International Journal of Zoology and Applied Biosciences **ISSN: 2455-9571**
Volume 2, Issue 2, pp: 53-57, 2017 **http://www.ijzab.com**
https://doi.org/10.5281/zenodo.1311614
Research Article

ESTUARY SOIL SEDIMENTS DERIVED ACTINOBACTERIAL ISOLATES FROM PUDUCHERRY REGION – IDENTIFICATION, CHARACTERIZATION AND ANTIBACTERIAL PROPERTIES OF *STREPTOMYCES* PAS 9

***[1]Priya, M. and [2]Anandaraj, B.**

[1]Department of Microbiology, Indira Gandhi College of Arts and Science, Puducherry-605 009, India
[2]Department of Microbiology, M.R Govt. Arts College, Mannargudi-614 001, Tamil Nadu, India

Article History: Received 31st January 2017; Accepted 23rd February 2017; Published 13th March 2017

ABSTRACT

The actinobacterial strains were isolated from the soil sediments of estuary region in Puducherry. The isolated strains of Actinobacteria were screened for antimicrobial activities and the strain (actinobacterial strain PAS9) which proved a broad spectrum activity was further selected for detailed biochemical characterizations. The findings confirmed that the selected strain belonged to the genus *Streptomyces* group. In this study, in addition to the evaluation of the antibacterial activity of the selected strain against a battery of common human pathogens, the strain is to be molecularly evaluated by sequencing the 16S rRNA gene.

Keywords: Actinobacteria, Streptomyces, Estuary, Antibacterial activities.

INTRODUCTION

The domain actinobacteria dominates in soil microbial population and consists of the most economically important prokaryotes which produce more number of active secondary metabolites including antibiotics, anticancer agents, antioxidants and biopolymers etc. The genus *Streptomyces* is responsible for a half of the bioactive compounds which are commercially available at present, but still the research is going on in actinobacteria for the production of novel secondary metabolites due to the development of multi drug resistant (MDR) human pathogens. The development of multidrug resistance creates an urge in the search of new antibiotics from new resources like estuary (Sheng Qin *et al.,* 2009) with potent antibacterial activities. Prokaryotes in marine environments draw researchers' attention for their adaptability to extreme environments as well as for novel secondary metabolites (Jagan Mohan *et al.,* 2013). In recent years, the frequency of screening for novel secondary metabolites – novel antibiotics from actinobacteria of the terrestrial environment has switched over the attention of researchers to such isolates from various / diverse environments for their ability to produce the hyperactive secondary metabolites (Deepa *et al.,* 2013).

Streptomyces are aerobic, Gram- positive, non-acid-fast bacteria and chemo-organotrophic bacteria which have branched and aerial mycelia. The aerial mycelium forms three or more spores in chains at maturity and currently the genus *Streptomyces* have 787 validly published species listed in LPSN site (http://www.bacterio.net/streptomyces.html). Generally, the streptomycetes are catalase positive, reduce nitrates, in addition to adenine and L-tyrosine, can degrade gelatin, casein, hypoxanthine and starch. For energy and growth, wide a range of compounds are used as sole carbon source. The optimum temperature range for growth is 25°C - 35°C, however some species are even psychrophilic and thermophilic in nature. The optimum pH range for the genus *Streptomyces* is 3.5 – 8.0. The average DNA G+C content for the genus is 66-78%. The present study was carried out as to screen the estuary soil sediments derived *Streptomyces* isolates for their antibacterial

*Corresponding Author: Priya, M., Assistant Professor, Department of Microbiology, Indira Gandhi College of Arts and Science, Puducherry-605 009. India, Email: abiram1978@gmail.com

activities while subjecting the strain with potential antibacterial activity against a wide range of human bacterial pathogens checking the compounds broad spectrum.

MATERIALS AND METHODS

Study site and Sample collection

The sediment soil sample was collected at 15 cm depth (Gulve *et al.,* 2012) from Estuary of Sankarabarani River near boat house – Ariyankuppamin Pondicherry. The sample location was designated as 11°53'36.8"N 79°46'16.7"E. The collected Estuarian soil samples were transferred to sterile polythene bags and stored for further use.

Isolation of Actinobacteria

The soil sample (1g) was subjected aseptically to tenfold of serial dilution in sterile physiological saline (0.8%) form 10^{-1} to 10^{-10} and 1ml from the dilution 10^{-4} was used for pour plate by Starch casein agar / Potato Dextrose Agar medium as growth substrate with 30% seawater, providing substitute for its natural physiological conditions. In addition, the medium were supplemented with nalidixic and cycloheximide (50 µg/ml) to prevent bacterial and fungal contamination respectively. The inoculated plates were incubated at 30˚C and observed for growth. After 15 days, the suspected actinomycete colonies were sub- cultured on PDA with 30% sea water (Sujatha Peela *et al.,* 2005) and the colony purity was rechecked by repeated streaking on PDA and then used for screening. Totally, 50 isolates of actinobacteria were obtained and were maintained in sterile PDA slants for further use. The isolates were named as PAS1 to PAS 50.

Screening for antimicrobial activity

All the 50 isolates of actinobacteria were subjected to screening for their antimicrobial activity preliminarily with Agar well diffusion method (Gramer *et al.*, 1976) by using PDA against bacteria and fungi. The test organisms were S*taphylococcus aureus (MTCC96), Pseudomonas aeruginosa (MTCC424), Bacillus subtilis (MTCC121), Proteus vulgaris (MTCC744), Klebsiella pneumoniae (MTCC4031), Salmonella typhi (MTCC3220), Vibrio cholerae (MTCC3906), Shigella flexneri (MTCC1457), Bordetella bronchiseptica (MTCC6837),* and the yeast *Candida albicans (MTCC183).* Then agar wells of 6 mm diameter were punched with the help of a sterile cork borer. The wells were filled with 50 µl crude culture filtrate and incubated under laboratory conditions for 24-30 hrs. The zones of growth inhibitions were recorded after 24-30 hrs.

Biochemical characterization

Physiological and biochemical properties - Enzymatic activity

Amylase: The nutrient agar with 0.2% of soluble starch was prepared, sterilized and then dispensed in to Petri plates and allowed to solidify. The plates were streaked with spore suspension of the test isolate PAS 9 and incubated at room temperature for 4-5 days. Then, the plates were flooded with iodine crystal vapour/iodine solution. Any clear zone around the colony indicated amylase activity (Hankin *et al.,* 1971).

Lipase: The Nutrient agar medium (99 ml) was autoclaved separately. 1ml of Tween 20 was autoclaved separately and then added to the sterile molten medium, mixed and poured into Petri plates. The plates were streaked with the spore suspension of the test isolate PAS 9 and incubated for three to eight days. The formation of clear zone around the colonies indicated lipase activity (Hankin *et al.,* 1971).

Gelatinase: The isolate PAS 9 was streaked and inoculated on gelatine slant surface and incubated at 28±2˚C. After 3-5 days, the slants were checked for liquification for confirming the production of gelatinase (Aneja, 1996).

Protease: Protease activity was determined using the casein agar medium and a clear zone around the colony revealed protease activity(Cappuccino and Sherman, 2014).

Cellulase: A loop full of PAS 9 culture was inoculated on carboxy methyl cellulose (CMC) amended medium and incubated for 5-8 days at room temperature. The Petri plates were flooded with 1% Congo red solution. Decanted and the plates were further treated by flooding with 1M NaCl for 15 minutes. The formation of clear zone around the colony indicated cellulose degradation (Ariffin *et al.,* 2006).

Phosphatase: Phosphatase activity was determined on Pikovskaya's agar petri plates streaked with the spore suspension of PAS 9 and incubated for eight to ten days at 28˚C. Any appearance of a clear zone around the colonies indicates phosphatase activity (Pikosvkaya, 1948).

Pectinase: A loop full of culture of PAS 9 was streaked on the surface of the pectin agar and incubated for seven days at 28±2˚C. The petri plates were then flooded with 1% hexadecyltrimethyl ammonium bromide for one hour and observed for development of clear zone around the colony (Hankin *et al.,* 1971).

Urease: Christensen's agar slants were prepared and streaked with spore suspension of isolate PAS 9 and incubated at room temperature for 2-4 days. A change in colour of the medium from orange to deep pink indicated production of urease (Aneja, 1996).

Catalase: A loop full of culture PAS 9 from culture plate was transferred on a clean glass slide and kept inside the petriplate. Then the slides were flooded with 1ml of 30% H_2O_2 solution and observed for effervescence. Evolution of effervescence indicates production of catalase (Gunasekaran, 1996).

Citrate utilization: The isolate PAS 9 was streaked on to Simmon's citrate agar slants and incubated at 37˚C for 5-7 days, change in colour of the medium from green to Persian blue revealed the ability of the strain to utilize citrate (Aneja, 1996).

Indole test: The isolate PAS 9 was inoculated in sterilized tryptophan broth and incubated at room temperature for 5 days. Production of cherry red color ring when adding 3 drops of Kovac's reagent indicated a positive result (Aneja, 1996).

Nitrate reduction activity: The isolate PAS 9 was grown in tryptone nitrate broth for 48 hr along with a control to which 0.5 ml of sulphanilic acid and α-napthylamine reagent were added, development of red colour indicated the ability of the organism to reduce nitrate (Cappuccino and Sherman, 2014).

H_2S production: Five ml of sterilized peptone broth in a test tube was inoculated with a loop full of spores of PAS 9 culture. A filter paper strip soaked in saturated solution of lead acetate was kept inserted in the test tubes above the surface of the broth. The tubes were incubated at room temperature (28±2˚C) for 7-10 days. Blackening of the filter paper confirm a positive reaction (Dubey and Maheswari, 2006).

Haemolysis: Sterile blood agar plates were prepared and were inoculated with the strain PAS 9 and kept for incubation to observe the results after incubation.

Effect of pH and Temperature on its growth: For pH endurance experiments with potato dextrose broth of different pH adjusted with 1N Hcl and NaOH to get 4, 5, 6, 7, 8, 9, 10, 11 and 12 was inoculated with spore suspension and incubated at 28˚C for 20 days. After 15 days tubes were scored for growth and recorded. Growth at different temperatures was tested by incubating PDA (pH 7) slants inoculated with spore suspension of the test isolate PAS 9 at 20˚C, 25˚C, 28˚C, 30˚C, 32˚C, 35˚C and 40˚C in an Incubator for 15 days.

Effect of Sodium chloride concentration for its growth: For salt tolerance studies. Potato Dextrose broth was used as the basic medium. The NaCl concentrations (w/v) used were: 0%, 2%, 4%, 6%, 8%, 10%, 12%, 14% and 16%. The slants were inoculated by streaking the agar surface with a loopful of spore suspension of the isolate PAS 9. These tubes were incubated at 28˚C. The growth response was recorded after 15 days.

Utilization of different carbon sources: Ability of isolate PAS 9 to use 21 different sugars as sole carbon sources for energy and growth was examined in carbon utilization medium suggested by Shirling and Gottlieb (1966). Different sugars impregnated in cellulose disc (Himedia, India) were added to 5 ml liquid medium to the final concentration of 1%. The tubes were inoculated with 50 µl of spore suspension of PAS 9. All the inoculated tubes were incubated at 28±2˚C for 10-16 days. The test strain was also inoculated in the basal medium without glucose as a negative control and medium with glucose (1%) as positive control. A positive result was recorded when growth was greater than that in the negative control and that equal to or less than that in the negative control as negative.

The biochemical tests, amylase, lipase, gelatin, pectinase, cellulose, urease, indole production test, nitrate reduction, citrate utilization, H_2S production, protease, phosphatase and pigment production brought out reliable findings. The PAS 9 isolate showed positive results in amylase, lipase, pectinase, nitrate reduction, urease, H_2S production, phosphatase and pigment production (Table 1). Starch is an insoluble polymer of glucose which acts as a source of carbon for microorganisms Actinobacteria posses the ability to produce amylase that breaks starch into maltose.

While PAS 9 strain could reduce nitrate, (Table 1) the reduction of nitrate (NO_3) to nitrite (NO_2) has been used as criterion *for* species differentiation. Either $NaNO_3$ or KNO_3 are used as electron acceptor by some organisms. NO_3 and NO_2 serve as sources of nitrogen for the synthesis of organic nitrogenous compounds or they may function as H^+ acceptors. Nitratase enzyme converts NO_3 to NO_2. It was found that the isolates of the strain PAS 9 had the ability to use starch, pectin, nitrate, urea, and phosphate as a sole carbon source for their growth.

Growth at different pH, Temperature and $NaCl_2$ Concentration

The strain PAS 9 showed optimum growth at pH 7 with pigment production (Table 2) and exhibited antimicrobial activity very strongly when compared with other pH levels. The isolate PAS 9 had a maximum growth at the temperature of 25˚C, 28˚C, 30˚C, 32˚C with antibiotic production. The isolate PAS 9 grew maximum at the level of 1% salt concentration in the potato dextrose broth which shows in the Table 2.

Table 1. Actinobacterial strain PAS 9 and the antimicrobial activity of the growth extract against standard bacterial and yeast pathogens.

Organism	Zone of inhibitions in mm	Result
Bacilus subtilis (MTCC121)	20	Sensitivity
Staphylococcus aureus (MTCC96)	18	Sensitivity
Proteus vulgaris (MTCC744)	24	Sensitivity
Pseudomonas aeruginosa (MTCC424)	21	Sensitivity
Klebsiella pneumoniae (MTCC4031)	16	Sensitivity
Salmonella typhi(MTCC3220)	17	Sensitivity
Vibrio cholerae(MTCC3906)	21	Sensitivity
Bordetella bronchiseptica(MTCC6837)	13	Less sensitivity

Candida albicans (MTCC183)	17	Sensitivity

Below 10mm – Resistant, 10-15 mm- Less Sensitive, Above 15 mm – Sensitive.

Table 2. Growth at different pH, Temperature and $NaCl_2$ concentration.

pH range	*Growth*	*Diffusible pigment production*	*Temperature range*	*Growth*	*$NaCl_2$ Concentration (%)*	*Growth*
5.0	++	pinkish orange	20°C	++	1	+++
6.0	++	pinkish orange	25°C	+++	2	++
7.0	+++	Violet pigment	28°C	+++	3	+
8.0	++	Violet pigment	30°C	+++	4	+
9.0	++	Violet pigment	32°C	+++	5	+
10	++	Violet pigment	35°C	++	6	+
11	++	Violet pigment	40°C	+	7	+ (No spore)
12	++	Brown pigment			8	+ (No spore)
					9	+ (No spore)
					10	Nil

+ - Poor growth, ++ - Moderate growth, +++ - Good growth.

RESULTS AND DISCUSSION

The present study was designed to investigate the estuarine soil sample (15 cm depth) of Sankarabarani River near boat house – Ariyankuppam for novel actinobacteria and their biochemical characterization. The isolates of the study PAS 1 to PAS 50 were morphologically distinct and were screened for novel antimicrobial metabolites . Among them, PAS 9 strain showed significant antimicrobial activity (Table 1). The actinobacterial strain PAS 9 was selected through sensitivity test and was further subjected to biochemical characterization. The highest antimicrobial activity were observed to be 24 mm against *Proteus vulgaris* followed by *Pseudomonas aeruginosa* (21 mm), *Vibrio cholerae* (21 mm) and *Bacillus subtilis* (20 mm) with PAS 9 strain. The least activity was noted against *Bordetella bronchiseptica* (13 mm) by the PAS 9 strain. This strain has an optimum temperature range of 25-32°C and optimum pH of 7.0. The growth was recorded to be good at 9% NaCl (W/V) while there was no growth when the concentration exceeded 9%.

Utilization of different carbon sources

Out of 21 carbon sources, the strain PAS 9 shows maximum growth in cellibiose, Dextrose, Galactose, Sucrose, Inulin, Lactose, Manitol, and Raffinose. The maximum antibiotic production occurs in utilization of Dextrose and Galactose (Table 3)

Table 3. Utilization of different carbon source.

S.No.	Utilization of different carbon sources	Growth
1.	Adonitol (Ad)	++
2.	Arabinose (Ar)	+
3.	Cellobiose (Ce)	+++
4.	Dextrose (De)	+++
5.	Dulcitol (Du)	No growth
6.	Fractose (Fc)	++
7.	Galactose (Ga)	+++
8.	Inositol (Is)	++
9.	Inulin (In)	+++
10.	Lactose (La)	+++
11.	Maltose (Ma)	+
12.	Manitol (Mn)	+++
13.	Mannose (Mo)	++
14.	Melibiose (Mb	++
15.	Raffinose (Rf)	+++
16.	Rhamnose(Rh)	++
17.	Salicin (Sa)	+
18.	Sorbitol (Sb)	No growth
19.	Scrose(Su)	+++
20.	Trehalose (Te)	+++
21.	Xylose (Xy)	+

+ - Poor growth, ++ - Moderate growth, +++ - Good growth.

Beyond its biochemical assay, the study was initiated to ascertain whether the Estuary region was the habitat of Actinobacteria (*Streptomyces* sp). Interestingly, the biochemical characteristics such as pH range and temperature suggested that freshwater and terrestrial were its environment. Most species of *Streptomyces* produce many secondary metabolites including streptomycin. Even though the aquatic ecosystem was not major habitat, the halotolerance made it to thrive and many species were reported from marine and deep sea sediments (Jensen *et al.*, 1991; Moran *et al.*, 1995; Mincer *et al.*, 2002). Based on these biochemical studies from soil sediment of

Estuary, the cultured stain PAS9 was ascertained as actinobacteria that belongs to the genus *Streptomyces*. Further, the strain was sequenced and the phylogeny data of 16S rRNA gene sequence (KR296715) revealed that the strain PAS 9 was *Streptomyces* and as it shared 99.59% pair wise similarity with standard public database, the isolate was found to be *Streptomyces tuirus*.

ACKNOWLEDGMENT

The authors express sincere thanks to the Principal and HOD of Microbiology Indira Gandhi College of Arts and Science, Puducherry and M.R Govt. Arts College, Mannargudi for the facilities provided to carry out this research work.

REFERENCES

Aneja, K.R., 1996. Experiments in Microbiology, Plant Pathology, tissue culture and mushroom cultivation. 2nd ed. *Wishwa Prakashan*, 2, 248-250.

Ariffin, H., Abdullah, N., Umikalsom, M.S., Shiraiy and Hassan, M.A., 2006. Production of and Characterization of cellulase by Bacillus pumilus EB3. *Int. J. Eng. Tech.*, 3(1), 47-53.

Cappuccino, J.G. and Sherman, N., 2014. Microbiology a Laboratory Manual, Tenth edition, *Pearson Education*. pp. 156-157.

Deepa, S., Kanjimozhi, K. and Panneerselvam, A. 2013. 16S rDNA phylogenic analysis of actinolycetes isolated from marine environment associate with antimicrobial activities. *J. Drugs Med.,* 5(2), 43-50.

Dubey, R.C. and Maheswari, D.K. 2006. *Practical Microbiology*. 2nd ed., S. Chand and Co. Ltd, pp. 193-194.

Gramer, A., 1976. Antibiotic Sensitivity and Assay Test. In: *Microbiological Methods*, Colllins, C.H. and P.M. Lyne (Eds.). Butterworth and Co., London, p. 235.

Gulve, R.M. and Deshmukh, A.M., 2012. Antibacterial activity of Marine Actinomycetes. *Int. Multi. Res. J.*, 2(3), 16-22.

Gunasekaran, P., 1996. Laboratory Mannual in Microbiology. 1st ed., *New Age. International United*, 9-13.

Hankin, L., Zuker, M. and Sands, D.C., 1971. Improved solid medium for the detection and enumeration of pectinolyticbacteria. *Appl. Microbiol.*, 22, 205-209.

Jagan mohan, Y.S.Y.V., Sirisha, B., Haritha, R., Ramana, T., 2013. Selective screening, isolation and characterization of antimicrobial agents from marine actinomycetes. *Int. J. Pharm. Pharm. Sci.,* 5, 443-449.

Jensen, P.R., Dwight, R. and Fenical, W., 1991. Distribution of actinomycetes in near-shore tropical marine sediments. *Applied Environ. Microbiol.*, 57, 1102-1108.

Luis, E., Espinoza, Anita, L., Davelos Baines and Lowe, K.L., 2013. Biochemical, nutrient and inhibitory characteristics of Streptomyces cultured from a hypersaline estuary, the laguna madre (texas). *Online J. Biol. Sci.*, 13(1), 18-27.

Mincer, T.J., Jensen, P.R., Kauffman, C.A. and Fenical, W., 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Appl. Environ. Microbiol*., 68,5005-5011.

Moran, M.A., Rutherford L.T. and Hodson, R.S. 1995. Evidence for indigenous Streptomyces populations in a marine environment determined with a 16S rRNA Probe. *Applied Environ. Microbiol*., 61, 3695-3700. ,

Pikovskaya, R.I., 1948. Mobilization of Phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya*, 17, 362-370.

Sheng Qin, Jie Li, 1 Hua-Hong Chen, Guo-Zhen Zhao, Wen-Yong Zhu, Cheng-Lin Jiang, Li-Hua Xu, and Wen-Jun Li, 2009. Isolation, Diversity, and Antimicrobial Activity of Rare Actinobacteria from Medicinal Plants of Tropical Rain Forests in Xishuangbanna, China. *Appl. Environ. Microbiol.*, 75, (19), 6176–6186.

Shriling, E.B. and Gottlieb, D., 1966. Methods of Characterization of Streptomyces species. *Int. J. Syst. Bacteriol.,* 16, 313-340.

Sujatha Peela, Bapiraju Kurada, V.V.S.N. and Ramana Terli, 2005. Studies on Antagonistic marine actinomycetes from the Bay of Bengal. *World J. Microbiol. Biotech.,* 21, 583-585.