed the highest activity when starch is present as

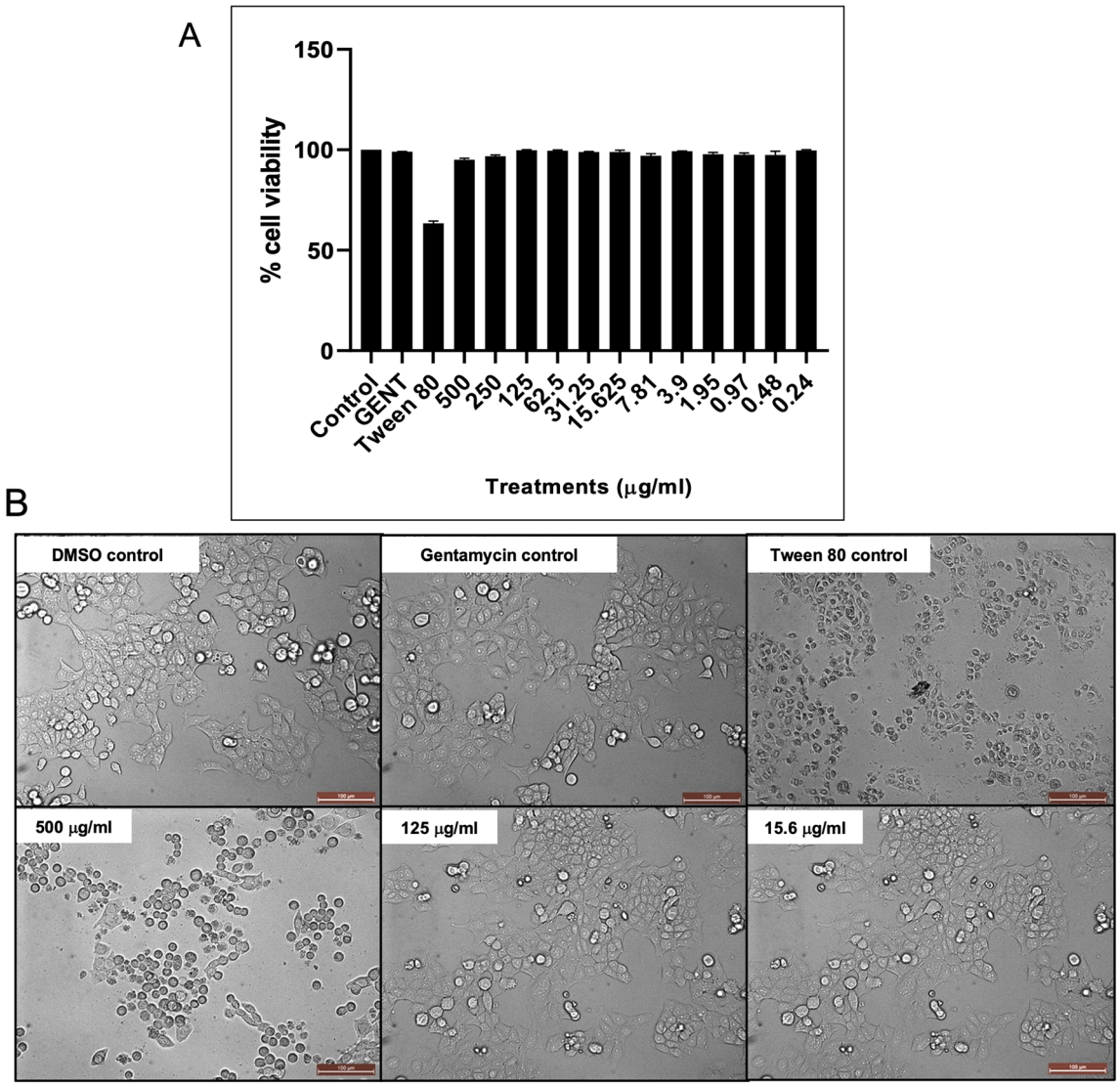
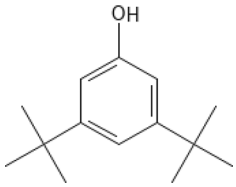
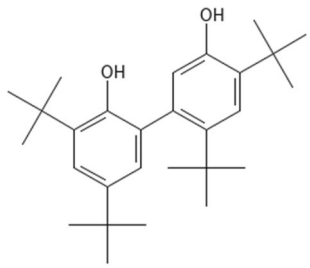


Fig. 6 Cytotoxicity of EA-MNP32 against animal cells (CC1 hepatocyte cell line). **A** Shows the % cell viability which was determined through the reduction of Alamar blue dye by cells when treated with various concentrations of EA-MNP32. It was observed that EA-MNP32 at had negligible cytotoxicity even in high concentrations. **B** Shows the visual representation of the negligible loss of cell viability when treated with near MIC concentrations of EA-MNP32. 0.1% DMSO (Control), Gentamycin 10 µg/mL (GENT) and 5% Tween 80 (Tween 80) were taken as negative and positive controls respectively

the carbon source and peptone as the nitrogen source. Similar findings were reported by Sarika et al. (2021), and Das et al. (2022), where antimicrobial activity was shown by *Actinomycetia* strain when cultured on media containing starch. The high alkaline pH at which MNP32 has shown the highest activity suggests that the strain can thrive in highly alkaline conditions. This is consistent with the studies of El-Naggar et al. (2016), where alkaliphilic *Streptomyces fradiae* NEAE-82 showed prominent L-asparaginase activity at pH of 8.5. To verify that the antibacterial activity was not due to highly alkaline media components,a control of uninoculated broth of pH 9 and pH 10 were taken as control where it was observed that highly alkaline media components were not responsible for the antibacterial activity.

After optimization of the culture conditions, the ethyl acetate extract was analyzed for its activity. It was found to be most active against *S. aureus*, MRSA and *A. baumannii*. This indicates that MNP32 has high potential against clinically important drug-resistant pathogens such as MRSA and *A. baumannii* which falls under critical priority and high priority groups of pathogens listed out by WHO (https://www.who.int/news/item/27-02-2017). The MIC values of EA-MNP32 against these two pathogens also suggest a considerable amount of antibacterial activity.

Table 3 Chemical compounds detected in enFr2 by GC–MS analysis

Sl no	Retention time	Area %	Height %	Base m/z	Compound	Structure	Bioactivity
1	19.44	18.97	20.85	163.05	Phenol, 3,5-bis(1,1-dimethylethyl)-		Reported antimicrobial activity (Dhanya et al. 2016; Sharma et al. 2016)
2	28.43	17.10	21.78	339.80	[1,1′-Biphenyl]-2,3′-diol, 3,4′,5,6′-tetrakis(1,1-dimethylethyl)-		Reported antimicrobial activity (Yahya Al-Ghamdi 2022)

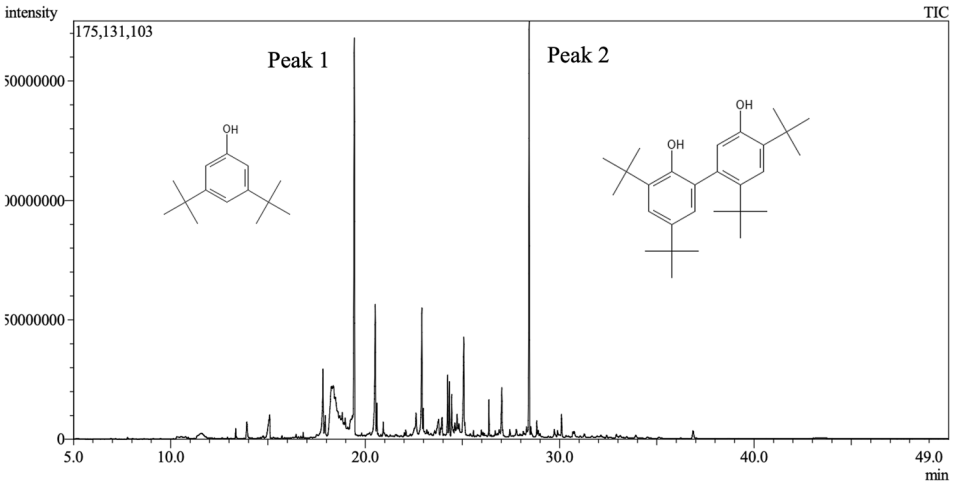


Fig. 7 Total Ion Chromatogram of GC–MS analysis showing 2 major peaks at retention times 19.44 and 28.43, with their corresponding illustrated chemical structures. The first compound was detected by comparison with NIST library, as Phenol, 3,5-bis(1,1-dimethylethyl)- and the second compound as [1,1'-Biphenyl]-2,3′-diol, 3,4′,5,6′-tetrakis(1,1-dimethylethyl)-

Further experiments with the EA-MNP32 revealed its membrane disrupting capability against both MRSA (gram-positive) and *A. baumannii* (gram-negative) which was confirmed by visualization through SEM. The electron micrograph showed that a considerable amount of cell constituents leaked out of the cells with disrupted membranes. This finding is similar to a study by Paderog et al. (2020) where they reported the cell membrane damage by the crude extracts of *Streptomyces griseorubens* strain DSD069 in MRSA cells through transmission electron microscopy.

Furthermore, the membrane damage of the MRSA and *A. baumannii* cells were confirmed through membrane leakage assay and confocal laser scanning microscopy. To determine the cellular contents, Padegorg et al. (2020), measured DNA at 260 nm and proteins at 280 nm. Similar procedures were followed in our experiments where protein estimation was directly measured using its peak absorbance at 280 nm instead of colorimetric measurements. In addition, a dose-dependent effect of the EA-MNP32 was observed where most of the cellular contents were leaked out of the cells at the MIC concentration, which gradually deceased as the concentration of the extract decreased. Tween 80, which was taken as a positive control, also had a similar effect of membrane leakage which was comparable to the 1 X MIC concentration. To further confirm the notion of membrane disruption, CLSM was performed where it was found that MRSA and *A. baumannii* cells treated with EA-MNP32 showed loss of membrane integrity. The cell viability was assessed using fluorescent dyes such as SYTO9 and Propidium Iodide (PI) (The LIVE/DEAD® BacLightTM Bacterial Viability Kit) that indicates the cell viability as a function of membrane integrity. Both dyes are nucleic acid intercalating dyes that enhance the fluorescence signal when it binds to nucleic acids, but they differ in membrane permeability.

SYTO9 (excitation/emission maxima ~480/500 nm) can cross all bacterial cell membranes that help in whole cell counts, but PI (excitation/emission maxima ~490/635 nm) can only enter cells with disrupted membranes that facilitate the differentiation between live and dead cells (Stiefel et al. 2015; Robertson et al. 2019).

One of the major problems of membrane-active antimicrobial compounds such as antimicrobial peptides and essential oils, is that it has high cytotoxicity towards host cells. This limits their potential as pharmaceutical drugs (Scazzocchio et al. 2016; Edwards et al. 2016; Yap et al. 2021). To evaluate the cytotoxicity of EA-MNP32 against animal cells, we tested its effect on a primary hepatic cell line from normal Wistar rat liver (CC1 hepatocytes). Interestingly, we observed that the concentrations of EA-MNP32 nearing its MIC value did not affect the cell viability, confirming its low cytotoxicity against animal cells.

During the identification of the chemical compounds in the active fraction, we found the presence of two major compounds in GC–MS analysis. Among them, Phenol, 3,5-bis(1,1-dimethylethyl)- is a known antimicrobial compound. Several studies have reported the finding of Phenol, 3,5-bis(1,1-dimethylethyl)- from *Streptomyces* sp. showing antimicrobial or antioxidant properties (Sharma et al. 2016; Wahaab and Subramaniam 2018; Sharma and Thakur 2020; Chakraborty et al. 2022). The other major compound that was identified as [1,1′-Biphenyl]-2,3′-diol, 3,4′,5,6′-tetrakis(1,1-dimethylethyl)- has not been reported previously in *Streptomyces* sp. as an antimicrobial compound. Since phenolic compounds are potent antimicrobial and antioxidant agents because of their hydrogen-donating capability to reduce free radicals, we propose that phenolic compounds identified in the active enriched fraction maybe responsible for the antimicrobial activity of EA-MNP32. Further chemical purification, structural elucidation, and other biological activities of the EA-MNP32 is a subject of future study.

Proposed mechanism for the mode of action of EA-MNP32

Based on the experimental observations it was determined that EA-MNP32 has membrane-disrupting capabilities, and its chemical constituents were identified as compounds containing phenol and biphenyl groups. The exact mechanism of how the phenolic compounds interact with the membrane was elucidated through a literature survey. It was found that the phenolic compounds, derived from plants or other sources, have various modes of action which includes inhibition of bacterial biofilm, inactivation of important virulent enzymes and toxins, and damage to the bacterial cell membrane (Miklasińska-Majdanik et al. 2018). Phenolic acids and phenolic compounds have strong nucleophilic properties owing to their hydroxyl groups, allowing them to interact with electrophilic groups of the plasma membrane proteins by donating a pair of electrons, leading to membrane dysfunction. They may also interfere with the respiratory process of the bacteria, as the proteins required for respiration are present in the plasma membrane. Additionally, the importance of the hydroxyl group was observed when its presence in flavonoids or chalcones increases the affinity towards the cytoplasmic membrane and thus increases the antibacterial activity. The position of the hydroxyl group in the ring structure also plays a role in facilitating antibacterial activity, as evidenced by the structure–activity relationship regarding plant phenol compounds and the importance of the phenolic hydroxyl group in antibacterial activity (Liu et al. 2008).

Therefore, it can be concluded that the hydroxyl groups present in the phenol and biphenyl compounds identified in EA-MNP32 contribute to the attachment to the cell membrane or cytoplasmic proteins, leading to membrane disruption and leakage of cell constituents (Fig. 8).

Conclusion

Exploration of the *Actinomycetia* population from unexplored ecosystems has been of immense importance in the age of antibiotic resistance. Few of the important new antimicrobial compounds have been isolated from these unexplored ecosystems recently and provides us with the hope of finding potent antimicrobials from the protected forests of northeast India. In this study, we were able to isolate a *Streptomyces* sp. from the Manas National Park of Assam, India which has significant antimicrobial activity against both gram-positive and gram-negative bacterial pathogens. We further observed that the ethyl acetate extract of the fermentation broth was active against two of the important pathogens i.e. MRSA and *A. baumannii* with a membrane disruption capability. Since resistance to membrane-disrupting compounds is difficult to develop, the phenolic compounds present in the active fraction can be a potent antimicrobial drug. Also, the low cytotoxicity value of EA-MNP32 against animal cells further supports the development of the compounds as antimicrobial drug.

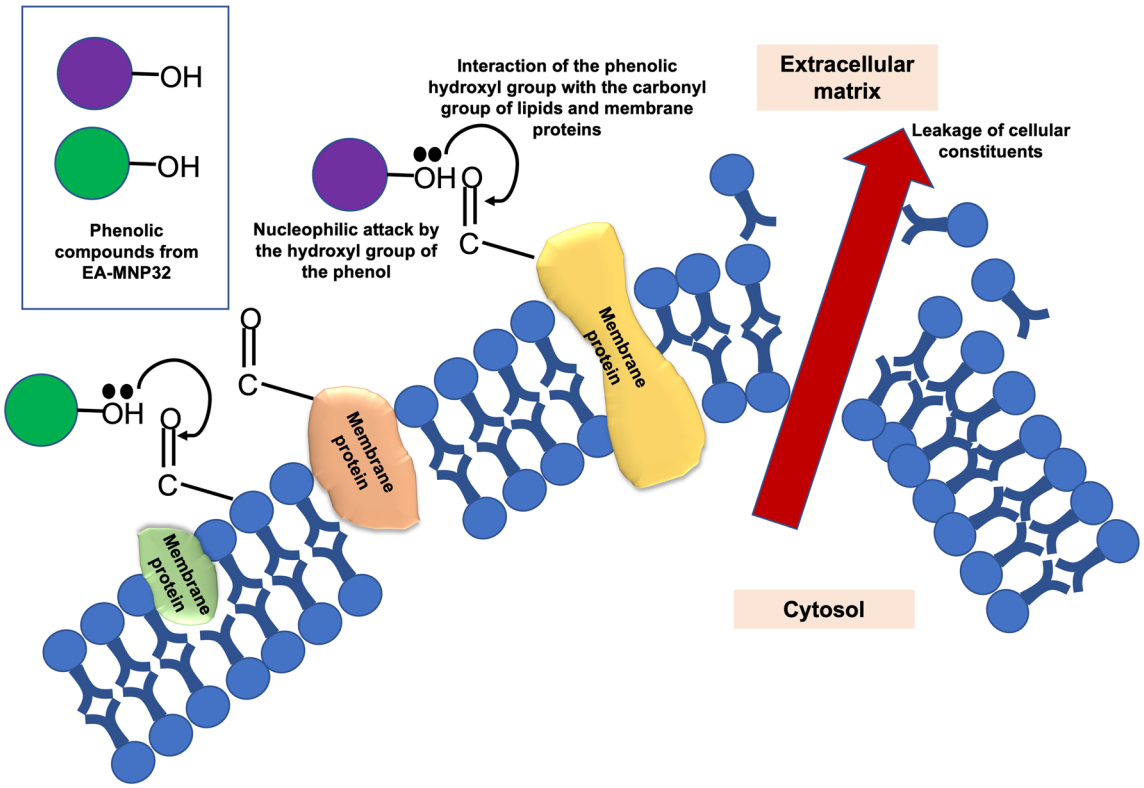


Fig. 8 A graphical representation of the proposed mechanism of action by EA-MNP32. The hydroxyl group of the phenolic compounds plays a crucial role in the interaction with the cytoplasmic membrane proteins and lipids. The hydroxyl group acts as a nucleophile and donates a pair of electron to the carbonyl group of the proteins and lipids. This interaction causes dysfunction in the membrane and causes the cell constituents to leak out of the cell and causing cell death

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Data availability The partial 16S rRNA gene sequence has been submitted to NCBI GenBank database and is available under the accession number - OP278937.

Declarations

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

Human and animal rights The work did not involve any experiments which included human or animal subjects.

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