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ANTIBACTERIAL EFFICACY OF STREPTOMYCES FRADIAE BUCBT-PVD9 FROM MARINE SOURCE AGAINST CARBAPENEM RESISTANT KLEBSIELLA PNEUMONIA

Kayalvizhi Arunnachalam — Research Scholar, Clinical Biotechnology Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore.

Santhosh Manikandan Kumaravel — Research Scholar, Clinical Biotechnology Laboratory, Department of Microbial Biotechnology, Bharathiar University, Coimbatore.

Brindha Priyadarisini Venkatesan* — Assistant Professor, Department of Microbial Biotechnology, Bharathiar University, Coimbatore. *Corresponding Author

ABSTRACT The emergence of drug resistance is one of the major threats which is now becoming an alarming situation around the world. The UTI causing organisms are also becoming resistant towards the available treatment protocols. Hence the discovery of new, efficient antibacterial metabolites is in need. Actinobacteria has its proven records in the field of pharmaceutical industry, the secondary metabolites are potent towards the resistant strains. The aim of this study is to identify the antibacterial metabolite producing actinobacterial strains from estuary soil. Study focused on collecting soil samples from estuary in Kerala and Tamil Nadu. From the collected soil samples around nine actinobacterial strains were isolated. Among the nine different strains one strain BUCBT-PVD9 was found to be effective against all the UTI MDR strains which was taken for further study. On characterizing the potent strain it was evident that the strain belongs to Streptomyces fradiae, based on biochemical, spore arrangements and molecular characterization. Further the one factor optimization was performed to check the influencing factor for enhanced antibacterial metabolite production. Factors like pH, temperature, Nitrogen & Carbon sources along with incubation period on SSY media which showed increased production of metabolite was optimized to increase the yield of the active metabolite. MIC of the ethyl acetate fraction was determined and found to be 31.25 µg/ml against CRKP and 250 µg/ml against ATCC 1705. Further studies are required to characterize the active metabolite which is responsible for the antibacterial activity.

KEYWORDS : Actinobacteria ,Streptomycessp, Antibacterial activity, Klebsiella pneumonia.

INTRODUCTION

Soil microorganisms provide an excellent resource for isolating and identifying therapeutically valuable products. Among them Actinomycetales are an important group. The order actinomycetales is composed of approximately 80 genera, nearly all from terrestrial soils, where they live primarily as saprophytes, water and colonizing plants showing marked chemical and morphological diversity, but from a distinct evolutionary line. Actinobacteria are Gram positive bacteria with high G+C content over 55% in their DNA, which have been recognized as source of several secondary metabolites, that can act as a bioactive compound inhibiting microbial growth and possessing other potential therapeutic values. Actinomycetes particularly genus Streptomyces is widely reported for the production of various antibiotics which are used therapeutically (Argoudelis et al., 1987). It has been estimated that approximately one-third of the thousands of naturally occurring antibiotics have been obtained from actinomycetes.

The emergence of Multi Drug Resistance is one of the leading health care problems with high morbidity and mortality in the developing countries (Black et al., 1982). Among the various strains the urinary tract infecting organism are prone to frequent resistance, which are making the available antibiotic therapy a failure one. The predominant UTI pathogens belong to Enterobacteriaceae family (E. coli, Klebsiellasp, Citrobactersp, etc). In recent years, Gram negative bacilli strains exhibits resistance towards minimum of one drug to multi drug resistance. The loss of drug potency or efficacy is one of the common problems hence there is need of new drug compounds to overcome the resistance, and for an effective therapeutic potential (Archana Singh and Padma Singh., 2021). This study was aimed to isolate a potent actinobacteria (Streptomyces sp) from estuary soil, which possess good activity against drug resistant UTI pathogens.

MATERIALS AND METHODS

Collection Of Marine Soil Samples

The soil samples were collected from marine sediments of tropical estuary in the South Indian region such as Alappuzha (Kerala), Thazhancheri,andPazhaverkad(Tamilnadu). The top soils of up to 3.0 cm were removed from the surface, and the samples were collected below 5-10 cm soil depth. The soil samples were taken into sterile polyethylene bags, sealed, and transferred to the laboratory for further studies.

Clinical Strains

The MDR UTI pathogens (such as E. coli, Klebsiella pneumoniae, Enterobacter aerogenes, and Citrobacter sp) were procured from the Microbiological Laboratory, RS Puram,Coimbatore. The standard strain, Klebsiella pneumonia (ATCC-1705) was procured as a lyophilized ampoule from American Type Culture Collection (ATCC Rockville, MD, USA). All the strains were subcultured and maintained in 40% (v/v) glycerol stocks for further use.

Isolation Of Actinobacteria

Initially, the soil samples were shade dried and heated (50˚C) for 1 hour to prevent the growth of non-target microorganisms. One gram of pretreated soil was added to a flask containing 100 ml of sterile water and kept under shaking for 1 hour and subjected to serial dilution. Further, 100µl of suspension was drawn from diluent 10^{-2} to 10^{-4} and spread over starch casein agar plates The inoculated plates were kept for incubation at 28 ±2°C for 7 days. After incubation, the plates were observed for the growth of actinobacteria later confirmed through morphological characteristics. (Haefner.,et al.,2003).

Screening Of Actinobacterial Strains Against Uti Pathogens:

Antagonistic activity of actinobacterial isolates were examined based on Agar well diffusion assay by adopting the procedure from NCCLS 1993. The isolates were inoculated in starch casein nitrate broth and incubated for 7 days at 28±2˚C under shaking conditions. Following incubation, the culture broth was centrifuged (at 8,000rpm for 15mins) and the supernatant was collected and used for screening the antibacterial property. Selected test pathogens were swabbed on Muller Hinton agar (MHA) plates, using the sterile cork borer 6mm wells were punched and loaded with 100µl of actinobacteria culture filtrate. The plates were then incubated at 37˚C and the results were noted.

Characterization And Identification Of Potential Actinobacterial Strain:

Biochemical Characterization

Identification of the actinobacteria was done on the basis of microscopic examination and biochemical tests. The genus level identification of potential isolates was performed using IMVIC test.

ISP Identification

The morphological characteristics of the potential isolates were also studied using the recommended procedures of International Streptomyces Project (ISP) according to Sherling and Gotlieb 1966. The potential isolates were streaked on ISP agar media (ISP-1 to ISP-7) and incubated for 7 to 14 days at 30 ±2˚C. After incubation, the colony of potential strains were observed for its growth intensity, pattern, color of aerial and substrate mycelium and the released diffusible or melanin pigments were also documented.

Spore Morphology

Characterization of spores was done based on its arrangement on aerial mycelia, shape and surface ornamentation which is an in dispensible category of classifying actinomycetes. The spore morphology of the potent strain BUCBT- PVD9, was identified through microscopic observation following the procedure of Sherling and Gotlieb (1966) and the images were recorded using FE-SEM (MIRA 3.TESCAN-Gold sputtered).

Molecular Characterization

The genomic DNA of potential strain BUCBT-PVD9 was isolated and subjected to 16S r RNA gene amplification for the species level identification. The primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' were used for the PCR amplification. Sequencing was performed at Macrogen, South Korea. The obtained sequences were subjected to multiple alignments to generate the phylogenetic tree.

Optimization Of Antibacterial Activity:

Optimization studies are widely used to maximize the yield of the desirable product which is mainly influenced by various physical and chemical parameters (Selvinet al 2009, Vijayakumaret al., 2010).Initially production media was selected among the diverse media composition(SCNB,SSY,ISP-4,GGS,DYM).The critical factors influencing the production of antimicrobial metabolite was evaluated through one-factor-at-a-time (OFAT) approach, by changing one factor each time while keeping other factors constant. Different culture conditions, including pH (4,5,6,7,8 and 9), temperature (30°C, 35°C, 40°C and 45°C), incubation period (1st to 7th day at one day interval) alternative carbon sources (Arabinose, Xylose, Sucrose, Fructose, Glucose, Mannose) and nitrogen sources (ammonium chloride, yeast extract, peptone and ammonium sulphate, ammonium nitrate and sodium nitrate) in the media were considered to establish the optimum antimicrobial activity in the culture filtrate of BUCBT-PVD9 strain. The potent strain was inoculated into the medium and kept in shaking condition at different above-mentioned conditions for seven days. Following incubation, the culture supernatant was collected after centrifugation and the antimicrobial activity was performed using Agar well diffusion assay according to CLSI 2006. The experiments were done in triplicates and the mean was taken as final result. The statistical analyses for the obtained values were carried out by analysis of variance (ANOVA) using IBM SPSS Software 23.0. Also, the values are shown in mean ± standard error of three independent experiments. The Duncan's Multiple Range Test (DMRT) was used to determine means at 0.5% level of significance.

Extraction Of Antibacterial Compound

The antibacterial efficacy of the selected strain was investigated against ATCC strain, Klebsiella pneumonia (ATCC-1705) and various UTI pathogens. The antimicrobial compounds were extracted from bioactive strains using liquid-liquid extraction method in accordance with Selvinet al(2009). The potent isolate BUCBT-PVD9 was inoculated in SSY broth and incubated in shaking condition at 28 ± 2°C, 125 rpm for 7 days. After incubation, the cultures were harvested by centrifugation (10,000 rpm for 20 minutes) and the resulted supernatant was collected. The supernatant was transferred aseptically into sterile separator funnel and equal volume of ethyl acetate was added and kept in shaken condition for 2 to 3 hours. The bioactive compound from the supernatant was extracted twice with ethyl acetate and the crude extract was completely dried using vacuum evaporator.

Minimal Inhibitory Concentration By Broth Dilution Assay:

The minimum inhibitory concentrations (MICs) of bioactive compounds were evaluated using broth micro dilution assay as described by Veigaet.al. (2019). The various concentration of crude extract (ranged from 500µg/ml to 3.9µg/ml) was prepared from 1mg/ml stock dissolved in methanol. Initially, crude extract of 500µg/ml was transferred into the wells of 96 well plate in containing 90µl of broth. Then, two-fold serial dilution was made in subsequent wells until the concentration reached to 3.9µg/ml and similar procedure was followed for standard antibiotic neomycin sulphate (Himedia) which served as positive control. Further, 10 µl of 0.5 McFarland adjusted UTI pathogens were added and the plates were incubated at 37ºC for 18-24 hours. Following incubation, 20µl of TTC solution 0.125% (w/v) was added to each well, and the plates were further incubated for 20 minutes. MIC result was determined with spectrophotometer measurement at 540 nm using microtitre plate. The experiments were performed in triplicates and the mean value was taken as final result.

RESULTS

Isolation Of Actinobacteria

Isolation plates were observed for distinct colony morphology in order to select different genus of actinobacteria. Colonies were selected based on characteristics features such as powdery appearance, with diverse colors. Thus, based on the varied colony appearance, a total of nine isolates were selected and maintained in starch casein nitrate agar plates for further study. All the isolates were subjected for screening antibacterial efficacy against various MDR UTI pathogens.

Screening Of Actinobacterial Strains Against Uti Pathogens:

For the discovery of antibiotics, screening of industrially significant actinomycetes is crucial. Clear zone around the wells indicates the antibacterial activity exerted by the actinobacteria isolates against the test pathogens. The results tabulated in table 1states that each isolates showed variation in their antibacterial activity against different pathogens. Among the different actinobacterial isolates, strain PVD9 showed better antibacterial activity against all the four UTI pathogens and next to that strain TNCH6, TNCH7showed good activity against three clinical strains. Thus, the strain PVD9 was selected as a potential bioactive strain and taken for further characterization studies.

Table 1: Antibacterial screening of actinobacterial isolates

S. no	Isolates	Pathogens			
		E. coli	K.pneumoniae	E.aerogenes	C.diversus
1	THCH4	13mm	14mm	-	-
2	TNCH5	-	-	-	-
3	TNCH6(a)	15mm	16mm	-	-
4	TNCH6(b)	14mm	16mm	-	12mm
5	THCH7	12mm	12mm	14mm	
6	TNCH8	-	12mm	12mm	
7	PVD9	14mm	16mm	16mm	13mm
8	ALP	14mm	-	13mm	
9	KVM	-	-	17mm	16mm

Identification Of Bioactive Strains:

Actinobacteria were identified on their morphology, biochemical and 16S rDNA sequencing, hence PVD9 was subjected to characterization.

A) Biochemical Methods:

The potent isolate, PVD9 showed positive to test such as Triple sugar iron, Simmon citrate and Gram's staining and negative in Methyl red, VP and Indole. By comparing the results, it is found that the isolates strain may belongs to Streptomyces sp.

Table 2: Biochemical analysis of the potent strain:

S.No	BIOCHEMICAL TEST	STRAIN (PVD9)
1	Gram's stain	+
2	Triple Sugar Iron	+
3	Simmon Citrate	+
4	Methy Red	-
5	VP	-
6	Indole	-

(+) indicates Positive result; (-) indicates Negative result

ISP Identification

The color of the aerial and substrate mycelium and other diffusible pigments if any can be identified using ISP media. Potent strain showed no diffusible pigments production and no melanin pigments were also produced on ISP 6 and 7. The Growth and other morphological studies of the strain in ISP 1 to 7 is given in the table 3. Based on the observation the results were compared with the color wheel pattern reported by Tresner and Backus 1963. Presence of shell pink color of the arial mycelium confirms that the strain belongs to Streptomyces fradiae.

Table 3: ISP characterization of potent strain

Sl. No	ISP medium	ISOLATE - BUCBT-PVD9
1	ISP-1 (Tryptone yeast extract broth)	A: White S: Pale Brown G:+
2	ISP-2 (Yeast malt agar)	A: Pale yellow S: Brownish yellow G:+
3	ISP-3 (Oat meal agar)	A: Pinkish white S: Creamy white G:+++
4	ISP-4 (Inorganic salt starch agar)	A: White S: Pale yellow G:+++
5	ISP-5 (Glycerol asparagine agar base)	A: Pink S: Pinkish yellow G:+++
6	ISP-6 (Peptone yeast extract iron agar)	A: Pinkish white S: Pinkish brown G:+
7	ISP-7 (Tyrosine agar base)	A: Pink S: Brownish yellow G:+++

A: Aerial mycelium; S: Substrate mycelium; G: Growth range (+++ - Good growth, ++ - moderate growth, + - low growth)

Spore morphology:

Comparing the morphology of spore bearing hyphae along with the whole spore chain and structure with actinomycete morphologies as defined in Bergey's manual allowed researchers to identify active pure strains of actinomycete isolates up to the genus level. The SEM image of the spore reveals that it has rectus flexible, smooth and lack of hairy morphology. This evident supports the possibility that the strain belongs to the genus Streptomyces.

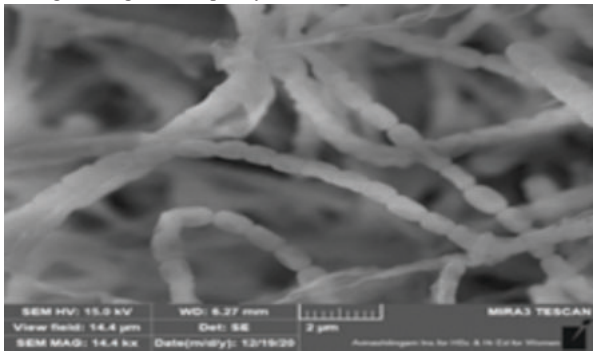

Figure – 1 Rectus flexible spore morphology of strain BUCBT-PVD9

Molecular Characterization:

The 1476 bp partial 16S rDNA sequence data of the strain PVD9 was submitted to the GenBank database assigned with accession number MW332495.Using the multisequence advanced BLAST comparison tool, which is available on the NCBI website, the partial sequence was aligned and compared with every 16S rRNA gene sequence present in the GenBank database. Phylogenetic analysis using the obtained nucleotide base pairs was then performed using Mega X version with CLUSTAL W programme, and the results were compared to the 16S rDNA gene sequences of closely related type strains of Actinomycetes (Figure 2). Thus, the 16S rDNA sequencing data validated the identification of this isolate BUCBT-PVD9 having highest sequence similarity value (99%) on *Streptomyces fradiae*.

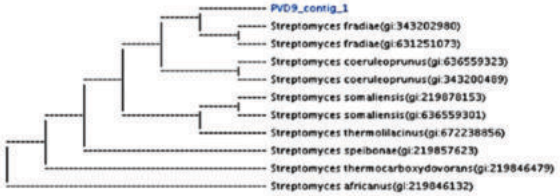

Figure – 2 Phylogenetic tree

Optimization Of Antibacterial Activity:

Environmental and cultural conditions play a crucial role in the growth of actinomycetes and the generation of bioactive compounds. Standing line of evidence suggests that the production of antibiotics was significantly influenced by the pH, temperature, medium composition, carbon and nitrogen sources, and incubation period. Moreover, these factors have profound biological effect on organisms especially with their morphology, physiology, and metabolites production. Therefore, the optimized conditions for antimicrobial production in the flask experiments was analyzed based on statistical methods such as univariate analysis and Duncan multiple range test (DMRT). The results were given in the Figure3.

Several literature surveys reveals that the optimum incubation time was the primary characteristics of actinomycetes for their regulatory control. It also suggests that the metabolite production of actinomycetes begins when the growth of an organism is declining or slowing down. In this study the antimicrobial metabolite production by the Streptomyces strain started after 3 days of incubation against CRKP and 5th day against ATCC. Following the 7th day of incubation, the peak level of production was attained. Hence it is apparent that for the production of antimicrobial metabolites the organism was allowed to incubate for 7 days. On the other hand, the composition of media, also act as a driving factor that shows variation in the antimicrobial metabolite production. Among the five tested media, the Streptomyces strain grown on SSY medium showed good antimicrobial activity against both CRKP and ATCC pathogens. Followed by SCNB media with moderate antimicrobial activity and all other media (ISP-4, GGS, DYM) showed no activity. These results indicate that production of the active metabolite has increased in SSY medium than other media tested. To further optimize the production of the active metabolite several physical parameter compositions of the SSY medium was altered.

The effect various physical and chemical parameters on antimicrobial metabolite production by Streptomyces strain was recorded. In case of pH the optimum pH for the antimicrobial metabolite production was found to be at pH 7.0. Moreover, the tendency of antimicrobial production was retained only when the pH of the medium resides between 5 to 8.

With another parameter the temperature, the production of bioactive metabolites and the growth of actinomycetes are both significantly influenced. The higher level of antimicrobial production was noticed when the culture medium was incubated at 30°C. And the order of producing antimicrobial metabolites decreased as the temperature increases from 35°C to 45°C. Henceforth the selected Streptomyces sp was strictly indicative of mesophilic strain for producing antimicrobial secondary metabolite.

A suitable carbon and nitrogen sources supplements promote antimicrobial production for different actinomycetes species. Besides some nitrogen or carbon sources are known to have interference with the production of specific antimicrobials too. Figure 4a demonstrates the effect of different carbon sources on antimicrobial production. Compared to other carbon sources used, antimicrobial activity was significantly higher in the presence of glucose. Similar pattern of antimicrobial activity was also promoted by arabinose, sucrose and fructose followed by xylose and mannose with least. The concentration of antimicrobial metabolite produced in the presence of all these carbon sources were effective against CRKP strains whereas the activity against ATCC was noticeable only when fructose and glucose was supplemented as a sole carbon sources. Figure 4b illustrates that different nitrogen sources have profound effect on the production of antimicrobial compounds by actinomycetes. Growing body of evidence suggest that organic as well as inorganic nitrogen sources are capable of regulating the secondary metabolite production. The highest concentration of antimicrobial metabolite was observed with the medium containing yeast extract as a sole nitrogen source. Medium supplemented with peptone and A.chloride showed similar results followed by A.sulphate, A.nitrate and S.nitrate.

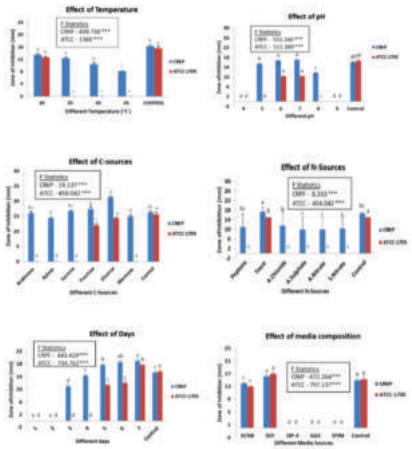

Figure 3: Optimization of antibacterial activity

Minimal Inhibitory Concentration By Broth Dilution Assay:

Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the crude extract that inhibited observable bacterial growth. The ethyl acetate extraction of antimicrobial metabolite, exhibit the antibacterial activity higher than the unextracted supernatant. The MIC value of the extract were determined by the red color development due to reduction of TTC (colorless) to formazan (red), the MIC was considered to be the least concentration where there was no color development. Moreover, for a specific antibiotic, the MIC is not constant because it depends on the type of pathogens that it encounters. Thus, the MIC value for extracted metabolite against CRKP is 31.25µg/ml, and for ATCC it is 250 µg/ml. In accordance to standard antibiotic neomycin sulphate has an MIC value of <3.9 µg/ml for both the strains. Results are given in table 4.

Table 4: Minimal Inhibitory Concentration of Ethyl acetate extract

S. No	Test Pathogens	Ethyl Acetate Fraction (µg/ml)	Standard (Neomycin Sulphate) (µg/ml)
1	CRKP	31.25	<3.9
2	ATCC-1705	250	<3.9

CONCLUSION

The aim of the study is to isolate the actinobacteria strains from estuary sources and to screen the isolated strains against various UTI causing drug resistant strains. Based on the isolation a total of nine different isolates were selected for further study. Among the nine different strains, BUCBT-PVD9 showed enhanced activity against all the tested UTI clinical isolates, hence BUCBT-PVD9 strain was selected for further study. The identity of the isolate determined based on the biochemical, morphological and molecular characteristic reveals that the isolate belongs to Streptomyces fradiae and the sequence was submitted with an accession number MW332495. The optimization study shows us that the optimum range for the enhanced metabolite production is along with the medium composition of SSY, pH of 7, temperature of 30°C, glucose as carbon source and yeast as nitrogen source. Optimum incubation period for the maximum production and activity was observed to be 7 days. The ethyl acetate extraction of active metabolite showed activity with a MIC of 31.25(µg/ml), and 250 (µg/ml) for Carbepenem resistant clinical isolate and ATCC – 1705 respectively. Standard antibiotic neomycin sulphate showed a MIC range <3.9(µg/ml). From this study we have identified a positive isolate BUCBT-PVD9 which belonged to Streptomyces fradiae. This potential isolate was able to inhibit standard and clinical strains of Klebsiella pneumoniae. Optimizing the conditions gave a increased production of the product which revealed in an increase of zone of inhibition from 17mm to 21mm for CRKP and 14mm to 18mm for ATCC – 1705. The antibacterial activity of ethyl acetate fraction revealed that the extract is having efficacy to inhibit the growth of drug resistant strains and could also pave a way for possible new drug candidate for further treatment strategies.

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