Environmental and Experimental Biology (2021) 19: 255–263
http://doi.org/10.22364/eeb.19.24

Original Paper

Exploring antagonistic actinobacteria from a mangrove ecosystem of the southern coast of India against multidrug-resistant pathogens

ISSN 2255-9582

Sam Albert Joshua[1], Nallathambi Sangeetha[1], Appadurai Muthamil Iniyan[1,2], Samuel Gnana Prakash Vincent[1]*

[1]International Centre for Nanobiotechnology (ICN), Centre for Marine Science and Technology (CMST), Manonmaniam Sundaranar University, Rajakkamangalam-629502, Kanyakumari District, Tamil Nadu, India
[2]York Bioscience Private Limited, Ambattur Industrial Estate, Chennai, Tamil Nadu – 600 058, India

*Corresponding author, E-mail: prakash.vincent@msuniv.ac.in

Abstract

There is an urgent need to find new ways to fight multidrug-resistant pathogens, and search for novel microbial metabolites is one of the common strategies. The aim of the current study was to screen actinomycete isolates from a marine-associated ecosystem, for active compounds against multidrug-resistant human pathogens. A total of 14 morphologically distinct isolates from coastal mangrove soil were screened against seven pathogens and were found to be active against at least two pathogens. The antagonistic metabolite production studies revealed that the initial antibacterial activity of isolates against methicillin-sensitive and resistant strains of *Staphylococcus aureus* was observed on the first day of visible growth. Two strains, ICN937 and ICN938, were active against *Acinetobacter baumannii*, and were selected for further molecular identification and quantitative antibacterial analysis. Phylogenetic analysis based on 16S rRNA showed association of ICN937 to *Streptomyces antibioticus* NBRC 12838^T and of ICN938 to *Streptomyces rubiginosohelvolus* CSSP731^T. This study provides an evidence that mangrove ecosystems in coastal areas could be used as a source for active microbial metabolite producers.

Key words: *Acinetobacter baumannii*, mangrove ecosystem, methicillin-resistant *Staphylococcus aureus*, multidrug-resistant pathogens, *Streptomyces* spp.
Abbreviations: AIM, actinomycete isolation medium; CFU, colony forming units; ICN, International Centre for Nanobiotechnology; ISML, ICN Small Molecules Library; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; Rf, retardation factor; TLC, thin layer chromatography.

Introduction

Infections of various categories caused by multidrug-resistant (MDR) bacteria continue to be a worldwide problem challenging clinicians. Society currently faces a rapidly growing problem of resistance of both gram-positive and gram-negative pathogens to multiple drugs, in infections both in the hospital environment and the general community (Dhingra et al. 2020; Hu et al. 2020). This multidrug resistance a wide range of drugs occurs due to the accumulation of multiple resistance genes, typically on the plasmid, and also due to the increased expression of acquired genes that code for multidrug efflux pumps (Abdi et al. 2020). Several MDR pathogens, especially *Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter aerogenes* are the major cause of fatal infections within the hospital environment (Wright 2012). Although last-stage drugs for almost all of the listed pathogens, studies on novel antibiotics to treat drug resistance of *Acinetobacter baumannii* are still very limited (Vázquez-López et al. 2020). This calls for an urgent need to seek new antibiotics for the fight against MDR pathogens.

It is apparent that most antibiotics in use originated from natural sources including microorganisms and medicinal plants. Microorganisms have played a great deal in providing us with valuable antibiotics in the past century. Among those, actinomycetes are a diverse family of filamentous bacteria that produce a variety of pharmaceutically significant products not limited to antibiotics, but including two-thirds of all antibiotics in current clinical use (Jagannathan et al. 2021). Of all the actinomycetes, *Streptomyces* are particularly prolific antibiotic producers, which grow as a branched multicellular network of hyphae called mycelium (Barka et al. 2016). A large number of *Streptomyces* species have been isolated and screened from soil in the past decades (Otoguro et al. 2001). About 500 species of *Streptomyces*

account for 70 to 80% of relevant secondary metabolites, which have a wide range of activities such as antibacterial, antifungal, antimalarial, antiviral, antioxidant and anti-angiogenetic (Jagannathan et al. 2021).

The discovery of several new marine actinomycete taxa with unique metabolic activities in their natural environment (Subramani, Sipkema 2019), and their ability to form stable populations in different habitats and produce novel compounds with various biological activities (Rahman et al. 2010; McCauley et al. 2020), clearly show that indigenous marine actinomycetes from oceans are an important source of novel secondary metabolites. Terrestrial actinomycetes have been screened for at least half a century and now much attention has been given to the marine ecosystem and is being extensively studied (Mitra et al., 2008; Das et al. 2018). Intermediate ecosystems between the terrestrial and marine systems are of special importance as playing a vital role in contributing to novel antimicrobials. From an evolutionary perspective, these intermediary ecosystems of estuaries with mangroves serve as an evolutionary battlefield of survival, molding the microorganisms to develop adaptive behaviours. The study aim was to screen for actinomycetes that are rich in antagonistic molecules against selected human pathogens. The actinomycetes were sampled from an estuarine ecosystem in the southernmost part of the Indian peninsula.

Materials and methods

Collection of samples and isolation of actinomycetes

Soil samples were collected from a mangrove habitat of the Rajakkamangalam coastal area, Kanyakumari, India. The samples were air-dried and then heat-treated at 50 °C for 1 h to kill fast-growing microorganisms. Soil samples (1 g) were serially diluted into 10 dilutions, from which 1 mL from each tube was spread-plated on a petri dish containing actinomycete isolation medium (AIM) with starch 20 g, agar 20 g, KNO_3 1 g, $MgSO_4$ 0.5 g, NaCl 0.5 g, $FeSO_4$ 20 µg, K_2HPO_4 0.5 g in 1000 mL distilled water at pH 7.2 to 7.4. After incubation at 28 °C for 7 days, colonies with dry, powdery texture were sub-cultured individually until pure colonies were obtained. The isolated colonies were stored in 2% glycerol for long term viability and catalogued in the Microbial Bank of the International Centre for Nanobiotechnology.

Morphological characterization of isolated strains

The actinomycete isolates were distinguished macroscopically by colony characteristics such as size, shape, colour, etc. Visual observation of a 15-day-old culture cultivated on AIM medium was used to assess the morphological characteristics of isolated strains in culture. Micromorphology, i.e., spore chain morphology and sporulation were observed under a light microscope by coverslip culture method (Shinobu et al. 1959) after incubation at 28 °C for 7, 14 and 21 days. Colour of aerial and substrate mycelia was determined and recorded.

Screening for antagonistic isolates by agar plug method

All isolated actinomycetes were grown on AIM medium containing soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $FeSO_4$ 20 µM and agar 15 g in 1000 mL distilled water. Petri dishes were incubated at 28 °C for 10 days. After incubation, agar plugs were removed in replicates of three and placed on separate Petri dishes. Freshly prepared Mueller Hinton agar inoculated with overnight broth culture of each pathogen (*Enterococcus faecalis, methicillin-resistant and methicillin sensitive Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter aerogenes*) was overlaid over the placed actinomycete agar plugs. The plates were incubated overnight at 37 °C and the inhibition zones were measured.

Selection of cultivation day for activity analysis

The day of cultivation with highest inhibitory activity of crude extracts for the selected strains was tested with the same procedure used for screening of isolates for antagonistic capability. The assay was carried out daily within seven days against methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus*. The zones of inhibition were measured for each day. Three replicates were used. The statistical significance of the results was determined using ANOVA.

Growth media utilization

Testing of growth media utilization was carried out in seven different media: peptone yeast extract agar (ISP 6), yeast extract malt extract (ISP 2), glycerol asparagine agar (ISP 5), tyrosine agar (ISP 7), inorganic salt starch agar (ISP 4), oat meat agar (ISP 3), actinomycete isolation medium (AIM) and starch casein agar. Carbon source utilization was carried out by substituting starch with one of the following; arabinose, cellulose, dextrose, fructose, galactose, glucose, mannose, maltose, mannitol and sucrose. Nitrogen source utilization was determined by substituting potassium nitrate with yeast extract, ammonium sulphate, ammonium chloride, ammonium nitrate, peptone, sodium nitrate in soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $FeSO_4$ 20 µM and agar 15 g in 1000 mL distilled water. Sodium chloride tolerance was checked from 0 to 8% on an increment of 2% in the media.

Production and secondary screening of active crude extracts

Active strains from primary screening were selected based on activity against pathogens and cultured in solid AIM agar media with starch 20 g, agar 15 g, KNO_3 1 g, $MgSO_4$ 0.5 g, NaCl 0.5 g, $FeSO_4$ 20 µg, K_2HPO_4 0.5 g in 1000 mL distilled water prepared for a volume of about 25 petri dishes, each containing about 20 mL of media. After 15 days of incubation at 28 °C, the cultures were aseptically cut into pieces and treated with hexane to remove non-polar lipids.

Extract was obtained by overnight cold percolation with ethyl acetate, chloroform and methanol. The organic crude extracts were filtered using Whatman No. 1 filter paper to exclude bacterial biomass. These extracts were dried, weighed and deposited at the Small Molecules Library of the International Centre for Nanobiotechnology and stored at –20 °C for further use. The activity of the crude extracts was measured using the well diffusion method against MSSA, MRSA and *Acinetobacter baumannii* pathogens with a concentration of 50 mg mL^{-1} for efficacy.

Minimal inhibitory concentration by broth microdilution method

Minimal inhibitory concentration (MIC) by broth microdilution method was performed to quantitatively measure the effectiveness of the crude extract. A susceptibility panel with a 96-well microtitre plate containing 0.5% dimethylsulphoxide in Luria broth, increasing concentration of crude (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 μg mL^{-1}) from the extracts of ICN937 methanol, ICN937 ethyl acetate, ICN938 ethyl acetate and 10 μL of *Staphylococcus aureus* bacterial suspension was prepared to make up a total volume of about the 200 μL in each well. The plates were sealed with plastic tape and incubated overnight at 37 °C.

The MIC value was observed as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation. The turbidity of the wells in the microtitre plate was interpreted as visible growth of the microorganisms. To confirm the presence of growth, 20 μL of 1% resazurin was added and colour change was noted.

Thin-layer chromatography

Thin-layer chromatography (TLC) technique was used to partially purify the active metabolite from the crude mixture of compounds in the extract. Silica gel 60 F254 sheets (Merck) were cut into 10 × 3 cm pieces and activated in dry heat of 50 °C in a hot air oven for 15 min. Crude extracts (10 μL) of ICN937 and ICN938 were spotted 1 cm from the bottom and air-dried. A mobile phase mixture of chloroform: methanol in a ratio of 9:1 in 10 mL was prepared and poured in a 100 mL glass beaker. A strip of filter paper is dipped touching the solvent to facilitate saturation and left undisturbed for 30 min.

The spotted silica gel was placed in the beaker with the mobile phase taking care not to immerse the spot inside the solvent phase. A chromatogram was developed for 30 min and the final solvent front was marked. Developed TLC plates were visualized under UV light at wavelength 250 to 300 nm and photographed.

TLC bioautography analysis

TLC bioautography analysis was used to identify the active compounds among those separated by TLC. The TLC plate was overlaid with 1×10^6 CFU mL^{-1} of MRSA pathogen suspension prepared in a fresh Muller-Hinton broth in 0.4% (w/v) agar. The plate was incubated overnight at 37 °C in a humidified environment for 14 h. After incubation, the plate was sprayed with a 2% solution of resazurin and incubated for 30 min. The inhibition zone was observed as a purple-coloured area against a pink background on the TLC plate.

16S rRNA-based identification

Identification of the selected actinomycetes was carried out using 16S rRNA gene amplification and sequencing. The total DNA was extracted and the 16S rRNA gene of ICN937 and ICN938 was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The sequencing of PCR product was done by the dideoxy chain termination approach using an ABI 3730 DNA Analyzer (Applied Biosystems, USA). The contig from processed forward and reverse reads was queried via the EzTaxon database (Yoon et al. 2017) to find the best match. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar et al. 2018)

Results

Characterization of isolated strains

The mangrove-associated soil yielded 14 unique dry powdery actinomycete colonies in serial dilution on actinomycete isolation medium (AIM). Most strains were grey to dirty white with only 6 isolates noted to be producing diffusing pigments. Production of pigments was seen after at least 15 days of growth in AIM. Colours of aerial and substrate mycelia of all the isolates were determined and are shown in Table 1.

On screening against the seven selected human pathogens, most isolates were active against methicillin-sensitive *Staphylococcus aureus* (MSSA) with the exception of ICN939 and ICN950 (Table 2). Following MSSA, susceptibility was higher for *Enterobacter aerogenes* with only three isolates not active against it. Two isolates were active against *Acinetobacter baumannii*. There were three isolates active against *Klebsiella pneumoniae*. No isolate produced active metabolite against *Pseudomonas aeruginosa* and only one isolate was found to be active against *Enterococcus faecalis*. The highest recorded zone of inhibition was of ICN937 (25 mm) against MSSA. The detailed activity of all isolates against the pathogens is shown in Table 2.

Active metabolites were produced against both types of pathogen at day 5 for all selected isolates. The activity was seen as soon as the second day after inoculation (Table 3) for three isolates ICN937, ICN941 and ICN946. The activity was continuously observed until day 7 after inoculation. Significant difference ($p < 0.01$) in activity for ICN937 against MSSA was observed between day 2 and day 4. Significant difference ($p < 0.01$) in activity for ICN941 was observed between day 2 and day 3 against MRSA, and

Table 1. Morphological characteristics of isolated actinomycete strains in AIM plates

No.	Strain	Aerial mycelial colour	Substrate mycelial colour	Pigment production	Metabolite exudation	Micromorphology
1	ICN937	Grey	Dirty white	Absent	Absent	Rectus flexibilis
2	ICN938	Grey	Grey	Present	Absent	Rectus flexibilis
3	ICN939	Grey	Grey	Absent	Absent	Open spiral
4	ICN940	Greyish orange	Greyish orange	Present	Absent	Open spiral
5	ICN941	Brick red	Grey	Absent	Absent	Open spiral
6	ICN942	Greyish orange	Greyish orange, purple	Present	Absent	Monoverticillate spiral
7	ICN943	Grey	Brick red	Present	Absent	Rectus flexibilis
8	ICN944	Dirty white	Dirty white	Absent	Absent	Spiral
9	ICN945	Greyish green	Green	Present	Absent	Open spiral
10	ICN946	Dirty white	Dirty white	Absent	Absent	Rectus flexibilis
11	ICN947	Pale orange	Reddish brown	Present	Absent	Rectus flexibilis
12	ICN948	Dirty white	Dirty white	Absent	Absent	Open spiral
13	ICN949	Greyish orange	Brick red, pale yellow	Absent	Absent	Open spiral
14	ICN950	Dirty white	Yellow	Absent	Absent	Open spiral

also between day 3 and day 4 for ICN946. The differences in activity were not significant between day 6 and day 7 for all the tested isolates (Table 3).

Of all the tested growth media, both ICN937 and ICN938 had a similar preference for different media (Table 4). Prolific growth was observed in AIM, ISP 6 and ISP 7 media. Very mild growth was observed in ISP 5 medium. No growth was observed in ISP 2, ISP 3 and ISP 4 media until 15 days of observation. ICN937 utilized seven of the given carbon sources and four of the given nitrogen sources, whereas, ICN938 utilized only four of the provided carbon sources and four of the given nitrogen sources. The NaCl tolerance of ICN937 was 4% and that of ICN938 was 2% (Table 4).

Antagonistic efficacy of ICN937 and ICN938

The selected actinomycete ICN937 yielded about 1.2 g of dry extract with ethyl acetate extraction from the solid media. The extract appeared to be dark yellow in colour and had the highest zone of inhibition against MRSA (23.33 ± 1.52 mm) and MSSA (20.66 ± 0.57 mm) in the well diffusion assay. Similarly, about 1.7 g of golden brown coloured dry extract was obtained from methanol extraction with an activity of 21.33 ± 1.15 mm and 22.33 ± 0.57 mm for MRSA and MSSA, respectively. The ICN938 crude extract using ethyl acetate gave about 1.447 g of dry extract and with chloroform – 0.958 g of crude ectract. The antagonistic activity was greater in MRSA with a 20.66 ± 0.57 mm zone and a 19.66 ± 1.52 mm zone was observed

Table 2. Screening of selected actinomycetes against multi drug resistant pathogens by agar plug method. –, no activity; +, less than 20 mm zone of inhibition; ++, more than 20 mm zone of inhibition

No.	Strain	*Enterococcus faecalis*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*	Methicillin-sensitive *Staphylococcus aureus*	Methicillin-resistant *Staphylococcus aureus*
1	ICN937	–	–	+	–	+	++	+
2	ICN938	–	–	+	–	+	+	+
3	ICN939	–	+	–	–	+	–	+
4	ICN940	–	–	–	–	+	+	–
5	ICN941	–	–	–	–	+	+	+
6	ICN942	–	–	–	–	+	+	–
7	ICN943	–	–	–	–	+	+	–
8	ICN944	–	+	–	–	–	+	–
9	ICN945	+	–	–	–	–	+	–
10	ICN946	–	–	–	–	+	++	+
11	ICN947	–	–	–	–	+	+	+
12	ICN948	–	–	–	–	+	+	–
13	ICN949	–	–	–	–	+	+	–
14	ICN950	–	+	–	–	–	–	+

Table 3. Day of active secondary metabolite production analysis of selected actinomycete strains. (zone of inhibition in mm, $n = 3$). –, no activity. MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*. The data was statistically analyzed by two-way ANOVA followed by Tukey's post-hoc test: c, compared with day 3; d, compared with day 4; e, compared with day 5; f, compared with day 6; g, compared with day 7. *, $p < 0.05$; **, $p < 0.01$

Strain	Pathogen	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
ICN937	MRSA	–	–	15.00 ± 0.00 e*f*g*	16.66 ± 0.57 e*f*g*	21.00 ± 1.00	21.00 ± 1.00	21.00 ± 1.00
	MSSA	–	15.66 ± 0.57 d**e*f*g*	17.33 ± 0.57 e*	17.66 ± 0.57 e*f*g*	21 ± 1.00	21.33 ± 1.15	21.33 ± 1.15
ICN938	MRSA	–	–	17.33 ± 0.57	17.33 ± 0.57	18.66 ± 0.57	18.66 ± 0.57	19.00 ± 0.00
	MSSA	–	–	–	12.00 ± 0.00	13.33 ± 1.15	13 ± 1.73	13.33 ± 1.15
ICN941	MRSA	–	–	13.00 ± 2.00	13.33 ± 1.52	13.66 ± 1.15	14 ± 1.00	14 ± 1.00
	MSSA	–	12.00 c**d*e*f*g*	13.00 ± 0.00 e*f*g*	15.66 ± 0.57 e**f**g**	16.66 ± 0.57 f**g**	18.66 ± 0.57	18.66 ± 0.57
ICN946	MRSA	–	11.33 ± 0.57 d*e**f**g**	16.33 ± 1.15 d**e**g**	23.33 ± 1.15	23.66 ± 0.57	23.00 ± 1.00	23.66 ± 0.57
	MSSA	–	15.66 ± 1.15 f*g*	15.66 ± 1.15 f*g*	16.66 ± 0.57 e*	21.33 ± 1.15	21.66 ± 1.52	21.66 ± 1.52
ICN947	MRSA	–	–	11.00 ± 0.00 e*f*g**	11.00 ± 0.00 e*f*g**	15.33 ± 0.57	15.33 ± 0.57	16.00 ± 0.00
	MSSA	–	–	–	–	12.66 ± 0.57	12.66 ± 0.57	12.66 ± 0.57

for MSSA with ethyl acetate extract. Chloroform extracts showed significantly lower activity ($p < 0.01$) of 13.33 ± 1.52 and 11.33 ± 0.57 mm zones, for MRSA and MSSA, respectively, and no activity for *Acinetobacter baumannii*, compared to the positive control with gentamicin

TLC separation of ICN937 ethyl acetate and methanolic extracts provided two separate profiles when viewed under different wavelengths of light (Fig. 1). The methanolic extract had two distinct active spots when bioautographed with an overnight culture of *Staphylococcus aureus*, which was seen more prominently after the addition of resazurin. The ICN938's ethyl acetate extract had three active zones, while no active areas were noted for the chloroform extract. The detailed results are shown in Table 5.

MIC of ICN937 was calculated to be 256 μg mL^{-1} for the methanol extract and 64 μg mL^{-1} for the ethyl acetate extract, where at this concentration, complete visible growth was inhibited after overnight incubation at 37 °C. For ethyl acetate crude extract of ICN938, the MIC was 128 μg mL^{-1} (Fig. 2).

16S rRNA-based identification of ICN937

16S rRNA sequencing analysis of ICN937 showed phylogenetical similarity to the *Streptomyces antibioticus* NBRC 12838^{T} strain after BLAST (identity of 99.27%) and EzTaxon analysis (identity of 95.13%) (Fig. 3). For ICN938, BLAST analysis showed that the top hit was the *Streptomyces rubiginosohelvolus* CSSP731 strain with identity of 99.60% and EzTaxon analysis showed *Streptomyces nashvillensis* NBRC 13064^{T} as a top hit with identity of 99.44%. The 16S

Table 4. Media utilization characteristics of ICN937 and ICN938

Characteristics	ICN937	ICN938
Growth media	Actinomycete isolation medium, ISP7, ISP6, ISP5	Actinomycete isolation medium, ISP7, ISP6, ISP5
Activity	Actinomycete isolation medium, ISP7, ISP6	Actinomycete isolation medium, ISP7, ISP6
Carbon sources	Glucose, sucrose, cellulose, mannose, maltose, mannitol, dextrose	Arabinose, fructose, glucose, mannitol
Nitrogen sources	Peptone, yeast extract, ammonium nitrate, sodium nitrate	Peptone, yeast extract, ammonium chloride, sodium nitrate
NaCl tolerance (%)	4%	2%
Catalase test	+	+
Urease test	+	+
Oxidase test	+	+
Nitrate reductase test	+	+
H2S production	+	–
Citrate utilization test	–	–
Starch hydrolysis test	+	+

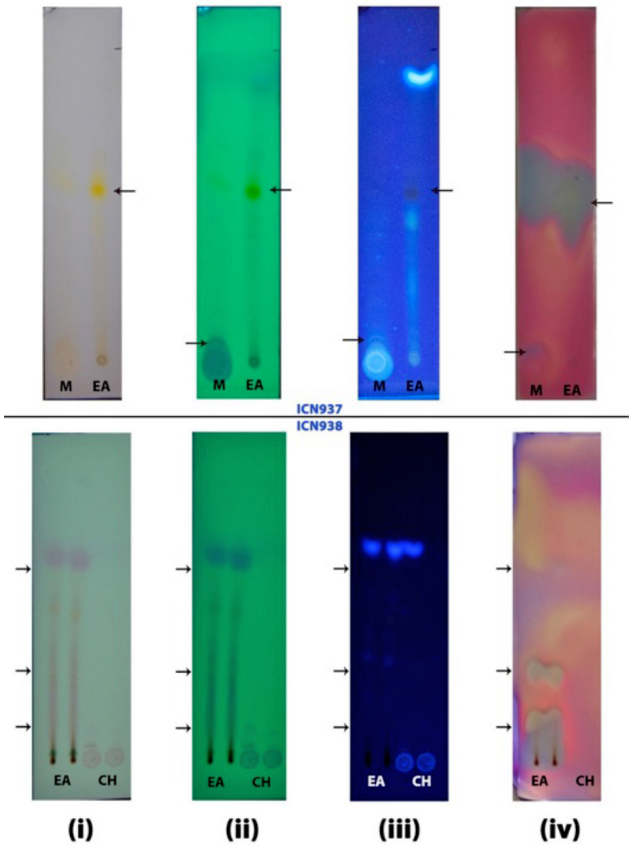

Fig. 1. Thin layer chromatography of ICN937 methanol and ethyl acetate extract; ICN938 ethyl acetate and chloroform extract. (i) as seen with the naked eye, (ii) as seen through UV-B spectrum [~312nm], (iii) as seen through UV-C spectrum [~250nm], (iv) visualization of active zones via bioautography after addition of resazurin dye.

rDNA sequences of ICN937 and ICN938 were submitted in the NCBI nucleotide database with the accession numbers MZ413588 and MZ496457, respectively.

Discussion

The emergence of challenging MDR pathogens has led to increasing interest in the search for effective antibiotics from actinomycetes in diverse ecological niches. Even now, the marine ecosystem has not been fully explored and our previous discovery proves that it is still the greatest source of natural products (Iniyan et al. 2019; Fenical 2020; Iniyan et al. 2021; Wang et al. 2020). MRSA strains have developed a thick cell wall with high resistance against almost all β-lactam antibiotics, such as penicillin, methicillin, amoxicillin and oxacillin (Al-Ansari et al. 2020). The present study was undertaken to isolate and screen culturable actinomycetes from a marine associated environment, in search of novel active compounds against MDR human pathogens. The successful isolation of actinomycetes from environmental samples requires an understanding of the potential sampling areas and environmental factors affecting their growth. In our previous screening programs, the selection of rhizosphere soil samples was found to be successful for isolating antibiotic-producing actinomycetes (Kannan et al. 2011; Iniyan et al. 2017). All of the isolated actinomycetes had antagonistic activity against at least two of the seven tested pathogens. It is known that most actinomycete species can produce a diverse range of secondary metabolites and the prospects of marine actinomycetes have previously been reported (Jensen et al. 2005; Stincone, Brandelli 2020).

Previous studies have shown that the production of secondary metabolites is dependent on cell growth (Hoskisson et al. 2006), the availability of specific nutrient

Table 5. Characteristics of crude extracts obtained from ICN937 and ICN938 after fermentation and extraction. –, no activity. #, not applicable. Well diffusion data was statistically analyzed by one-way ANOVA followed by Tukey's post-hoc test: a, compared with ICN937 ethyl acetate; b, compared with ICN937 methanol; c, compared with ICN938 ethyl acetate; d, compared with ICN938 chloroform; e, compared with gentamicin. MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*. *, $p < 0.05$; **, $p < 0.01$

Name	Solvents for extraction	Extract colour	Dry weight of the crude extract (g)	Activity against test pathogens by well diffusion (mm, $n = 3$)			Rf values of active zones
				MRSA	MSSA	*Acinetobacter baumannii*	
ICN937	Ethyl acetate	Dark yellow	1.248	23.33 ± 1.52 d**	20.66 ± 0.57 d**e**	17.66 ± 1.15 d**	0.44, 0.72
	Methanol	Golden brown	1.783	21.33 ± 1.15 d**	22.33 ± 0.57 c*d**e**	18.33 ± 0.57 d**	0.50
ICN938	Ethyl acetate	Reddish brown	1.447	20.66 ± 0.57 d**e*	19.66 ± 1.52 d**e**	18.00 ± 0.00 d**	0.16, 0.31, 0.63
	Chloroform	Light yellow	0.958	13.33 ± 1.52 e**	11.33 ± 0.57 e**	–	–
Gentamicin	#	#	#	24.33 ± 0.57	26.33 ± 0.57	18.33 ± 0.57	#

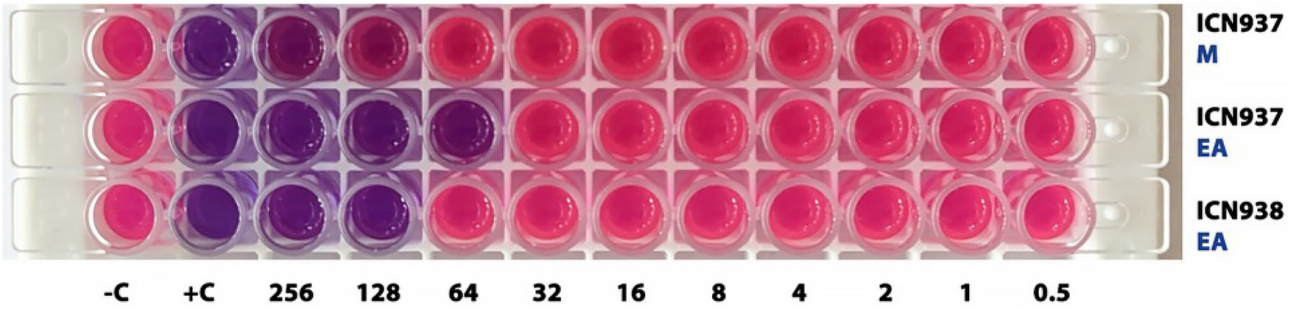

Fig. 2. Minimal inhibitory concentration of ICN937 and ICN938 extracts with positive (+C) and negative (–C) controls.

sources *in vivo* (mainly carbon and nitrogen sources), phosphate starvation, as well as amino acid and iron availability (Ruiz et al. 2010; van der Heul et al. 2018). The utilization of a wide range of carbon sources by ICN937 suggests that it could survive in a wide range of environments. However, ICN938 utilizes only four carbon sources, suggesting adaptation to more specific environments. Both actinomycete strains tested for the day of cultivation with highest inhibitory activity were able to produce metabolites considerably fast within three days after inoculation. However, it is well known that the optimal duration of metabolite extraction for actinomycetes is usually between 7 to 14 days (Pimenta et al. 2010), and the peak activity can occur as early as day 4 (Sharma, Manhas 2019). In our study, after day 5, all of the isolates maintained the activity level till day 7. Salt tolerance of several actinomycetes has been reported to be more than 15%, and these are known to be extremophiles (Sengupta et al. 2015). However, most *Streptomyces* species can only tolerate about 4% (Okazaki, Okami 1972), which is consistent with our results.

Streptomyces antibioticus, which is closely related to

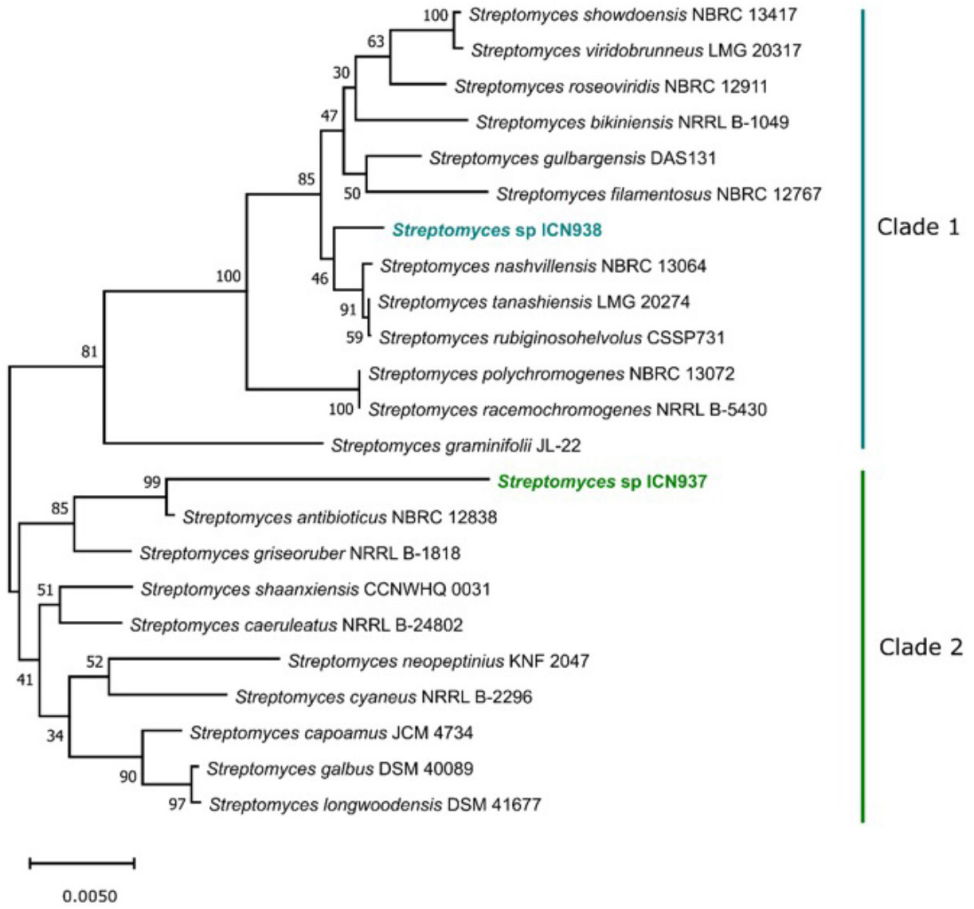

Fig. 3. Combined phylogenetic tree constructed from the 16S rRNA sequence of ICN937 and ICN938 shown as separate clades.

ICN937, was first reported by Waksman and Woodruff (1941), isolated from a garden soil. This strain was reported to produce an array of compounds commonly labelled as "actinomycins" (Sharma, Manhas 2019). *Streptomyces antibioticus* can produce siderophores like ferrioxamine E and desferrithiocin (Mulqueen et al. 1993). Further structural identification might shed more light on the molecule responsible for the antagonistic property exhibited by the ICN937 isolate against the tested pathogens. In an evolutionary perspective, ICN937 seems to be more recently evolved than its closest neighbor – *Streptomyces antibioticus* NBRC 12838 strain.

Streptomyces rubiginosohelvolus, which is closely related to ICN938, produces rubomycin (Lapchinskaia et al. 1975), an anti-tumour agent (Shishatskaya et al. 2008). It is also reported to produce daunomycin (also known as "daunorubicin") with cytotoxic properties and other structurally related compounds (Huk, Blumauerova 1989). The other closely related species of ICN938, *Streptomyces nashvillensis*, is reported to produce tetrodecamycin and dihydrotetrodecamycin, which are active against Gram-positive bacteria including MRSA and 12 strains of *Pasteurella piscicida* (Tsuchida et al. 1995).

All of the isolates that were phylogenetically closer to ICN937 and ICN938 have been isolated from terrestrial soil, except for the *Streptomyces shaanxiensis* CCNWHQ 0031 strain, which was isolated from wastewater. Since the sampling site was a fusion of terrestrial and marine ecosystems, it is no wonder that terrestrial isolates were found. In this transition zone, novel compounds could be produced because of the nature of the sampling site and possible adaptations to the ecosystem through genetic modifications. The phylogenetic analysis of the ICN937 showed different results in NCBI-BLAST and EzTaxon database searches, where only 95% identity was achieved. Thus, further investigations on the identity of the isolated strain through polyphasic taxonomic analysis (Iniyan et al. 2021) is needed. Also, for the isolated strains, further investigation of the identity and importance of the produced active compounds in needed.

Acknowledgements

The authors declare no conflict of interest. The support of Science and Engineering Research Board, Govt. of India [F.No. SR/SO/HS-104/2012] to SGPV is gratefully acknowledged The first author thankfully acknowledges the BSR fellowship provided by the University Grants Commission, New Delhi, India.

References

Abdi S.N., Ghotaslou R., Ganbarov K., Mobed A., Tanomand A., Yousefi M., Asgharzadeh M., Kafil H.S. 2020. *Acinetobacter baumannii* efflux pumps and antibiotic resistance. *Infect. Drug Resist.* 13: 423–434.

Al-Ansari M., Kalaiyarasi M., Almalki M.A., Vijayaraghavan P. 2020. Optimization of medium components for the production of antimicrobial and anticancer secondary metabolites from *Streptomyces* sp. AS11 isolated from the marine environment. J. King Saud Univ. Sci. 32: 1993–1998.

Barka E.A., Vatsa P., Sanchez L., Gaveau-Vaillant N., Jacquard C., Klenk H.-P., Clément C., Ouhdouch Y., van Wezel G.P. 2016. Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* 80: 1–43.

Das Ranjita, Romi W., Das Rictika, Sharma H.K., Thakur D. 2018. Antimicrobial potentiality of actinobacteria isolated from two microbiologically unexplored forest ecosystems of Northeast India. *BMC Microbiol.* 18: 71.

Dhingra S., Rahman N.A.A., Peile E., Rahman M., Sartelli M., Hassali M.A., Islam T., Islam S., Haque M. 2020. Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. *Front. Public Health* 8: 535668.

Fenical W.B. 2020. Marine microbial natural products: the evolution of a new field of science. *J. Antibiot.* 73: 481–487.

Hoskisson P.A., Sharples G.P., Hobbs G. 2006. Differentiation and protease production in *Micromonospora echinospora* (ATCC 15837). *Antonie van Leeuwenhoek* 89: 191–195.

Hu X.Y., Logue M., Robinson N. 2020. Antimicrobial resistance is a global problem – a UK perspective. *Eur. J. Integr. Med.* 36: 101136.

Huk J., Blumauerova M. 1989. Streptomycetes producing daunomycin and related compounds: Do we know enough about them after 25 years? *Folia Microbiol.* 34: 324–349.

Iniyan A.M., Kannan R.R., Vincent S.G.P. 2017. Characterization of culturable Actinomycetes associated with halophytic rhizosphere as potential source of antibiotics. *Proc. Natl. Acad. Sci. India B Biol. Sci.* 87: 233–242.

Iniyan A.M., Sudarman E., Wink J., Kannan R.R., Vincent S.G.P. 2019. Ala-geninthiocin, a new broad spectrum thiopeptide antibiotic, produced by a marine *Streptomyces* sp. ICN19. *J. Antibiot.* 72: 99–105.

Iniyan A.M., Wink J., Landwehr W., Ramprasad E.V.V., Sasikala C., Ramana C.V., Schumann P., Spröer C., Bunk B., Joseph F.-J.R.S., Joshua S.A., Shyl E.-N.S.-G.D., Kannan R.R., Vincent S.G.P. 2021. *Streptomyces marianii* sp. nov., a novel marine actinomycete from southern coast of India. *J. Antibiot.* 74: 59–69.

Jagannathan S.V., Manemann E.M., Rowe S.E., Callender M.C., Soto W. 2021. Marine Actinomycetes, new sources of biotechnological products. *Mar. Drugs* 19: 365.

Jensen P.R., Mincer T.J., Williams P.G., Fenical W. 2005. Marine actinomycete diversity and natural product discovery. *Antonie van Leeuwenhoek* 87: 43–48.

Kannan R.R., Iniyan A.M., Prakash V.S.G. 2011. Isolation of a small molecule with anti-MRSA activity from a mangrove symbiont *Streptomyces* sp. PVRK-1 and its biomedical studies in Zebrafish embryos. *Asian Pac. J. Trop. Biomed.* 1: 341–347.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35: 1547–1549.

Lapchinskaia O.A., Saburova T.P., Siniagina O.P., Konstantinova N.V, Filicheva V.A. 1975. Spontaneous and induced variability in *Actinomyces rubiginosohelvolus*, a new producer of the antibiotic rubomycin. *Antibiotiki* 20: 1061–1065. /in Russian/

McCauley E.P., Piña I.C., Thompson A.D., Bashir K., Weinberg M., Kurz S.L., Crews P. 2020. Highlights of marine natural products having parallel scaffolds found from marine-derived bacteria, sponges, and tunicates. *J. Antibiot.* 73: 504–525.

Mitra A., Santra S.C., Mukherjee J. 2008. Distribution of actinomycetes, their antagonistic behaviour and the physico-chemical characteristics of the world's largest tidal mangrove forest. *Appl. Microbiol. Biotechnol.* 80: 685–695.

Mulqueen G.C., Pattenden G., Whiting D.A. 1993. Synthesis of the thiazoline-based siderophore (S)-desferrithiocin. *Tetrahedron* 49: 5359–5364.

Okazaki T., Okami Y. 1972. Studies on marine microorganisms. II: Actinomycetes in sagami bay and their antibiotic substances. *J. Antibiot.* 25: 461–466.

Otoguro M., Hayakawa M., Yamazaki T., Iimura Y. 2001. An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. *J. Appl. Microbiol.* 91: 118–130.

Pimenta E.F., Vita-Marques A.M., Tininis A., Seleghim M.H.R., Sette L.D., Veloso K., Ferreira A.G., Williams D.E., Patrick B.O., Dalisay D.S., Andersen R.J., Berlinck R.G.S. 2010. Use of experimental design for the optimization of the production of new secondary metabolites by two *Penicillium* species. *J. Nat. Prod.* 73: 1821–1832.

Rahman H., Austin B., Mitchell W.J., Morris P.C., Jamieson D.J., Adams D.R., Spragg A.M., Schweizer M. 2010. Novel anti-infective compounds from marine bacteria. *Mar. Drugs* 8: 498–518.

Ruiz B., Chávez A., Forero A., García-Huante Y., Romero A., Sánchez M., Rocha D., Sánchez B., Rodríguez-Sanoja R., Sánchez S., Langley E. 2010. Production of microbial secondary metabolites: Regulation by the carbon source. *Crit. Rev. Microbiol.* 36: 146–167.

Sengupta S., Pramanik A., Ghosh A., Bhattacharyya M. 2015. Antimicrobial activities of actinomycetes isolated from unexplored regions of Sundarbans mangrove ecosystem. *BMC Microbiol.* 15: 1–16.

Sharma M., Manhas R.K. 2019. Purification and characterization of actinomycins from *Streptomyces* strain M7 active against methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus. BMC Microbiol.* 19: 44.

Shinobu R., Kawato M. 1959. On *Streptomyces massasporeus* nov. sp. *Shokubutsugaku Zasshi* 72: 283–288.

Shishatskaya E.I., Goreva A. V., Voinova O.N., Inzhevatkin E. V., Khlebopros R.G., Volova T.G. 2008. Evaluation of antitumor activity of rubomycin deposited in absorbable polymeric microparticles. *Bull. Exp. Biol. Med.* 145: 358–361.

Stincone P., Brandelli A. 2020. Marine bacteria as source of antimicrobial compounds. *Crit. Rev. Biotechnol.* 40: 306–319.

Subramani, Sipkema 2019. Marine rare actinomycetes: a promising source of structurally diverse and unique novel natural products. *Mar. Drugs* 17: 249.

Tsuchida T., Iinuma H., Nishida C., Kinoshita N., Sawa T., Hamada M., Takeuchi T. 1995. Tetrodecamycin and dihydrotetrodecamycin, new antimicrobial antibiotics against *Pasteurella piscicida* produced by *Streptomyces nashvillensis* MJ885-mF8: I. Taxonomy, fermentation, isolation, characterization and biological activities. *J. Antibiot.* 48: 1104–1109.

van der Heul H.U., Bilyk B.L., McDowall K.J., Seipke R.F., van Wezel G.P. 2018. Regulation of antibiotic production in Actinobacteria: new perspectives from the post-genomic era. *Nat. Prod. Rep.* 35: 575–604.

Vázquez-López R., Solano-Gálvez S.G., Juárez Vignon-Whaley J.J., Abello Vaamonde J.A., Padró Alonzo L.A., Rivera Reséndiz A., Muleiro Álvarez M., Vega López E.N., Franyuti-Kelly G., Álvarez-Hernández D.A., Moncaleano Guzmán V., Juárez Bañuelos J.E., Marcos Felix J., González Barrios J.A., Barrientos Fortes T. 2020. *Acinetobacter baumannii* resistance: a real challenge for clinicians. *Antibiotics* 9: 205.

Waksman S.A., Woodruff H.B. 1941. Actinomyces antibioticus, a new soil organism antagonistic to pathogenic and non-pathogenic bacteria. *J. Bacteriol.* 42: 231–249.

Wang Y.-N., Meng L.-H., Wang B.-G. 2020. Progress in research on bioactive secondary metabolites from deep-sea derived microorganisms. *Mar. Drugs* 18: 614.

Wright G.D. 2012. Antibiotics: a new hope. Chem. Biol. 19: 3–10.

Yoon S.H., Ha S.M., Kwon S., Lim J., Kim Y., Seo H., Chun J. 2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67: 1613–1617.

Received 27 July 2021; received in revised form 12 December 2021; accepted 15 December 2021