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In vitro biomedicinal properties of Pyrrolidine-2,4-Dione derived from a novel actinobacterium *Streptomyces rochei*, a green approach

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ABSTRACT

A novel actinobacterium, *Streptomyces rochei* (MSA-14) was isolated from the mining sediments of Indian Rare Earth (IRE) Ltd. southwest coast of India. Secondary metabolites were extracted from *S. rochei* using ethyl acetate. The crude ethyl acetate extract exhibited prominent antibacterial property with wide spectral inhibitory zones (3–15 mm) having least Minimum Inhibitory Concentration (1–50 μg/ml) and Minimum Bactericidal Concentration (10–100 μg/ml) against the tested bacterial pathogens. The extract evidenced remarkable antioxidant (91%) and hemolytic (45.52%) properties at 800 μg/ml. In addition, the extract recorded good cytotoxic effect against *Artemia salina* nauplii with the LC_{50} value of 106.75 μg/ml. Further, the extract was purified by normal phase silica column chromatography using step gradient solvent method. Among the column fractions, 14th fraction rendered good biomedicinal properties. The bioassay guided 14th fraction was further purified by preparative TLC. The lead compound was analysed using FT-IR and GC-MS analysis, which ascribed a prominent bioactive compound Pyrrolidine-2,4-Dione. *In silico* analysis on the biodegradability of Pyrrolidine-2,4-Dione using BIOWIN and ECOSAR signified that the identified bioactive compound is more plausible for the environment, eco-friendly, easily degradable within days to week under aerobic and anaerobic conditions. Also, it is non-toxic against chosen bacterial pathogens when compared with the standard antibiotic Streptomycin.

1. Introduction

Microbes are ubiquitous in the world. The diversity and abundance of microbes is determined by the biogeographical habitat where they survive. Biological diversity renders chemical diversity which is a sustainable source of clues for the novel and robust biomolecules. On the current pharmaceutical scenario, insists for new antimicrobial agents to control the emerging diseases or pathogenic drug resistant microbes prompted a number of researchers to explore natural and novel bioactive compounds from the ocean. Globally, many researchers are formulating extensive screening programs and thus implying greater efforts with intend of isolating novel bioactive compounds from marine microbes throughout the year (Debbab et al., 2010). Marine microbes are one of the prolific sources of natural products. They are the origin of many novel chemically diverged bioactive molecules that paves way for the development of lead bioactive molecules (Molinski et al., 2009; Mayer et al., 2011). Remarkably, the marine microbes scored the maximum hit rates of marine compounds for the drug development and thus they are highly attractive. Amongst marine microorganisms, the natural products from actinobacterial group has gained special trademark in the pharmaceutical industry (Imhoff et al., 2011). Actinobacterial genera such as *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Marinospora*, *Micromonospora*, *Nocardiopsis*, *Saccharopolyspora*, *Salinospora*, *Verrucosispora* and *Streptomyces* are the major resources of several

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commercially important bioactive natural products (Manivasagan et al., 2014). Among these, *Streptomyces* are one of the widely distributed species in the marine environment and acquired first rank as the latent producer of new bioactive molecules (Manivasagam et al., 2010). Most important biological properties of *Streptomyces* spp. includes antibacterial, antifungal, cytotoxicity, anticancer, anti-inflammatory, antimalarial, antiviral, antioxidant and antifouling activities (Renner et al., 1999; Maskey et al., 2004; Kumar et al., 2006; Wu et al., 2006; Oh et al., 2008; Saurav and Kannabiran, 2012; Prakash et al., 2013, 2015).

On detailed literature review, it was revealed that the actinobacteria play a vital role in leaching of rare earth metals. Indeed, the studies on microbial consortia and their metabolic activities within the deep mineral deposits (mining sites) improved our knowledge on geomicrobiological for better management of hazardous wastes and also endowed attractive opportunities to explore novel pharmaceuticals (Zhang et al., 2005). Supportively, Islam et al. (2014) and Dhal and Sar (2014) explored the microbial diversity of Uranium mines along Singhbhum thrust belt of Jharkhand, India by 16S rRNA gene sequencing (culture dependent) and other molecular tools and inferred that these bacterial genera belong to the groups of *Proteobacteria*, *Acidobacteria*, *Bacteroidetes* and *Firmicutes*. Chronakova et al. (2010) assessed the total actinobacterial distribution and diversity in the mining sediment of Bohemia, Sokolov Coal Basin and evinced that *Streptomyces* spp. were the most predominant genera in the middle layers of soils (mineral layer). Nonetheless, *Streptomyces* spp. has also been recognized for their potent antagonistic properties against too many dreadful bacterial pathogens including MRSA. Adding cream to the top, the secondary metabolite production of actinobacteria is elicited by rare earth metals (Kawai et al., 2007). Currently, very little information is available on the identification of biomedical compounds from actinobacterial strains isolated from mining sites. In light of the above, present study is an attempt to isolate, screen, identify and purify potent bioactive compound from a novel actinobacterium *Streptomyces rochei* MSA-14 isolated from the marine mining sediment of Indian Rare Earth (IRE) Ltd., Tamilnadu, India.

2. Materials and methods

2.1. Description of the study area

In this study, the marine actinobacterial strains were isolated from the sediment samples of Indian Rare Earth (IRE) Ltd., Manavalakurichi, southwest coast (77°5′ and 77°40′ E long; 8°20′ and 8°50′ N lat.) of Kanyakumari District, Tamilnadu, India. Rare Earth Elements (REEs) are precious gift that improve the properties of soil and enhance the microbial diversity and density. Every year, approximately 1,16,500 tones of different REEs are separated from Manavalakurichi coastal sediments (Mukherjee, 2004). For the present investigation, eight sampling points were fixed and each sampling point was ~400 m distance starting from the eastern to western region of IRE. The first sampling (S1) point was pointed near river mouth; sampling points S2–S4 were along the REE soil dumping area. The samples S5–S7 were collected from the REE mining and mining effluent areas. The last sampling point (S8) was near the natural deposit of REEs sediment sample (Fig. S1).

2.2. Collection and pretreatment of sediment samples

The samples from all the sampling points were collected in sterile polythene bags and brought to the laboratory in an ice cold box. The collected samples were then air dried at room temperature for a week in aseptic condition and pretreated by hot water bath at 55 °C for 45 min.

2.3. Isolation and identification of marine actinomycetes groups

From the pretreated sediment samples collected from the eight sampling points, 1 g each was taken and the individual sample was serially diluted up to 10^{-6} using sterile 50% seawater. Total Heterotrophic Actinomycetes (THA) was isolated using three different isolation media *viz*. Starch Casein Agar (SCA), Actinomycete Isolation Agar (AIA) and Yeast Malt Extract agar (ISP-2) (Himedia, India). All the isolation media were individually prepared in 50% filter sterilized seawater and autoclaved at 121 °C for 15 min. After sterilization, the media were cooled and 100 μg/ml of Cycloheximide (antibacterial) and 20 μg/ml of Nalidixic acid (antifungal) were added to suppress the growth of gram negative bacteria, fungi and yeast (Sivakumar et al., 2005). An aliquot of 0.2 ml from each sample was spread individually on the surface of plates using sterile L rod and incubated at 28 ± 2 °C. The plates were observed for the actinobacterial growth from 7th day onwards til the end of month. Finally, the THA density and the percentage distribution were calculated using standard formulae. Further, the actinobacterial isolates were purified and stored in SCA medium at 4 °C. The isolated actinomycetes were identified on the basis of culture, morphological (micro and macro) and physiological characteristics described by Shirling and Gottlieb (1966). Further, all these isolates were identified up to genus level based on the reference keys of Nonomura (1974) and Holt et al. (1994).

2.4. Selection of pathogenic bacterial strains

Totally, eleven different disease causing clinical bacterial pathogens such as *Streptococcus mutans* (MTCC 890), *Pseudomonas aeruginosa* (MTCC 2642), *Enterococcus faecalis* (MTCC 439), *Bacillus subtilis* (MTCC 1134), *Salmonella typhi* (MTCC 733), *Proteus vulgaris* (MTCC 744), *Lactococcus lactis* (MTCC 440), *Escherichia coli* (MTCC 1671), *Streptococcus pneumoniae* (MTCC 1936), *Klebsiella pneumoniae* (MTCC 7407) and *Staphylococcus aureus* (MTCC 96) were obtained from Microbial Culture Collection Center, Department of Microbiology, Center for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari district, Tamil Nadu, India.

2.5. Screening of antagonistic properties of isolated marine actinomycetes

In total, 35 morphologically distinct actinomycetes (MSA1to MSA35) isolates were selected for screening the antagonistic properties. Primarily, the actinomycetes isolates were screened against selected clinical bacterial pathogens through cross streak method (Sivakumar et al., 2005). From this, only 10 actinobacterial isolates exhibited maximum growth inhibitory activity (MSA1-4, 7, 8, 9 and 12–14) were subjected for secondary screening by agar well diffusion method (Saadoun and Muhana, 2008). Based on the primary and secondary screening results, only one potent actinobacterial isolate MSA14 with excellent antagonistic activity was selected. Further, the culture characteristics of MSA14 was determined by chemotaxonomy and different ISP (International Streptomyces Project) and non- ISP media (Aparana et al., 2013). Also, the isolate MSA14 was identified by classical methods such as by examining aerial and substrate mycelium colour, diffusion of pigments, spore morphology, melanoid pigment production, physiological characters and also molecular taxonomy (Prakash et al., 2013). Based on the above characteristics, the strain MSA14 was identified as *Streptomyces rochei*.

2.6. Fermentation and extraction of secondary metabolites from S. rochei

To extract the secondary metabolite, the potent isolate *S. rochei* (MSA-14) was mass cultivated in Starch Casein Broth (SCB) at 37 °C for 4 days. Briefly, 2% of prepared seed culture was inoculated into 10 L flask containing SCB and incubated in an orbital shaker (150 rpm) for 7 days at 30 °C. Then the fermented culture broth was centrifuged (10,000 rpm) at 4 °C for 20 min and the collected supernatant was filtered using 0.45 μ cellulose filter paper. The obtained cell free culture

supernatant was divided in two equal portions and individually mixed with the organic solvents such as ethyl acetate and chloroform and placed in a shaker at 120 rpm for 72 h. The active metabolite localized in the organic phase was separated, concentrated by rotary vacuum evaporator and the obtained crude extract was tested for its biological activities.

2.7. Antibacterial property, MIC and MBC of crude extract of S. rochei

Antibacterial property of the ethyl acetate and chloroform based crude extracts of *S. rochei* was studied against the test bacterial pathogens. Before initiating the assay, all the test pathogenic bacterial strains were seeded (2×10^8 cells/ml) individually on Muller Hinton Agar (MHA) plates. Then, wells of 6 mm diameter were made using a sterile cork borer and each well was then filled with 100 µg/ml concentration of both crude extracts individually and incubated for 24 h at 30 °C (Ramasubburayan et al., 2015). The assay was carried out in triplicate. Streptomycin (50 µg/ml) was used as a positive control. Growth inhibitory activity in terms of zone of inhibition (mm) was measured from the edge of the well. Based on the results of antibacterial activity, the ethyl acetate extract of *S. rochei* with potent activity was used for further studies.

The MIC of ethyl acetate crude extract of *S. rochei* was determined by micro dilution method in 96 multi well microtiter plate (Prakash et al., 2015). In brief, different concentrations (0.01–100 µg/ml) of the crude ethyl acetate extract was prepared using carrier solvent [ethyl acetate: methanol (3:1)] and added in to the microtiter plate and allowed for complete evaporation of solvent. To this, 100 µl each of the test bacterial culture (2×10^8 cells/ml) was added in each well. Wells containing only the bacterial suspension was used as a negative control; whereas broth containing standard drug (Streptomycin) was used as a positive control. Then the microtiter plate was incubated at 37 °C for 24 h. The assay was carried out in six replicates. The MIC was the lowest concentration of crude extract which showed no turbidity after incubation. The turbidity in the well is the result of visible growth of microorganisms. The MBC was determined by inoculating a loop full of bacterial culture from each well into Nutrient Agar (NA) plates and incubated. In this, the least concentration of extract which did not show any visible growth in NA plates was taken as the value of MBC (Ramasubburayan et al., 2017b).

2.8. In vitro antioxidant property of crude extract of S. rochei

In vitro antioxidant ability of the crude extract of *S. rochei* was determined by assessing Total Phenolic Content (TPC), Total Antioxidant Activity (TAA) and DPPH free radical scavenging activities by the methods described by Prakash et al. (2016).

2.9. Total phenolic content (TPC) assay

The TPC of the extract was estimated by Folin- Ciocalteau method. In brief, an aliquot (400 µg/ml) of crude extract of *S. rochei* was added to 2 ml of Folin-Ciocalteu reagent and 1 ml (75 g/L) of sodium carbonate. The reactant tube was vortexed for 15 s and allowed to stand at 40 °C for 30 min in dark condition. Finally, the coloured reaction mixer solution was read at 760 nm using UV-VIS Spectrophotometer (Per Kin Elmer Lamda 25, Waltham, MA- 02451, USA). The total phenolic content in the crude extract of *S. rochei* was expressed as Gallic acid per gram (GAE/g). The assay was carried out in three separate tubes.

2.10. Total antioxidant activity (TAA)

TAA of the extract was examined by using phosphomolybdenum method. For this, 400 µg/ml of crude extract of *S. rochei* was taken in a test tube and mixed with 1.8 ml of reagent solution (0.6 M Sulfuric acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate) and incubated at 95 °C for 90 min. The test tube was allowed to cool at room temperature and the absorbance was read at 696 nm. TAA was expressed as equivalents of Gallic acid (mg/ml) of the extract.

2.11. DPPH - free radical scavenging property

1 ml of different concentrations (50–800 µg/ml) of crude extract of *S. rochei* was taken in test tubes. To this, 2 ml of DPPH solution (0.16 mM DPPH prepared in methanol) was added and vortexed well and allowed to stand for 30 min under dark condition at room temperature. Gallic acid (400 µg/ml) was used as a positive control. The optical density (OD) of different concentrations of the extract and positive control were read at 517 nm and its ability to scavenge DPPH radical was calculated by the following formula

$$\text{DPPH Scavenging activity } (\%) = 1 - \left(\frac{\text{OD of the test sample}}{\text{OD of the control}}\right) \times 100$$

2.12. Hemolytic activity of crude extract of S. rochei

Human blood samples from healthy volunteers were collected in vacuum tubes containing 2.7% EDTA as anti-coagulant. The erythrocytes were pooled out by centrifugation at 2000 rpm for 10 mintues and was washed thrice with Phosphate Buffered Saline (PBS). Then, the PBS was added to the pellet to yield 10% (v/v) erythrocytes/PBS suspension (0.844 OD). Then 10% suspension was diluted (1:10) in PBS. From this, 100 µl of suspension was added in triplicate to 96-well microtiter plates containing 100 µl of different concentrations (25–1600 µg/ml) of crude extract. Methanol: ethyl acetate (3:1) were used as carrier solvents. PBS was used as a negative control and 0.1% Triton X-100 was used as a positive control. Then, the reaction mixture in the microtiter plate was incubated at 37 °C for 1 h and then centrifuged at 2000 rpm for 10 min. The absorbance of supernatant was read using the ELISA reader (Robonik India Pvt. Ltd., Maharashtra, India) at 540 nm (Suthindhiran and Kannabiran, 2010). Then the percentage of hemolysis and hemolytic index were calculated by ASTM standard practice F 756-00; assessment of hemolytic properties of materials.

2.13. Cytotoxic effect of crude extract of S. rochei against Artemia salina nauplii

A. salina nauplii cytotoxicity assay is one of the simplest *in vitro* screening techniques to test the toxicity of secondary metabolites/drug obtained from the plants and microbes (Ramasubburayan et al., 2017b). For this, varying concentrations (12.5–400 µg/ml) of crude extract was prepared and transferred in to 24 well microtiter plate (Flat bottom polystyrene) using carrier solvent [methanol: ethyl acetate (3:1)] (Zhang et al., 2012). Then, live *A. salina* nauplii (10 nauplii in each well) were added to the extract coated wells with 500 µl of filter sterilized seawater. After 24 h of incubation at 25 °C, live and dead napulii were counted in each test concentration. Only seawater was used as a negative control. The assay was tested in triplicate. After 24 h of incubation, the result was recorded as the percentage of mortality and 50% Lethal Concentration (LC_{50}) value of the crude extract was calculated through probit analysis.

2.14. Purification and characterization of bioassay guided active fraction

The crude extract (1.75 g) of *S. rochei* was purified by normal phase column chromatographic techniques (60–200 µm mesh size) through stepwise gradient solvent system i.e. hexane, chloroform, ethyl acetate, methanol, warm and cold water. Totally, 25 fractions (20 ml each) were collected and dried under dark condition at room temperature. All the fractions (100 µg/ml) were screened individually against test bacterial

pathogens in microtiter plate assay (Prakash et al., 2015, 2016). Based on the result, the most active fraction (fraction No.-14th-Ethyl acetate: Methanol - 80:20) was selected for further purification.

TLC bioautography, antioxidant and primary chemical constituents of bioassay guided fraction.

The bioactive metabolite present in the bioassay guided column fraction (126 mg) was dissolved in 1 ml of methanol and made up to a volume of 126 mg/ml. From this, 100 µl was taken and spotted on TLC plates (TLC aluminum sheets, silica gel $60F_{254}$, Merck Co., USA) with a mobile phase chloroform: ethyl acetate: methanol (30:50:20). The developed TLC plates were dried at room temperature and observed under UV/Vis absorption (Bio-Rad; AlphaImager™ 3300) for detection of bands at different wavelengths (254 and 366 nm). The R_f values of bands on the TLC plate was then calculated and recorded.

To detect the bioassay guided active spot of *S. rochei*, the TLC plates were tested for antibacterial activity against selected pathogenic bacterial strains using TLC bioautography method (Prakash et al., 2013). The antioxidant potential of the active spot in TLC plate was checked out by spraying 2% DPPH solution (2 mg/ml) and observed for colour change from yellow to purple after 30 min of expoure (Kannan et al., 2010). Also, the hemolytic property of the active fraction was assessed by Amini et al. (2014).

2.15. Chemical characterization of the active fraction

The active spot (R_f 0.69) was separated from the TLC plate by the modified method described by Prakash et al. (2015). Concisely, after developing TLC with appropriate organic solvent systems, they were air dried and visualized under UV- transilluminator. The active TLC spot was scraped off using sterile spatula and redissolved in the same solvent system and filtered through Whatman No.1 filter paper (Himedia, India) for the complete removal of silica. Finally, the solvent containing bioactive metabolite was concentrated at room temperature. The partially purified active residue was tested further for the presence of primary chemical constituents *viz* alkaloids, carboxylic acid, glycosides, coumarins, flavonoids, quinone, phenolics, saponins, protein, resin, steroids, tannin and sugars (Ahmed et al., 2007).

2.16. Determination of active principle compound through FT-IR and GC-MS analysis

The possible functional groups present in the active spot was assessed by Fourier Transmission Infra Red (FT-IR, Shimadzu FTIR-820 IPC, Japan) spectrum, where the frequency set was between 4500 and 300 cm^{-1} wave number and the vibration spectrum was recorded. The GC-MS analysis of active spot was analysed by using Agilent GC-MS 5975 Inert XL MSD (United States) gas chromatography (equipped with J & W 122–5532G DB-5 ms 30 × 0.25 mm x 0.25 µm) and mass detector was operated in EMV mode. Helium was used as a carrier gas with a flow rate of 1.0 ml/min. The injection port temperature was operated at 250 °C. The column oven temperature was set at 80 °C for 2 min programmed at 10–250 °C/min, which was held for 0 min. Then at 5 °C/min to 280 °C, which was held for 9 min. Electron impact spectra in positive ionization mode was acquired between *m/z* 40 and 450. For more accuracy, the peaks with prominent area and quality (above 80%) were alone considered and the constituents were identified by comparison with the internal standards of the instrument and spectral match with NIST library. From the FTIR and GC-MS studies, the active principle compounds was identified as Pyrrolidine-2,4-dione (Tetramic acid).

Biodegradability of Pyrrolidine-2,4-dione and Streptomycin sulphate (*In Silico*).

To understand the biodegradability pattern of the identified bioactive compound, Pyrrolindine-2,4-dione (Tetramic acids) and positive control Streptomycin sulphate (antibiotic) were tested *in silico*. The log K_{ow} value (log octanol-water partition coefficient) was calculated using the HOWWIN™ and biodegradability prediction by BIOWIN™ (biodegradation probability program) Models: Biowin-1: Linear; Biowin-2: non-Linear; Biowin-3: ultimate; Biowin-4: Primary; Biowin-5: linear Ministry of International Trade and Industry (MITI); Biowin-6: non-linear MITI and Biowin-7: anaerobic. The result was evaluated by Criteria A: model-2 & 6 > 0.5 and model-3 > 2.2; Criteria-B: model-3 as “Week or faster” and model-5 > 0.5. In addition, the natural organic SAR baseline toxicity concentration against aquatic organisms was estimated using the Ecological Structure Activity Relationship Programme (ECOSAR™) tools from the Estimation Program Interface (EPI-4.2) software developed by the Environmental Protection Agency's Office of Pollution prevention and Toxics an Syracuse Research Corporation (SRC) (http://www.epa.gov/opptintr/exposure/pubs/episuitedl.html).

2.17. Statistical analysis

Data obtained in the present study were expressed as Mean ± SD. Two-way ANOVA was performed using MS Office-2007. The percentage mortality of *A. salina* nauplii between the test concentrations and negative control was compared by *post hoc* multiple range test using Dunnett's test with different significant levels ($P < 0.05$; $P < 0.001$; $P < 0.0001$). Student- Newman Keuls (SNK) test ($P < 0.05$) was applied for the antioxidant and hemolytic properties using SPSS 16.0 (SPSS Inc. Chicago, USA). Probit analysis of toxicity data on the lethal concentration (50%) was materialized with EPA probit analysis software (version 1.5), Cincinnati, Ohio, USA.

3. Results and discussion

3.1. Marine actinobacterial density and distribution

Actinobacteria are generally present in extreme environment, especially the genera *Streptomyces* are recognized for the pharmaceutical importance. Recent studies opined that the investigation on the density and diversity of microbial population from mining sediments was much poor compared to that of other environment (Rastogi et al., 2010; Dhal et al., 2011; Dhal and Sar, 2014 and Islam et al., 2014; Jensen et al., 2005; Sogin et al., 2006; Prakash et al., 2013, 2015). In fact, only a countable number of informations are available on the actinobacterial communities from marine mining sediment (Akob et al., 2007). Therefore, in the present study, the actinobacterial diversity from IRE sediments along Manavalakurichi coastal belt was investigated. The result inferred that the total viable count (TVC) of actinobacterial density in the sediment samples significantly ($P < 0.05$) varied in different culture media as well as different sampling sites. Here, the maximum actinobacterial density ($15 \pm 0.06 \times 10^6$ CFU/g) was observed in the sampling point S5 of fresh mining sediment inoculated in SCA, whereas minimum actinobacterial density was recorded in the sediment collected from the sampling site S7 in all the tested media. The TVC in other sampling sites displayed marginally lesser actinobacterial density and it ranged between 3 and 12×10^6 CFU/g. The actinobacterial density from different sampling sites inoculated was in the following order: S5 > S8 > S1 > S2 > S3 > S4 > S6 (Table 1) in all the media. The higher actinobacterial density observed in the sampling site S5 might be due to the incidence of higher concentrations of minerals and other micro- and macro elements (Chronakova et al., 2010).

In the present study, the diversity of actinomycetes isolated from the IRE sediments displayed a remarkable variation with respect to different sampling stations. Altogether, 35 morphologically different actinobacterial strains were isolated based on spore morphology, aerial and substrate mycelium colour, diffusion of pigments, shape of the colony, etc. Of the 35 isolates, the genera *Streptomyces* was the most dominant (51.47%) represented with 18 isolates. The colony nature was leathery, powdery and softy and the spore morphology was

Table 1
Sampling points, sediment nature, geographical position and density of actinobacterial groups (CFU gm^{-1}).

Sampling Code	Name of sampling points	Sediment Characteristics			Density of actinomycetes (CFU gm × 10^{-6})**		
		Colour	Nature	Depth	SCA	AIA	ISP-2
S1	River Mouth area	Blackish yellow	Moisture sand	Surface	10 ± 0.47^{e}	8 ± 0.23^{e}	8 ± 0.47^{d}
S2	Rocky shore area	Black	Fine sand	Surface	8 ± 0.43^{d}	6 ± 0.37^{d}	5 ± 0.47^{c}
S3	Mining sand dumping area	yellow	Losse sand	Surface	8 ± 0.31^{d}	5 ± 0.47^{cd}	4 ± 0.47^{bc}
S4	Old mining sand collecting area	Blackish Red	Wet fine sand	One meter below	4 ± 0.25^{b}	3 ± 0.25^{ab}	3 ± 0.94^{ab}
S5	Fresh Mining sand collecting area	Reddish black	Moisture sediment	One meter	15 ± 0.06^{g}	10 ± 0.35^{f}	14 ± 0.10^{f}
S6	Mining effluent deposit area	Reddish brown	Red Sand	Surface	6 ± 0.26^{c}	4 ± 0.30^{bc}	4 ± 0.47^{bc}
S7	Mining waste dumping area	Yellowish	Fine losse sand	Surface	2 ± 0.33^{a}	2 ± 0.74^{a}	1 ± 0.47^{a}
S8	*Marine sediment	Blackish red with yellow	Fine sand	Surface	12 ± 0.51^{f}	10 ± 0.64^{f}	10 ± 0.62^{e}

observed as spiral with different coloration. The second dominant genera was *Actinopolyspora* and their occurrence was 11.42% (4 isolates); colony was powdery with long chain spore morphological characteristics. *Nocardiopsis* spp. and *Sacchropolyspora* spp. contributed 8.57% (3 isolates each) and their colony nature was powdery possessing small long chain spores (more than 15 spores in each sporophore) with varying colours. Finally, the least occurrence (1–2%) was *Actinobispora* sp., *Nocardia* sp., *Micromonospora* sp. and *Rhodococcus* sp. with different colony morphology and spores (Table S1 & Fig. S2). The density and distribution of actinomycetes in the present studied mining area varied among different sampling stations. This might be due to the influence of physical and chemical factors in the mining sediment samples favored some actionobacterial species to be dominant (Wolfaardt et al., 2008; Khan et al., 2013).

3.2. Taxonomic and culture characteristics of the potent strain MSA14

Based on the primary and secondary screening results, it could be authenticated that strain *Streptomyces* sp. (MSA14) was the most potent strain. The chemotaxonomy of MSA14 showed positive for the presence of LL- Diaminopimelic acid (LL-DAP- amino acids) and negative for whole cell wall sugar hydrolysate i.e. cell wall type-I. Further studies on the culture distinctiveness of *Streptomyces* sp. (MSA14) showed whitish grey, grey coloured spore and good growth on all the tested media. The diffused yellowish brown pigment in ISP-3 media was powdery. The growth of the strain MSA14 was moderate in ISP-3 and ISP-6 agar media and poor in ISP-1 and ISP-5 agar media. This is in good concurrence with the previous findings of based on the chemotaxonomy, the potent strain was confirmed as *Streptomyces rochei* (Tables S2 and S3) (Reddy et al., 2011; Aparana et al., 2013; Kanini et al., 2013; El-Hussein et al., 2014).

Antibacterial activity, MIC and MBC of crude extract of S. rochei (MSA14)

Nowadays, the secondary metabolites of actinomycetes from the extreme environment have lot of significance in the biomedical applications. Among these, *Streptomyces* was found to be the excellent antibiotic producer with multifaceted properties such as antimicrobial, antifungal, antimalarial, anticancer and antioxidant activities (Manivasagan et al., 2014; Valliappan et al., 2014). In the present study, the antibacterial activity of ethyl acetate and chloroform derived crude extracts of *S. rochei* significantly ($P < 0.0001$) varied (Fig. 1). The ethyl acetate extract rendered pronounced inhibitory effect against the tested bacterial pathogens with the zone of inhibition ranging between 5 ± 0.09 and 13 ± 0.25 mm. In particular, it exhibited the highest inhibitory zone against *E. faecalis* (13 ± 0.25 mm) and *E. coli* (13 ± 0.12 mm). The lowest zone of inhibition (5 ± 0.09) was recorded against *S. aureus* with varying MIC (1–50 µg/ml) and MBC (10–100 µg/ml). On the other hand, the chloroform extract of *S. rochei* exhibited moderate inhibitory activity with zone of inhibition varying from 10 ± 0.12–3 mm against the tested bacterial pathogens. The antibacterial activity of the ethyl acetate extract was comparably better

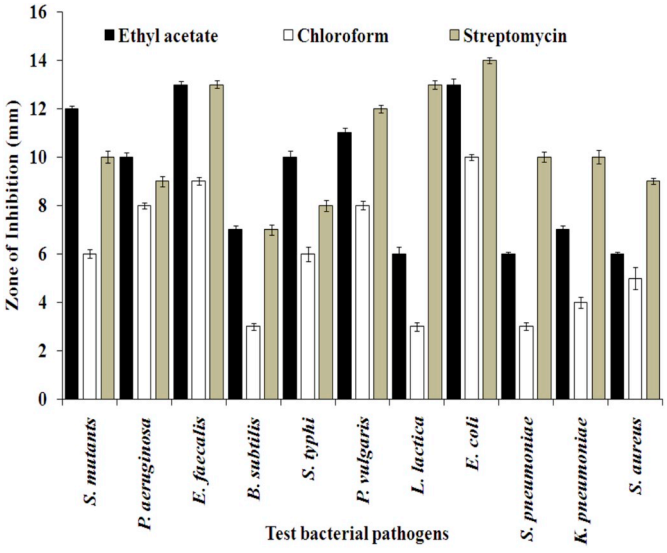


Fig. 1. Antibacterial activity of ethyl acetate and chloroform based crude extracts of *S. rochei* MSA14 and Streptomycin.

than the chloroform extract. The positive control, Streptomycin showed very good antibacterial property and it ranged from 7 ± 0.21 to 14 ± 0.12 mm with MIC between 1 and 10 µg/ml and MBC between > 1.0 and > 50 µg/ml against the tested bacterial pathogens (Table 2).

There are several reports which strongly authenticated the antimicrobial properties of crude extract of *S. rochei* against fish, phytopathogens (Kanini et al., 2013; El-Hussein et al., 2014; Srivastava et al., 2015), human pathogenic bacteria and fungi (Augustine et al., 2005; Reddy et al., 2011; Prakash et al., 2013). Thus, the above mentioned statements coincide well with the present result and thereby validate *S. rochei* as a potent actinobacterium from uranium contaminated marine sediment.

3.3. Antioxidant properties of S. rochei (In vitro) crude extract

Antioxidants are the essence that may keep away the cells from oxidative damage by free radicals. Natural products from terrestrial and marine habitats have strong antioxidant activity and have potential beneficial effects on human health (Sowndhararajan and Kang, 2013). The secondary metabolites from actinomycetes showed the existence of novel bioactive compounds with potent antioxidant property (Praveen Kumar et al., 2014; Janardhan et al., 2014; Kamala et al., 2015). Surprisingly, so far there is no report on the antioxidant property of secondary metabolites from *S. rochei*. In the present study, the maximum phenolic content (2.287 ± 0.012 µg/ml) was recorded in the crude ethyl acetate extract than the positive control Gallic acid (1.910 ± 0.016 µg/ml). The crude extract of *S. rochei* also showed

Table 2
MIC and MBC of crude ethyl acetate extract of *S. rochei* MSA14 against bacterial pathogens.

Bacterial Pathogens		Test concentrations (µg/ml)						Streptomycin (µg/ml) (Positive control)	
		0.01	0.1	1.0	10.0	50.0	100	MIC	MBC
S. mutants	MTCC 890	+ + +	+ + +	+ +	+ +	+	+	> 1.0	> 10
P. aeruginosa	MTCC 2642	+ + +	+ + +	+ + +	+ +	+	+	> 1.0	> 10
E. faecalis	MTCC 439	+ + +	+ + +	+ +	+	+	+	< 10	> 10
B. subtilis	MTCC 1134	+ + +	+ + +	+	+ +	+	+	> 10	> 10
S. typhi	MTCC 733	+ + +	+ + +	+	+ +	+ +	+	> 1.0	> 10
P. vulgaris	MTCC 744	+ + +	+ + +	+ +	+	+	+	< 10	< 10
L. lactica	MTCC 440	+ + +	+ + +	+ + +	+ +	+ +	+	> 1.0	> 1.0
E. coli	MTCC 1671	+ + +	+ + +	+ +	+ +	+	+	> 1.0	> 1.0
S. pneumoniae	MTCC 1936	+ + +	+ + +	+ + +	+	+ +	+	< 10	> 10
K. pneumoniae	MTCC 7407	+ + +	+ + +	+ + +	+ +	+ +	+	< 10	> 10
S. aureus	MTCC 96	+ + +	+ + +	+ + +	+	+ +	+	> 10	> 50

+ + + good Growth; + +: MIC value; +: MBC value.

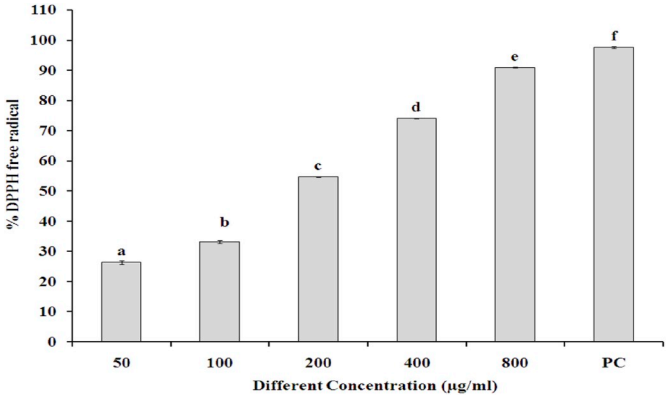


Fig. 2. DPPH – free radical scavenging activity of crude ethyl acetate extract of *S. rochei* MSA14
PC: Positive control (Gallic acid); Each value is the Mean ± SD of three replicates; bars with different superscript letters are statistically significant ($P < 0.05$) from each other and subsequent *post hoc* multiple comparisons with SNK test.

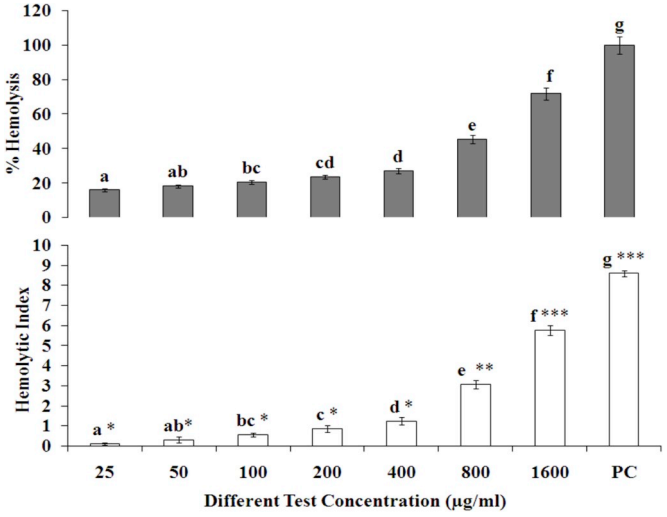


Fig. 3. *In vitro* antihemolytic property and hemolytic index of crude ethyl acetate extract of *S. rochei* MSA14 on human erythrocyte cells
PC: Pos
tive control (Triton-X); Each value is the Mean ± SD of three replicates; bars with different superscript different letters are statistically significant ($P < 0.05$) from each other compared with PC: Positive control (Trion X-100; 0.1%) and subsequent *post hoc* multiple comparisons with SNK test. Hemolytic index was recorded with *No hemolysis; **moderate hemolysis; *** high hemolysis.

good antioxidant property (0.095 ± 0.001 µg/ml at 400 µg/ml), which corroborate with the antioxidant activity of Gallic acid (0.080) (Fig. 3). In the present study, the total antioxidant activity was determined by the reduction of phosphomolybdenum (MoIV) complex to green coloured phosphate (MoV) at low pH.

DPPH free radical scavenging assay has been broadly used to calculate the antioxidant ability of potent crude extract/active compounds from microbial, plant and animal resources. In the present study, the crude extract from *S. rochei* displayed DPPH free radical scavenging activity with maximum of 91 ± 0.16% at 800 µg/ml extract. At the same time, the positive control gallic acid registered 98 ± 0.25% DPPH radical scavenging property The free radical scavenging activity was dose dependent. Increasing concentration of crude extract recorded a substantial increase in radical scavenging activity was recorded as follows as 26 ± 0.568 < 33 ± 0.41 < 55 ± 0.10 < 74.0.13% at 50–400 µg/ml concentrations respectively (Fig. 2). These results clearly evidenced the presence of bioactive molecules with radical quenching properties in *in vitro* conditions. Researchers have recorded several organic extracts/compounds such as alkylated phenol and its derivatives, terpeniods, long chain fatty acids and alkaloids from actinomycetes claimed to have striking antioxidant properties (Saurav and Kannabiran, 2012; Narendhran et al., 2014; Kamala et al., 2015).

3.4. Hemolytic activity of S. rochei (In vitro) crude extract

Hemolysis is concerned with the rupture of red blood cells (RBC's) and thus releasing the hemoglobin and other internal mechanism into the surrounding fluid by organic chemicals/drugs. Hemolysis can be visually detected by a pink to red colour drop in the blood serum or plasma (Arzoumanian, 2003). In the present investigation, *in vitro* hemolytic property of the crude extract of *S. rochei* was tested using healthy human red blood cells and the result is shown in Fig. 3. From the result, there was a significant increase in the percentage of hemolysis (15.95 ± 1.08% to 71.97 ± 2.55%) with respect to progressive increase in the concentration (25–1600 µg/ml) of crude extract of *S. rochei*. The positive control Triton X (10 µg/ml) which disclosed 100% hemolysis.

The crude extract of *S. rochei* exhibited < 5 hemolysis index and thus possessed excellent hemolysis of RBCs. From the result, up to the concentration 400 µg/ml, there was no hemolysis against chosen blood cells. Further increase in the test concentration i.e. from 800 to 1600 µg/ml evidenced moderate index of hemolysis (3.08 ± 0.2 and 5.4 ± 0.25). But, the positive control Triton X 100 showed maximum hemolytic (8.6 ± 0.4) index even at very less concentration (10 µg/ml) than the test concentrations of *S. rochei*. From the hemolysis result, it can be ascribed that the secondary metabolites present in the crude extract of *S. rochei* exhibited hemolytic activity from 800 to 1600 µg/ml

Table 3
Cytotoxic property of crude ethyl acetate extract of *S. rochei* MSA14 against *A. salina* nauplii.

Test Concentration (µµg/ml)	Total no of nauplii	Total Response	Mortality (%)	LC_{50} value (µµg/ml)
12.5	10	0	0	106.75
25	10	2	20	
50	10	3	30	
100	10	4	40	
200	10	7	70	
400	10	8	80	
800	10	10	100	
NC	10	0	0	

concentration, is above the threshold concentration of antibacterial and antioxidant activities. Thus, the present result of hemolysis study strongly concluded that the bioactive metabolites existing in the *S. rochei* extract is non-toxic in nature (Fig.3). Previously, there was a report on the existence of less hemolytic purified compound 5-(2,4-dimethylbenzyl) pyrrolidin-2-one from *Streptomyces* spp. VITSVK (Saurav and Kannabiran, 2012).

3.5. Brine shrimp cytotoxicity assay

The brine shrimp (*A. salina* nauplii) cytotoxic assay is a simplest screening method to find out the toxicity level of active metabolites/drugs from plants, animals and microbial sources (Ramasubburayan et al., 2017a,b). In general, the lethal concentration (LC_{50}) of any metabolites/drug is related to cytotoxicity and antitumor properties (Baravalia et al., 2012). In the present study, the complete mortality of *A. salina* nauplii was recorded at 800 µg/ml concentration of *S. rochei* crude extract. The mortality of *A. salina* nauplii significantly ($P < 0.05$) increased with increasing concentration of extract. The lethal concentration (LC_{50}) was recorded at 106.75 µg/ml (Table 3). Previously, Ogugu et al. (2012) recorded the LC_{50} value < 100 µg/ml of bioactive compound derived from marine *Streptomyces* spp. against *Artemia* nauplii and was affirmed as low/non-toxic. In the present study also, LC_{50} low toxic to the non-target organisms. Thus, the overall results of cytotoxic and hemolytic properties of crude extract of *S. rochei* strongly implied low/non-toxic on the human red blood cells and *A. salina* nauplii at lower concentration.

3.6. Purification, TLC bioautography, separation and biomedicinal properties of ethyl acetate extract of S. rochei

Crude extract of *S. rochei* was purified by normal phase silica gel column chromatography. In total, 25 fractions (each 20 ml) were collected and tested for antibacterial activity against the pathogenic bacteria. Among these, 14th fraction showed an excellent antibacterial activity against the chosen pathogenic bacterial strains. This bioassay guided column fraction was further purified and characterized by TLC plate, which displayed three spots with the R_f values of 0.52, 0.69 and 0.77 under the UV light with short and long wavelength. Among these spots, the spot with 0.69 R_f value rendered maximum inhibitory activity against the tested bacterial strains in TLC bioautography. Moreover, this active spot displayed positive reaction with DPPH reagent, thus confirmed good antioxidant activity and also further confirmed as non-toxic to human erythrocytes. The chemical constituents of this bioassay guided spot showed dark brown colour under UV light of long wavelength (365 nm), after sprayed with Dragen-draff reagent. This indicates the presence of alkaloids. The active spot R_f value 0.69 was referred positive for alkaloids in the previous reports also (Narayanasamy and Ragavan, 2012; Sammani et al., 2013; Thenmozhi et al., 2015). Also the active spot explicated diverse biomedicinal properties *viz*. antibacterial, hemolytic and antioxidant activities were authenticated by several authors (Jiao et al., 2013; França et al., 2014; Wang et al., 2014).

3.7. Chemistry and spectral view of the active spot

The chemical characterization of the active spot (R_f 0.69) was evaluated through two main spectroscopic analysis i.e. IR *ν*-max (KBr) and GC-MS spectra. Following the spectral key results, the bioactive compound present in the active spot was identified as Pyrrolindine-2,4-Dione, which is also known as Tetramic acid. The result of IR spectrum is as followed: (KBr - cm^{-1}) 3431 (OH str(b)); 2947 and 2841 (C–H str (m)); 1720 (C = O str(m)); 1647 (-C = C- str(m)); 1454 (C–C interring-str (m)); 1379 (C–H rock - str(sm)); 1259 (C–N aromatic ring str(sh)) and the other IR spectral peaks might be due to the solvent impurities (Fig.4). GC-MS result showed the peak area of 2.61 with RT (7.37) and the quality of compound was 86%, as well as *m/z* (%): 99 (M^+), 93, 83, 74, 71, 59, 56; Anal. calculation for $C_4H_5NO_2$ (99.09) found in C 48.48; H 5.09; N 14.14 and O 32.29% (Table 4., Fig.5, Figs. S2 and S3). Based on FT-IR and GC-MS analysis, the active compound was identified as

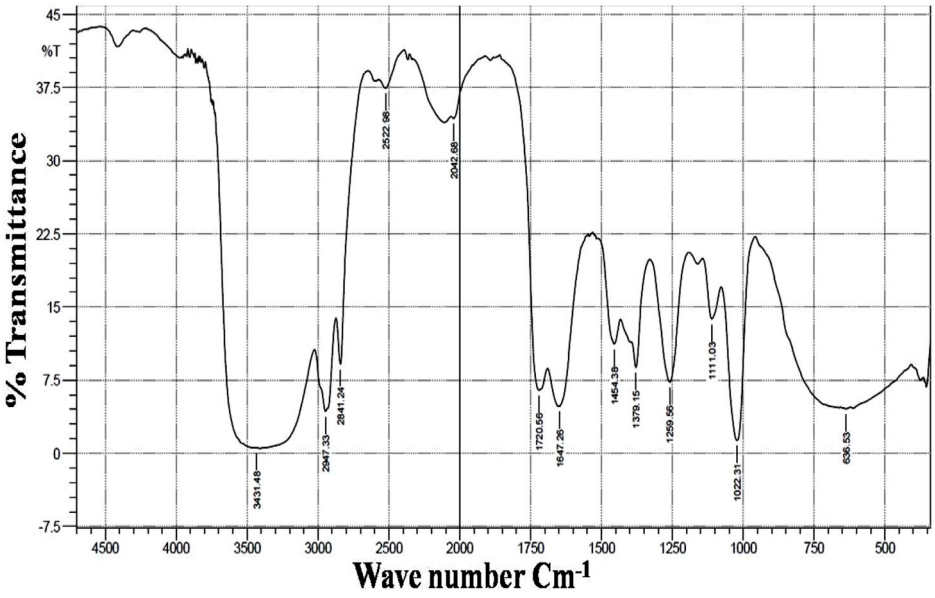


Fig. 4. FT-IR spectral analysis showing the functional groups in bioassay guided fraction of *S. rochei* MSA14.

Table 4
GC-MS spectral characteristics and the chemical constituents of bioassy guided active spot of *S. rochei* (MSA14).

Name of the Compound	RT	Molecular formula	Molecularwight	Peak Area	(%) quality of Peak	Chemical nature	Bioassays of the active spot*		
							AB	AO	H
Pyrroline-2,4-Dione (Tetramic acid)	7.37	C_4 H_5 NO_2	99.09	2.61	86	Alkaloids	HI	Y	NI

RT: Retention time; AB: Antibacterial activity; AO: Antioxident activity; H: Hemolysis; HI: Highly Inhibition; Y: Yes; NI: No Inhibtiion.

Pyrrolidine-2,4-Dione. Similar reports have also been recorded by Zhu et al. (2010). Liu and Walsh (2009) isolated Pyrrolidine-Dione ring from marine bacteria and evidenced that it was originally synthesized by PKS-I & II and NPRS genes that are in fact responsible for releasing polyketides and non-ribosomal natural products. Kouadri et al. (2014) strongly authenticated that the presence of PKS-I & II genes are very much essential for the synthesis of marine natural products (MNPs) as compared to NPRS genes from marine *S. rochei*.

3.8. Biodegradability study of Pyrrolidine-2,4-Dione and Streptomycin sulphate

More recently, the microbiologists, pharmacologists and environmentalists are struggling due to the rising problems of drug resistant pathogens, which are mainly antibiotics resistant microbial pathogens. These pathogens are self developed throughout the world due to vast accumulation of antibiotics/drugs in soil by unsafe deposing of vials and other drug related materials by manmade default. In view of

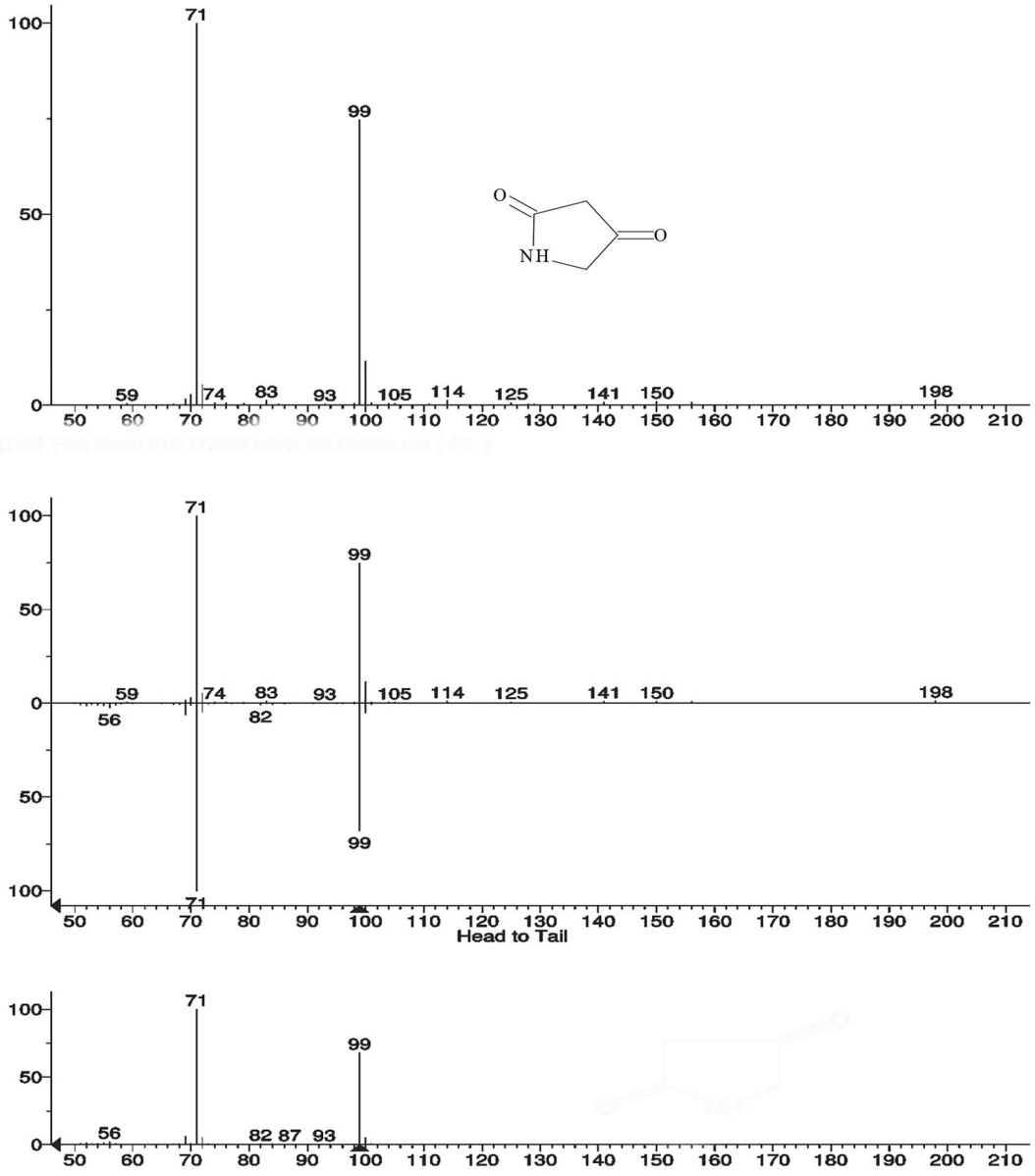
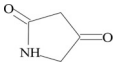
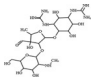


Fig. 5. GC-MS and head to tail match spectrum of Pyrroline-2,4-Dione.

Table 5
In silico Biodegradation and baseline toxicity of Pyrrolidine-2,4-dione (Tetramic acid) and Streptomycin sulphate model.

Test compounds			Log K_{ow}	Biodegradability by BIOWIN™								Netural Oraganic SAR - Baseline Toxicity by ECOSAR™ (mg/L)		
Name	Structure	Mol. Wt		BIO-1	BIO-2	BIO-3	BIO-4	BIO-5	BIO-6	BIO-7	Yes/No	Fish sps (LC_{50}:96hr)	Daphnia sps (LC_{50}:48hr)	Green Algae (EC_{50}:96hr)
Pyrrolidine-2,4-dione		99.09	−0.63	0.917	0.979	2.904	3.888	0.701	0.865	−0.364	A:Y B:Y	19.96	844.6	237.64
Streptomycin sulphate		581.58	−9.07	0.2881	0.0034	2.674	3.833	0.691	0.0008	0.988	A:N B:N	4.19e+012[a]	8.56e+011[a]	9.31e+009[a]

[a] Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation are reported to acute - chronic; A: Anaerobic degradation; B: biodegradation; Y: Yes; N: NO.

this, now studies are being focused on the degradation of new born antibiotics/drugs in soil from days to weeks. The BIOWIN models are frequently used in regulatory contexts for classifying the resolution of chemicals for the calculation of the biodegradability of the chosen chemicals (Posthumus et al., 2005; Papa et al., 2007). In this context, in the present study the identified compound Pyrrolidine-2,4-Dione in comparison with standard Streptomycin sulphate were checked for their biodegradability pattern by KOWWIN™, BIOWIN™ and ECOSAR™ program. The predicted bioaccumulation, biodegradability and baseline toxicity are shown in Table 5. From the Log_{KW} result, Pyrrolidine-2,4-Dione (Log_{KW}: −0.63) was found to be less accumulative in the environment than the antibiotic Streptomycin sulphate (Log_{KW}: −9.07). Further, the biodegradability of the fragment molecules of the identified compound and the reference compound were more significantly calculated by BIOWIN™ programme. Recently, the validity of the six BIOWIN ™ models was assessed on new industrial chemicals as well as pharmaceutical compounds with the combination of BIOWIN-3 and BIOWIN-6 program, which showed the highest score for overall degradable prediction (Posthumus et al., 2005). In the present study, biodegradation of Pyrrolidine-2,4-Dione and Streptomycin sulphate through BIOWIN-3 and 6 inferred that the bioactive compound present in *S. rochei* is rapidly degradable (0.8650; Non liner model prediction) within a day to weeks (2.9035; time frame biodegradation). Nevertheless, the reference antibiotic was found to be non-degradable (0.0008) over months. Moreover, our result suggested that bioactive compound Pyrrolidine-2,4-Dione was more safe and eco-friendly in nature with anaerobic degradability which was confirmed through BIOWIN-7 (−0.364). On the other hand, the reference compound Streptomycin sulphate displayed BIOWIN-7 (0.988), was found to be detrimental to the environment Thus, the *in silico* biodegradability prediction test for Pyrrolidine-2,4-Dione divulged that it is well suited for the environment (Table 5).

The structure activity relationship (SAR) was calculated by the baseline toxicity of test chemicals on the aquatic organisms based on their correspondence of previous reports (Sanderson and Thomsen, 2007; Cui et al., 2014; Chai et al., 2014; Ramasubburayan et al., 2017b). SAR baseline toxicity of the test compounds (mg/L) was calculated and recorded in freshwater species like Fish sp. (LC_{50} for 96 h); Daphnia sp. (LC_{50} for 48 h) and Green algae (EC_{50} for 96 h) in the ECOSAR programme. The test result suggested that the compound Pyrrolidine-2,4-Dione displayed low/non-toxic at the acceptable concentration against the chosen aquatic species such as *Daphnia* sp. (844.6 mg/L), green algae (237.64 mg/L) and fishes (19.96 mg/L). In contrary, the antibiotic Streptomycin sulphate showed toxicity to aquatic species which exceeded the water solubility of the chosen antibiotic. Coincidentally, Alighardashi et al. (2014) evaluated the environmental risk assessment of antibiotics used in the Iranian people and the SAR results evidenced that the chosen test antibiotic was more toxic at low EC_{50} and LC_{50} drug and chemical concentrations against aquatic organisms.

4. Conclusion

The present study disclosed the distribution of actinobacterial density and diversity in the rare earth metal sediments. The principle compound, Pyrrolidine-2,4-Dione identified from the secondary metabolite of potent actinomycete strain, *S. rochei* exhibited excellent biomedicinal properties with less toxicity. In addition, the potent bioactive compound was eco-friendly. Further studies are underway to develop a new class of human therapeutics using the identified bioactive compound for the benefit of human well being.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101244.

SCA- Starch Casein Agar, AIA- Actinomycetes Isolation Agar medium, ISP-2- International Streptomyces project Medium-2; *No mining activity area; **Each value is the mean ± SD of three replicates; value with in same column with different superscripts for each group are statistically significant (One way ANOVA test $P < 0.05$ and subsequent *post hoc* multiple comparisons with Tukey- HSD test).

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