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Actinobacterial Species Selection for Production of Antimicrobial Peptide using PROMETHEE GAIA

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Abstract
The investigation of robust antimicrobial peptides from the natural environment has promptly been boosting strength with the upsurge in multi-drug resistant pathogens. Actinobacteria are assorted cluster of Gram positive, high GC content and filamentous bacteria and considered as an admirable elaborators of therapeutic products. In the present study, antibacterial potential of crude peptides has been evaluated by initial screening of thirteen actinobacterial isolates. Isolate PPD 6 is the most promising candidate for the antimicrobial peptide production based on decision vector through key physical and chemical parameters as temperature, pH (Buffers) and antibiotic resistance as a competent of multi-criteria decision analyses (MCDA) and ranking with Preference Ranking Organisation Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) analysis. Biological activity was analysed in the presence of enzymes, chemicals, solvents and metals, no loss in their activity were recorded.

Keywords
Antibacterial profiling, Screening, Snail shell, *Streptomyces.*

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INTRODUCTION
Among the actinobacterial population, genus *Streptomyces* is considering as a rich source for their diversified activities. They are contemplating as biotechnologically vital due to the production of half of the secondary bioactive compounds [1]. Due to the provision of critical bioactive compounds with high commercial values pave the way to the periodic screening of new bioactive compounds. Several microbial communities inhibit in numerous habitats with their functional properties whereas some communities contend themselves by producing antimicrobial peptides, which are proteinaceous in nature. They provide an enormous variety of peptides including a signaling molecule, pheromone. Peptide biosynthesis can occur either ribosomally in bacterial cells acquires activity against other bacteria or across genera [2-3] and non-ribosomally paying to the chemical diversity of microbial metabolites. In the event of post-translational modification, there is an increased variation in the ribosomally synthesized and post translationally modified peptides.

Antimicrobial peptides are boosting further and further attention for not only as a therapeutics for clinical and also as preservatives in food industries to the avoidance of devastate and downturn of food [4]. It has been addressed to have a decisive role on performance, nutrient digestibility, the intestinal microflora and morphology and immune function in pigs [5]. It is essential to search for new, efficient and safe therapeutics from actinobacterial sources to overcome the hazard of multi drug-resistant infections. This study aims to explore novel, potent actinobacterial isolates from the snail shell using traditional and preferential methods for the antimicrobial peptide production. In this manuscript, an attempt was made to screen out the superiority nature of the potent isolate with the aid of PROMEETHEE and GAIA software.

MATERIALS AND METHODS

Snail Shell Collection

Cone snail shells were collected from the suriyur village pond by professional collectors with appropriate permits. Samples were aseptically collected to the laboratory. Shells were surface sterilized and crushed into the fine powder with the aid of mortar and pestle and it was subjected to refrigeration until the further process was done.

Isolation of Actinobacteria

Actinobacterial isolation was carried out by using Starch casein agar medium. The ingredients are as follows (g/l: Starch 10, Casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, MgSO4. $7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 18 and followed by addition of antibacterial and antifungal agent Griseofulvin and Cycloheximide (Himedia, Mumbai, India) 25 and 10 mg/ml) in order to avoid contamination [Kuster and Williams, 1964]. With the assistance of sterilized bent L- rod and the plate spinner the diluted sample (0.1 ml) was spreaded over the medium. The plates were incubated at 30°C for seven to ten days at pH. The single and morphologically distinct colonies were picked and performed streak plate and preserved for future use.

Production Kinetics for antimicrobial peptides

The isolates were grown in SCB broth (Hi-Media, Mumbai, India) seeded with 5% inoculum and maintained anaerobically at 30°C for 48 hrs. After incubation, cells were removed by centrifugation (12,000 rpm for 15 mins, 4°C). The cell-free supernatants were adjusted to pH 4.0 using 1N NaOH and which was used as crude peptides [6].

The screening property for the crude peptides was performed by well diffusion method. Aliquots of the sterile supernatant were placed in 4mm diameter wells in Mueller-Hinton agar plates by using sterile well borer and the indicator organisms/bacteria were previously seeded with the dishes. After 12-18 hrs, zone of inhibition was measured in diameter of the zones [7].

PCR amplification and sequencing of 16S rDNA gene

The genomic DNA was isolated by the modified method of [8]. A set of primers (i.e., forward (St-F):5´-AAGCCCTGGAAACGGGGT-3´, reverse (St-R):5´-CGTGTGCAGCCCAAGACA-3´) and Master Mix (Ampliqon, Odense, Denmark) Primer - 0.4 µM primer, chromosomal DNA - 40 ng which constitutes the 25µl final volume was used to amplify the 16SrDNA fragment. The thermal cycler was used for amplification (Verity Applied Bio systems, Lincoln Centre Drive Foster City, USA) program as follows: Primary denaturation for 94°C for 5 min, 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 105 sec and final extension was 72°C for 10 min. The PCR products were visualized using gel electrophoresis on 1% agarose (Qiagen, Hilden, Germany) and compared with 1 kb DNA ladder (Fermentas, Sankt Leon-Rot, Germany). The purified products were sequenced by (MacroGen, Seoul, Korea). Furthermore, using BLAST software, the determined sequence was compared with the sequences deposited in NCBI Gen Bank and the accession number was obtained.

Phylogenetic analysis

The 16S rRNA nucleotide sequence obtained from the PCR product. A BLAST of an acquired series was performed with that of available EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank and the 16S rRNA nucleotide sequence was subjected to phylogenetic analysis using bioinformatics tool available in online using software Mega.

Growth Kinetics and Ammonium Sulphate Fractionation

Two ml of sample was withdrawn everyday which was grown at 30°C for 48 hrs in SCB broth, and the cell-free supernatant was centrifuged at 12, 000 rpm for 15 mins at 4°C. The filtrate was precipitated by 80% ammonium sulphate [9]. Again centrifuged and the pellet was resuspended in 20mM Tris buffer (pH7.0) and was dialysed using 2 kDa molecular weight cut-off membrane (Spectrapor, Gardena, USA) against the same buffer for 24 hrs with four changes at 4°C and labelled as crude antimicrobial peptides. *E. coli* was used as an indicator organism in this experiment. The antimicrobial activity was expressed in terms of arbitrary units (AU) which was defined as the highest dilution of the sample that produced a zone of inhibition. The reciprocal of the

dilution was considered as the titre of antimicrobial activity (AUml−1). Simultaneously bacteria growth was recorded spectrophotometrically at OD 600 nm.

Preliminary Characterization of the crude antimicrobial peptide

Effect of Enzymes

Enzyme sensitivity assay was executed by [10] ultra-filtered supernatant was treated with filter-sterilized trypsin (0.5M Tris HCl buffer, pH 8.0), pronase E (10mM sodium phosphate buffer, pH 7.0), lipase (100mM phosphate buffer, pH 7.4), α-amylase (20mM phosphate buffer, pH 7.0) at a final concentration of 10 mg/mL at 37°C and proteinase K (50mM Tris HCl buffer, pH 7.5) at a final concentration of 10mg/mL at 55°C for 2 hrs. The concentrates with and without enzyme treatment and buffers as positive and negative controls, then the residual activity was determined by agar well diffusion method using the above mentioned bacterial pathogens.

Effect of Surfactants and Other Chemicals

Surfactants like urea, SDS, Tween 20 and Triton X-100 at a final concentration of 1% (v/v), trichloroacetic acid (10mg/mL (w/v)), β-mercaptoethanol at 10% concentration and EDTA at 2, 5 and 50mM concentration were incubating at 37°C for 5hrs and their effects were analyzed [11]. Chemicals at the rate of 1% and 10% in mTSB broth and untreated as negative and positive controls, respectively. After treatment with TCA, samples were centrifuged at 10,000 rpm for 5 mins and the supernatant was adjusted to pH 8.0 and checked for antimicrobial activity [12].

Effect of Organic Solvents and Metals

The concentrate was mixed with various organic solvents (ethanol, methanol, isopropanol, acetone, ethyl acetate, chloroform and isobutanol) at a final concentration of 50% (v/v). After incubation for 1 h at 25°C, the organic solvent was evaporated in a vacuum concentrator (Martin-Christ, Kingston, New York) and was dissolved in 20mM Tris HCl buffer pH 8.0. The concerned treated and untreated buffers were used as negative and positive controls [12] and antimicrobial activity was performed. Effect of metal salts ($AgNO_3$, MgSO4, MnCl2, $ZnSO_4$, $CuSO_4$, $CdCl_2$, $FeSO_4$, and $CaCl_2$) at a final concentration of 1mg/mL on concentrated supernatant was analyzed by incubating at 37°C for 1h. The untreated supernatant and solutions of metals salts were used as positive and negative controls [11].

Stability of the Antimicrobial Activity

To determine thermal stability, aliquots of concentrated supernatants were incubated at different temperatures such as -20°C, +5°C, +15°C, +20°C, +30°C, +40°C, +50°C, +60°C for 12 hrs. pH stability in different buffers were analyzed for 12hrs at 37°C with 0.1M buffers of different pH: Glacial acetic acid - pH 3.0, sodium acetate at pH 4.0, sodium citrate at 5.0, phosphate buffer - pH 6.0, Tris HCL - pH 7.0,HEPES buffer at 8.0,glycine NaOH buffer (pH 9.0) and incubated after that antimicrobial efficacy was checked. The following antibiotics amikacin, ampicillin, cefotaxime, erythromycin, tetracycline, gentamicin, penicillin was determined for its resistance.

MCDA analysis of antibacterial profiling

MCDA analyses exist from fundamentally to multifaceted methods like ELECTRE, PROMETHEE and REGIME [13]. When compared to others, Preference Ranking Organization Method for Enrichment Evaluation (PROMETHEE) and Graphical Analysis for Interactive Assistance (GAIA) have momentous assets because it aids lucid decision making *i.e.*, the decision vectors bounce towards the referred solution [14]. The multiple criteria like antibiotics optimization, temperature and pH (buffers) optimization were taken into the account for the selection process. This study followed the PROMETHEE- GAIA algorithm in order to find and rank the actinobacterial isolates suitable for antimicrobial peptide production.

RESULTS AND DISCUSSION

Actinobacteria is one among the assertive group of microorganisms and ubiquitous in nature having an extensive array of pharmacological interest. Of course, several habitats and biological activities were coming into the limelight by several studies still snail shell is also one of the notable and favourable microenvironment for actinobacterial communities. Totally, eighty-one actinobacterial isolates were isolated from the snail shell, out of which 13 morphologically distinct isolates were chosen for screening. The isolates were white, grey, pink, brown, yellow and red colour colonies (Figure 1). Majority of the colonies are Grey followed by white and red colour colonies. Yellow, pink, brown colonies were leastly recorded in the study. According to [15] stated that white color series of actinobacterial colonies are abundantly present in the soil profile whereas the results revealed grey colonies are majorly recorded in the snail shell environment. Usually, snail shell holds 98% $CaCO_3$ in the form of aragonite with small quantities of calcite, remaining 2% mass was probably compounds of Fe, Mg, Mn, Al, Na, and K. The color difference may due to the presence of trace amount like Fe and Mn with organic compounds.

The results of production kinetics stated that the isolate, designated as PPD2, PPD6, PPD8 and PPD13 had been exhibiting antibacterial activity against all the pathogens by the zone clearance meanwhile the isolates PPD7 and PPD10 showed minimum activity whereas remaining isolates shows moderate action and the readings were recorded in table 1.Isolate PPD6 scored maximum activity in both gram positive and gram negative pathogens, which was chosen as potent isolate and further studies were carried out. Succinctly, our results concordant with [16] stated that *Streptomyces* sp were highly susceptible to gram negatives (*P.aeruginosa* and *P. fluorescence*) and showed moderate activity against gram positive bacteria.

The genomic DNA was separated on 0.8% agarose gel, and the 16S rRNA gene of the actinobacterial isolate was partially amplified using specific universal 16S rRNA primers 27F (forward primer) and 765R (reverse primer). The PCR amplified product was suggested 700 - 800 base pairs nucleotide product, which was observed in 1.2% agarose gel electrophoresis (Figure 2). The PCR amplified partial 16S rRNA gene sequence was attained and subjected to BLAST analysis to identify the similarity between conserved sequences, the isolate was identified as *Streptomyces rochei*. The sequence was submitted to the Gen Bank with the accession number MG774931.Furthermore, the phylogenetic tree was constructed from the sequence and neighbour joining phylogenetic tree revealed that sequence was made the cluster with respective *Streptomyces rochei* with high bootstrap value (Figure 3). From the morphological, physiological, biochemical, chemotaxonomic (data's not shown here) and genotypic chacterization concluded that the potent actinobacteria belongs to family Streptomycetes and identified as *Streptomyces rochei* MG774931. Still, there is a massive breach between traditional approaches and modern methods for the classification and identification of Streptomycetes [17]. So their ranking is still an important issue for the taxonomists as well as for the researchers who are working with this genus.

The antimicrobials production was initiated at second day and supreme level was attained at seventh day of growth (Figure 4). From that, antimicrobial activity remained constant until the early death phase was attained, afterward it started diminishing. Everyday pH values were checked along with the growth and production of antimicrobial substance. It was identified that values increased progressively from the initial pH of 7.4 to 9.5 at the death phase. However, the pH value was found to be 8.0 during the maximum production (80AUml−1) of the antimicrobial substance. The kinetics results were agreed with [10], she worked in *Bacillus subtilis* to obtain broad spectrum antimicrobial activity against pathogens.

Preliminary characterization of crude antimicrobial peptides results revealed that there was no loss of biological activity in the enzyme sensitivity assay. Pronase scored 80% biological activity meanwhile alpha amylase acquire 20%. The remaining enzymes showed moderate biological activity (Figure 5a). The effect of crude peptides was evaluated by the presence of surfactants. Triton X showed good biological activity whereas SDS exhibited poor activity. Chemicals like trichloroacetic acid and β-mercaptoethanol scored moderate activity (Figure 5b). Organic solvents results revealed there was 80% growth was observed in acetone, adequate growth was perceived in isopropanol, ethyl acetate and chloroform and little growth was observed in ethanol (Figure 5c). $MgSO_4$ showed maximum activity and $FeSO_4$, $MnCl_2$ and $ZnSO_4$ scored meger activity. No growth was recorded in the presence of the metal $CaCl_2$ (Figure 5d). The ammonium sulfate precipitates treated with proteinase K enzyme showed antimicrobial activity which confirming the protein nature of the antimicrobial peptides and exhibited strong antimicrobial activity against sensitive strains [9].

Multicriterion decision making is a progression of evaluating tangible situations, based on various environments to propose a course of strategy among the available options. It represents a valuable tool in attaining lucid decisions about antibacterial investments in the light of multiple criteria. Formerly, this tool has been adopted for ranking for spore survivability [18], antifungal property of organotin (IV) compounds [19] and cyanobacterial biodiesel production [20]. In this work, we envisioned to rank the potent isolate from the parameters like antibiotics, pH (buffers), Temperature with their antibacterial efficacy. Therefore, the percentages of efficacy were fed to the visual PROMETHEE 1.4 Academic Edition software [developed by Dr. Bertrand Mareschal (2011–2015)] for MCDA analysis with the 'maximized' preference, because higher the activity, lower the resistance.

There was a 91% variance congregated the U and W components which are illustrated in the graphical analysis for interactive aid (GAIA) (Figure 6 and 7) [20] stated that direction and length of criteria vectors indicated the specified desirable action with the aid of the decision vector (red line). Moreover,

the actions in which the direction of decision vector and outermost criteria in that direction are aligned together to generate the utmost preferable factors [21]. Cefotaxime showed the higher rate of sensitive in *Proteus vulgaris* and *Klebsiella pneumoniae* but gentamicin, ampicillin, penicillin, amikacin bared the least survival towards all the cidal agents (Figure 6a). Additionally, the PROMETHEE II complete ranking based on the preference (Phi) net flow which is the balance (difference) between Phi^+ and Phi^- are shown in Figure.6b in which the preferred highest to least survival of bacterial pathogens against the tested antibiotics and *streptomyces rochei* are potent isolate(φ^+:0.8095) > cefotaxime(φ^+:0.7143) > erythromycin(φ^+:0.5000) > Amikacin(φ^-:0.0714) > Tetracycline(φ:0.3333) > Gentamicin(φ^-:0.3810) > penicillin(φ^-:0.5952) > Ampicillin(φ^-:0.7857). This results visibly picturized that potent isolate is more resistant to the tested bacterial cultures than the tested antibiotics whereas cefotaxime and erythromycin showed moderate activity and the remaining tested antibiotics scored the least resistance. The potent isolate ranked first, therefore we suggest that antimicrobial peptides from the potent isolate could possibly be a novel therapeutics to overcome multi drug resistant bacteria in the health care scenario.

Similarly, the results of pH (buffers) revealed glacial acetic acid buffer at pH 3 scored maximum activity against *Proteus vulgaris, Klebsiella pneumoniae* and *E. coli* followed by phosphate buffer at pH 6 which is illustrated in figure $7a_1$. Remaining buffers exhibited least activity. The complete ranking based on preference showed in figure $7a_2$ that Glacial acetic acid (pH3) (φ^+:0.9444)> Phosphate buffer (pH6) (φ^+:0.3056) > Tris HCL (pH7) (φ^+:0.0278) > Sodium citrate (pH5) (φ^-:0.0278) > HEPES buffer (pH8) (φ^-:0.2500) > Sodium acetate (pH4) (φ^-:0.4722) > Glycine NaOH (pH9) (φ^-:0.5278). Temperature optimization results showed in figure $7b_1$ at+40°C favoured the growth of *E. coli* and *Pseudomonas aeruginosa* followed by +60°C favoured the growth of Streptococcus *faecalis, Enterobacter aerogenes* and *Klebsiella pneumoniae*. The preference results based on ranking in figure $7b_2$ explored that +40°C (φ^+:0.7619) > +60°C (φ^+:0.2857) >+15°C (φ^+:0.1905) > +30°C (φ^-:0.0476) > +20°C (φ^-:0.0714)> +5°C (φ^-:0.1667)> +50°C (φ^-:0.3571) > -20°C (φ^-:0.5952). The results of pH (buffers) and Temperature obtained by PROMETHEE software is inversely proportional to the results of growth kinetics. The outcome of this study will be incorporated in the purification and characterization studies.

Since 1928 the discovery of penicillin by flemming, an enormous amount of antimicrobial pharm therapeutics have been identified, formulated and clinically used as an antibiotic. As a consequence, there was an advent of multidrug-resistant (MDR) microbes in the field of biomedicine and agriculture, it is mandatory for the search of alternatives. Snail shell serves as an actinohabitants and very few studies are reported. In order to explore the microenvironment and its potency in the field of peptide therapeutics, this piece of work was carried out. Traditional screening techniques led to the isolation of potent isolates by producing the common metabolites, which have well extensively studied and established already. With the aid of improved and current strategies the specific bioactive substances were screened out, in order to enhance the quality if the screened product. This study established a first pitch towards the screening of actinobacterial screening using PROMETHEE GAIA for antimicrobial production.

Table 1. Screening of Antimicrobial peptide potency by agar well diffusion method

Actinobacterial isolates	Indicator organisms (Inhibition Zone in cm)					
	Streptococcus faecalis	***E.coli***	***P.aureginosa***	***Proteus vulgaris***	***K.pneumoniae***	***Enterobacter aerogenes***
PPD1	**4.22±0.17**	-	**2.34±0.12**	**1.45±0.14**	-	**6.34±0.17**
PPD2	2.82±0.14	2.45±0.15	4.43±0.11	6.37±0.12	2.11±0.17	2.20±0.11
PPD3	-	8.33±0.17	-	2.65±0.10	5.34±0.15	4.45±0.12
PPD4	-	1.67±0.12	1.23±0.13	-	2.22±0.11	2.34±0.15
PPD5	-	2.43±0.14	3.45±0.17	4.64±0.11	2.43±0.24	1.0±0.11
PPD6	8.45±0.17	12.46±0.12	14.23±0.16	6.32±0.14	19.43±0.17	10.43±0.17
PPD7	-	2.58±0.17	2.45±0.14	-	3.56±0.11	-
PPD8	1.4±0.21	4.01±0.11	1.56±0.21	2.67±0.10	4.12±0.10	2.46±0.15
PPD9	3.33±0.12	1.09±0.14	4.23±0.17	-	1.40±0.21	4.45±0.11

PPD10	-	-	2.43±0.13	-	2.46±0.14	-
PPD11	1.32±0.14	1.47±0.21	-	7.86±0.12	4.33±0.17	3.33±0.12
PPD12	1.34±0.23	3.43±0.12	5.43±0.12	4.34±0.11	7.22±0.12	-
PPD13	4.65±0.17	1.41±0.17	5.46±0.11	3.42±0.17	1.34±0.11	2.43±0.15

Fig.1 Actinobacterial isolates based on the colours.

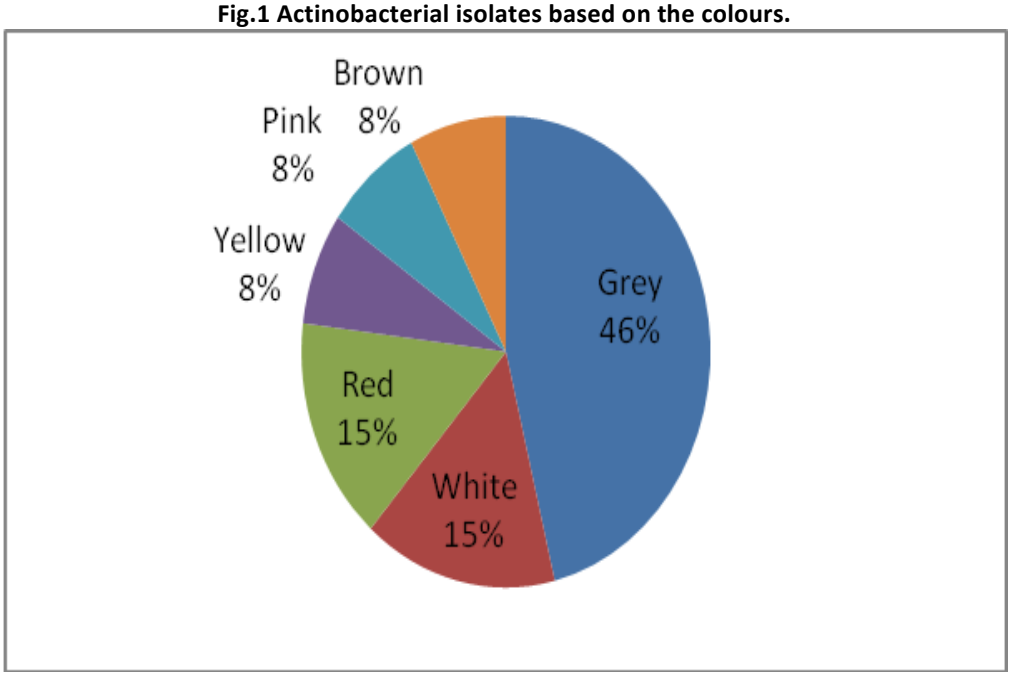


Fig.2 Amplified DNA and PCR Product of an isolate.

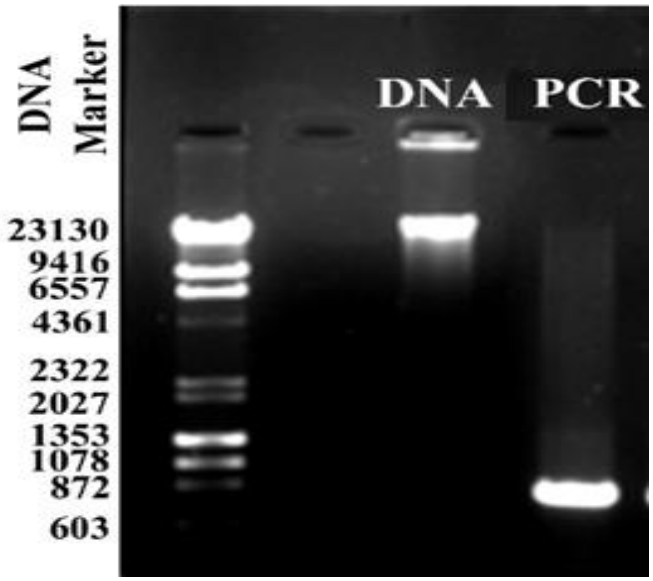


Fig.3 Phylogenetic tree construction of *Streptomyces rochei*

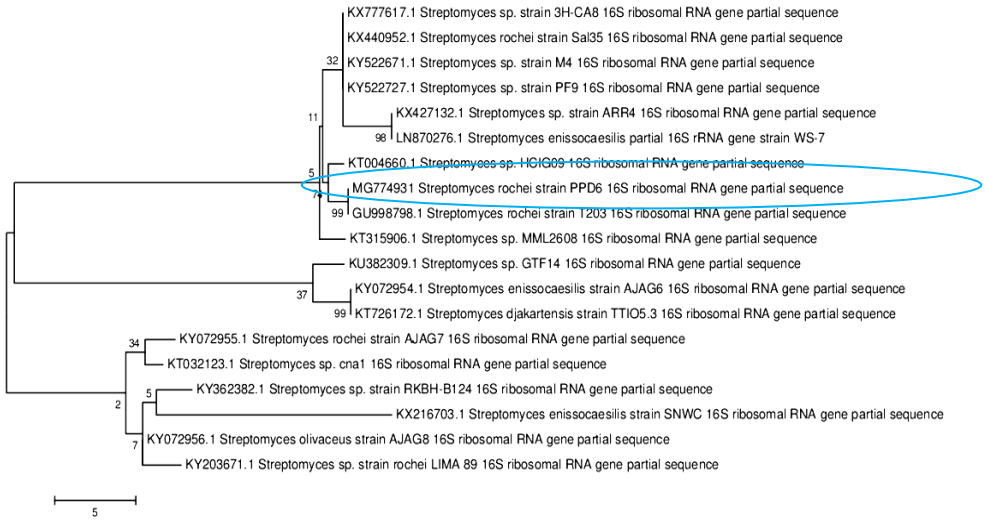


Fig.4 Growth kinetics of the potent isolate and antibacterial activity of cell-free culture supernatant was assayed by using *E. coli*.

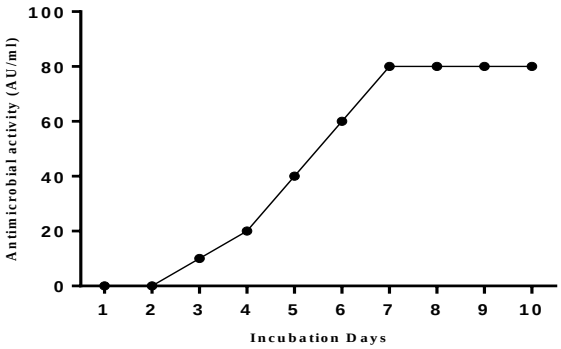


Fig.5 Preliminary characterization of the cell-free supernatant from the potent isolate. a) Effect of enzymes b) Effect of surfactants c) Effect of solvents d) Metals.

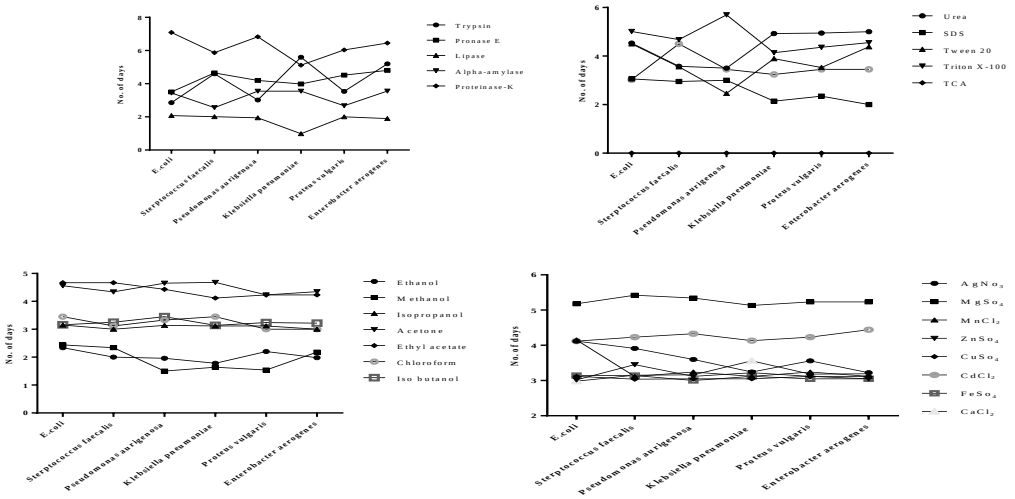
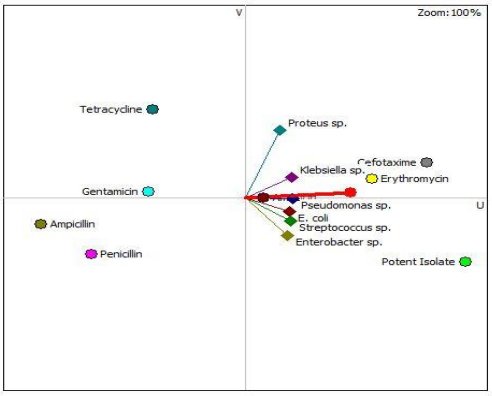
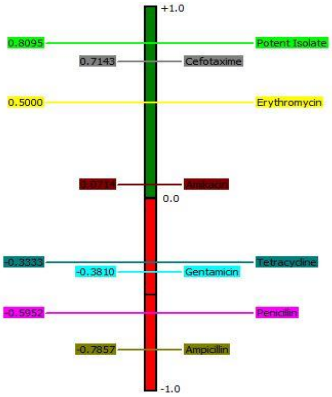


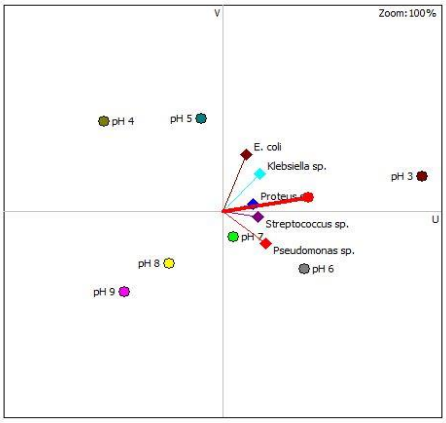
Fig.6 GAIA biplot and PROMETHEE II complete ranking. (a) GAIA biplot of antibiotics resistance against bacterial isolates; (b) Complete ranking of antibiotics based on their outranking flow.



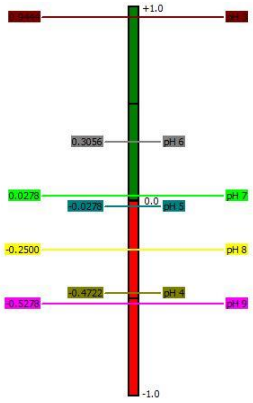
a



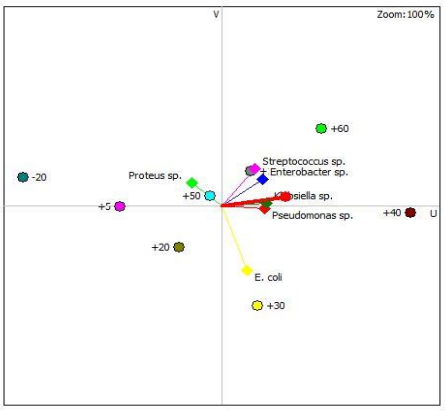
b



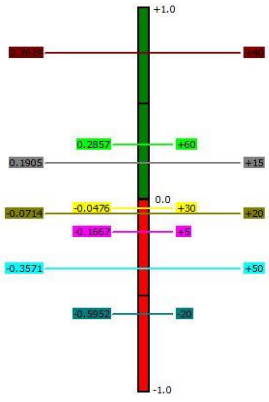
a_1



a_2



b_1



b_2

Fig.7 GAIA biplot and PROMETHEE II complete ranking. (a_1, b_1) GAIA biplot of pH (buffers) and temperature profile against bacterial isolates; (a_2, b_2) Complete ranking of pH and temperature based on their outranking flow.

CONCLUSION

To concluded, there is a massive evidence are available regarding *Streptomyces rochei* in marine and terrestrial environment still snail shell is also one of the notable environments for the tremendous contribution to the actinobacterial community. It is the first report to explore snail shell environment particularly for actinobacteria for the production of biologically active low molecular peptides. The crude peptide obtained from PPD6 was preliminary characterized with various enzyme, solvents, chemicals and metals indicated no loss of biological activity. The antibiotics, temperature and pH (buffer) optimization were subjected to multi criteria decision tool indicates maximum activity was found in the potent isolate PPD6. The present results strongly exhibited interesting characters for the screening which justifies its potential application in the antimicrobial production against multi drug resistance pathogens. However, further purification and characterization will be undertaken.

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