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Antimicrobial Profile of *Rhodococcus Erythropolis* JPVL-3 of Mangrove Origin

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Abstract: ***Actinobacteria*** **isolated from the mangrove ecosystem of Nizampatnam, located in Andhra Pradesh, India was analyzed for its antibacterial and antifungal activity. The soil samples were treated with calcium carbonate, followed by serial dilution and spread on the surface of International Streptomyces Project media (ISP). The strain JPVL-3 with potent antimicrobial activity was further studied for identification based on morphological, cultural, physiological and biochemical methods and found to be *Rhodococcus erythropolis*. Phylogenetic analysis of 16S r RNA gene sequence results obtained for strain JPVL-3 showed clade with the *Rhodococcus* 16S rRNA phylogenetic tree, which is closely related to *Rhodococcus erythropolis.***

Index Terms: **Mangrove ecosystem, *Rhodococcus erythropolis*, Phylogenetic analysis, antimicrobial profile, microorganisms**

I. INTRODUCTION

Actinomycetes found in different habitats are gram positive filamentous sporulating free living saprophytic bacteria with a G+C content of 55-75%. Actinobacteria are valuable sources for novel bioactive metabolites with potent applications in biotechnology and agriculture (1). It consists of biologically active compounds, nearly 9000 molecules and 60 pharmaceutical compounds (2). Marine actinomycetes are a source for antibiotic research with new compounds discovered with a good success rate.

Actinobacteria have the capability of producing antibiotics, pesticides, herbicides, and enzymes like cellulose and xylanase (3). The search for novel antimicrobial agents is very useful for coming generation to prevent the pathogenic diseases. Antibiotic resistance to be addressed and a global concern for disease prevention (4). It is important to follow ecofriendly methods for disease prevention and environmental protection. Drug resistant pathogens challenge drug molecules capable of pharmaceutical application. We have chosen different mangrove habitat of the south coast of Andhra Pradesh, India to isolate and screen actinomycetes as a source for potent bioactive compounds to act as bio-control agents for targeting multiple drug resistant disease causing organisms.

II. MATERIALS AND METHODS

Agarose gel DNA purification Kit (SoluteReady® Genomic DNA purification kit). PCR Master Mix, Primers Genie. Nutrient agar and Czapek-Dox (CD) agar and all chemicals used in experiments are Hi Media chemicals.

Test Organisms in agar-diffusion assay: Candida albicans (ATCC 10231), Pseudomonas aeruginosa (ATCC 9027), Bacillus subtilis (ATCC 6633), Escherichia coli (ATCC 35218), Staphylococcus aureus (MTCC3160).

Collection of soil sample and pretreatment with calcium carbonate:

Soil samples from the mangrove ecosystem of the south coast of Andhra Pradesh, India were randomly collected in sterile polythene bags. The soil samples are from different habitats. The soil samples were maintained separately and air dried for 3-4 days at room temperature and remoistened by mixing with calcium carbonate as a carbon source in 1:1 ratio and followed by incubation at 28ºC until further use (5).

Isolation of actinomycetes:

For isolation of *actinomycetes* 1 gram of the above soil sample was taken and properly air dried and diluted with sterilized distilled water for tenfold serial dilution. After serial dilution to isolate the actinomycetes colonies, 500 µl of supernatant from 10^{-4} to 10^{-10} dilution was evenly spread plated on Yeast extract malt extract dextrose (YMD) agar medium (pH 7.0) added with 25 µg/mL nystatin, 25 µg/mL tetracycline. Plates in triplicates are incubated for 10 -14 days and temperature maintained at 28-30°C. Actinomycetes were observed as dry colonies and all the strains were isolated and sub-cultured on YMD agar medium. Slants were prepared and preserved at 4°C for further study (6).

Morphological and biochemical characteristics:

Isolated strains were grown on seven International Streptomyces Project media and two non-ISP media (7). After 4 days of incubation, the morphological study was carried out of a scanning electron microscope (SEM: Model- JOEL-JSM 5600, Japan) at various magnifications.

Culture identification- 16s rRNA:

After 3 days of incubation culture was taken from YMB broth and centrifuged at 10000 rpm for 20 min. For isolation of DNA, pellet was collected (8). For PCR reaction in 10X buffer (2.5 µl), 25 mM - $MgCl_2$ (3.5 µl), 0.4 mM -dNTP (2 µl), 16S rRNA actino specific primer (1 µl) were taken. Forward: 16S rRNA actino specific Primer (1 µl of 10 pmol/µl). Reverse: Taq polymerase (2 U/µl) and template DNA (2 µl of 10 pmol/µl).

In PCR amplification initial denaturation step was carried out at 94ºC(3 min) then followed with 30 cycles of denaturation at 94°C(1 min), annealing was done at 65°C (1 min) and extension step was at 72°C (1 min), followed by extension at 72°C (5 min). Purification of PCR product was done by Agarose Gel Electrophoresis using an Agarose gel DNA purification Kit. Using the 16S rRNA actino specific forward and reverse primers, a 750 bp 16S rRNA sequence was determined. Amplification of the 16S rRNA gene fragment was done using Universal Primers (Actino specific forward Primer-5'-GCCTAACACATGCAAG TCGA-3' and Actino Specific Reverse primer - 5'-CGTATTAC CGCGGCTGCTGG-5') (9). The resulting 16s rRNA sequence was tested by using the Basic Local Alignment Search Tool (BLAST) with sequences in Gene Bank for similarity (http://www.ncbi. nlm.nih.gov/). By the Clustal *W* method, the related reference sequences were retrieved from NCBI Gene Bank databases. Molecular Evolutionary Genetic analysis (*MEGA)* version 5.0 was used for Phylogenetic and molecular evolutionary analyzes (10).

Gene Bank Acession Number:

The complete sequence of the 16S rRNA gene sequence of the strain was deposited at the National Center for Biotechnology Information (NCBI) with Acession Number MZ389874.

Strain JPVL-3 Growth:

Strain JPVL-3 culture was prepared in the seed medium for 48h. One ml of cultured inoculum was taken and added to sterilized YMD broth fermentation media (100 ml) and kept for incubation on a rotary shaker at 180 rpm (28°C). At every 24 h interval, the yields were taken by filtering the broth through What man no 1filter paper and dry weight was measured. The culture filtrate was extracted with ethyl acetate. The extracts were used to determine antimicrobial property.

Antimicrobial assay:

An agar well diffusion test was done to determine antimicrobial properties of crude extract of JPVL-3 (11). JPVL -3 was cultured as lawn on nutrient agar medium plates and wells (5 mm) were prepared with cork borer. Ethyl acetate extracts were added (500 µl of 50 ppm concentration) to each well. Control well was added with ethyl acetate alone. Plates incubated for 24hrs at 37ºC. Spore suspension of test fungi was mixed with sterilized and cooled to 40ºC Czapek-Dox (CD) medium and poured into petri dishes. Wells were prepared, and Ethyl acetate extracts were added (500 µl of 50 ppm concentration) in each well. Adding only ethyl acetate to the wells served as control. The plates were incubated (28ºC for 72 h) for fungal growth. The diameter of the inhibition zones was measured.

III. RESULTS AND DISCUSSIONS:

Isolation of actinomycetes

Actinomycete strains were isolated from air-dried soil samples pre-treated with calcium carbonate. Calcium carbonate pre-treatment was reported as the best technique for the potential isolation of actinomycetes. The calcium carbonate acts as a carbon source for the growth of actinomycetes (12, 13). A total of 10 isolates, JPVL-1 to JPVL-10 were isolated from Nizampatnam mangrove ecosystem and screened for antimicrobial properties by agar well diffusion test. Among 10 strains screened, one strain JPVL-3 had good growth inhibitory properties against tested bacteria and fungi such as *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. The strain with better antimicrobial spectrum was used for further studies.

Screening of potent actinomycete strain for bioactive metabolites

Rhodococcus erythropolis JPVL-3 was cultured from yeast extract, malt extract and dextrose broth. The potent strain JPVL-3 was tested for its growth pattern and antimicrobial activity (fig.1). After 24 h of incubation JPVL3 strain entered the log phase and showed exponential growth up to 72 h and stationary phase extended up to 120 h. At a 24-h interval, the fermentation broth was collected and extracted with ethyl acetate

Fig. 1. Growth pattern of JPVL-3

and antimicrobial activity was tested. Similarly this is compliance with the earlier reports (14& 15).

Identification of the strain JPVL-3

Isolate JPVL-3 was studied for cultural, morphological, and biochemical characteristics. Genomic analysis (16S rRNA) was done for phylogenetic analysis.

Cultural characteristics JPVL-3

For cultural characteristics, nine culture media were used for the growth of strain JPVL-3 (table I). Good growth was observed on ISP-1, ISP-2, ISP-4, ISP-6, Czapek-Dox agar and nutrient agar and moderate growth on ISP-3, ISP-5 and ISP-7. Aerial mycelium was creamy white and the substrate mycelium was pale yellow in all the culture media. Production of melanim pigment by the strain was not found in any medium.

Morphological characteristics of the strain JPVL-3

Strain JPVL-3 cultured in cover slip culture taken Scanning Electron Micrographs showed morphological characteristics similar to the genus *Rhodococcus* (16). The culture had extensively fragmented aerial mycelium and bear short rods. Its sporophore morphology had short rods has the fragmentation of hyphae (figure 2).

For identification, classification of actinomycetes, physiological tests are used as indispensable tools (17). The strain exhibited Gram positive reaction and growth betweem 25 ºC to 45ºC and optimum at 30ºC. Optimum growth pH 7 and could grow in the pH range of 5-10. . At 3% NaCl conc. strain recorded

Table I. Cultural characteristics JPVL3

S.No	Culture Medium	Growth
1	Tryptone yeast extract agar(ISP-1)	Good
2	Yeast extract -malt extract dextrose agar(ISP-2)	Good
3	Oat-meal-agar (ISP-3	Moderate
4	Starch Inorganic salts(ISP-4)	Good
5	Glycerol asparagine agar(ISP-5)	Moderate
6	Starchcasein agar(ISP-6)	Good
7	Tyrosine agar(ISP-7)	Moderate
8	Czapek-Dox agar	Good

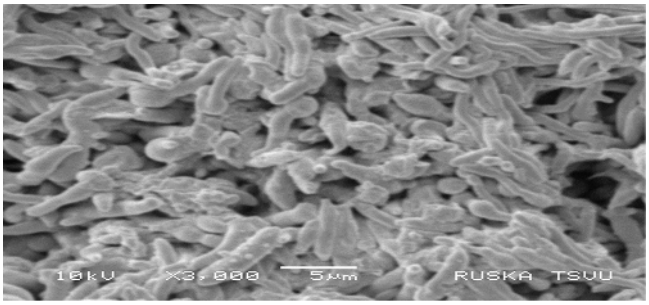

Fig.2. Scanning Electron microscopic photograph of as *Rhodococcus erythropolis* JPVL-3 (X 3000).

optimum growth. Henceforth the strain is moderately salt tolerant (18). Isolate JPVL-3 had the potential starch hydrolyzing ability and showed positive catalase and urease tests. Negative for methyl red, indole, Voges-Proskauer, nitrate reduction tests and gelatin liquefaction (table II). Amylase is positive with utilization of starch. The ability to utilize different carbon sources such as fructose and dextrose, moderate lactose, maltose, starch and sucrose indicate carbon acclimatization potential (table III). It could not utilize xylose and mannitol. Isolate JPVL-3 is positive for enzymes amylase, cellulase and L-asparaginase and lipase negative for nitrate reductase.

Table II. Biochemical characteristics of JPVL-3

S.No	Biochemical characters	Result
1	Starch hydrolysis	+
2	Catalase production	+
3	Urease production	+
4	Indole production	-
5	Methyl red test	-
6	Voges proskauer test	-
7	Nitrate reduction tets	-
8	Gelatin liquefaction	-

Table III. Utilization of carbon sources by JPVL-3

S.No	Carbon sources	*Growth
1	Lactose	++
2	Maltose	++
3	Xylose	-
4	Sucrose	++
5	Dextrose	+++
6	Fructose	+++
7	Starch	++
8	Mannitol	-

* +++ : Good growth, ++: Moderate growth, - :No growth

Phylogenetic analysis of the strain JPVL-3

Blast analysis done for the 16S rRNA gene sequence of isolated JPVL-3 nucleotide database of NCBI similarity search with a set of published sequences. The isolate is similar to *Rhodococcus erythropolis* according to the 16S rRNA gene sequence (figure 3). The sequence was deposited in NCBI database GenBank with accession number **MZ389874.** Based on Morphological, biochemical characters and molecular analysis, the isolate was identified as *Rhodococcus erythropolis* JPVL-3.

Antimicrobial activity

The extracts from the strain JPVL-3 showed the highest significant antimicrobial activity against test organisms (table 4). Extracts of isolates VLK-12 (14), *Streptomyces purpeofuscus* (19) and *S. albidoflavus* (20) were active against bacteria and fungi, while metabolites produced by *Streptomyces* sp. were found potent against test bacteria and fungi (21). Extracts of *Rhodococcus erythropolis* VL_RK-05 (22) and *Pseudomonas* sp.VUK-10 (23) were exhibited good antimicrobial spectrum against tested microorganisms.

Table IV. Biochemical characteristics of JPVL-3

S.No	Biochemical characters	Result
1	Starch hydrolysis	+
2	Catalase production	+
3	Urease production	+
4	Indole production	-
5	Methyl red test	-
6	Voges proskauer test	-
7	Nitrate reduction tets	-
8	Gelatin liquefaction	-

Table V. Antimicrobial activity of the strain JPVL-3

S. No	Age of culture (h)	Antimicrobial activity (zone of growth inhibition mm)				
		*Sa	Bs	Ec	Pa	Ca
1	24	10	10	7	9	10
2	48	14	15	11	16	13
3	72	19	17	14	18	17
4	96	19	20	16	18	17
5	**120**	**20**	**22**	**18**	**19**	**19**
6	144	13	13	13	11	12
7	168	9	10	8	5	10
8	192	7	7	3	6	8

**Staphylococcus aureus (Sa)*, *Bacillus subtilis (Bs)*, *Escherichia coli (Ec), Pseudomonas aeruginosa (Pa), Candida albicans (Ca)* (Data statistically significant at 5%)

CONCLUSION

Rhodococcus erythropolis JPVL-3 had good growth inhibitory properties against tested bacteria and fungi such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans.* The strain with better antimicrobial spectrum can be used further for exploring its inhibitory properties and study the culture extracts for effective use as antimicrobial agents.

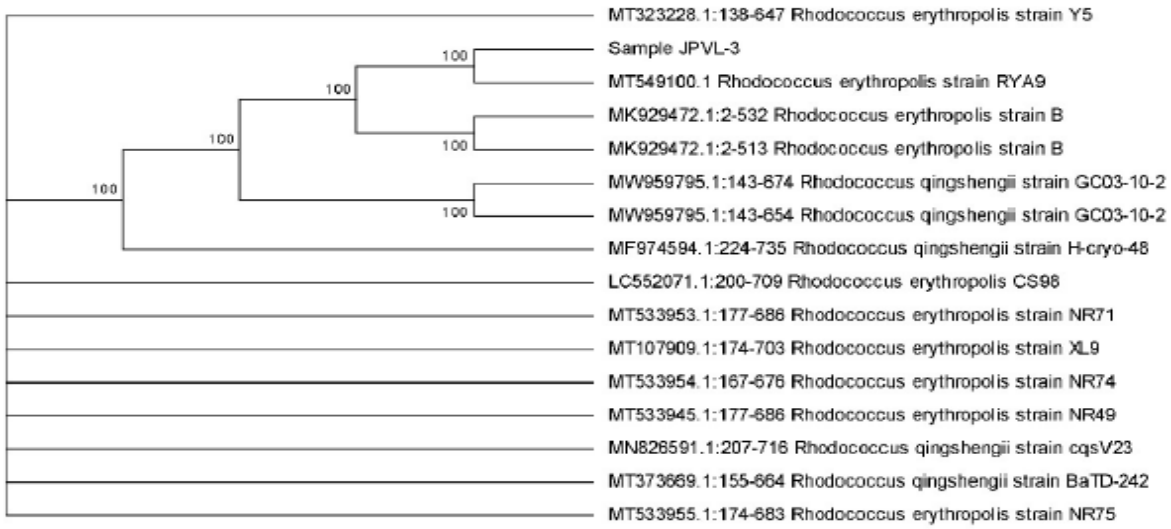

Fig 3. The Phylogenic analysis of 16S rRNA gene sequence of JPVL-3 similarity with *Rhodococcus erythropolis and* members of the genus *Rhodococcus* .

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