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Isolation, Identification, Characterisation and *In silico* Analysis of *Pseudomonas stutzeri* VITNR-1 from Amirthi Forest Soil, India

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The aim of this study was to isolate, identify, Characterize and in *silico* analysis of a novel bacterial strain from the Amirthi forest that is located in Vellore district of Tamil Nadu. The soil sample were serially diluted and plated on Nutrient agar. The inoculated plates were incubated at 37 °C for 24-48 hrs. A total of 22 isolates were obtained, out of which 12 were bacteria. From these ten different colonies of various morphology and pigmentation was selected for the study. Then the isolates were characterised by morphological, biochemical and molecular techniques. One of the isolate showed wrinkled dry morphology on Nutrient agar. It was further subcultured on selective medium. The microbiological analysis, 16 S rRNA followed by *in silico* analysis showed that the isolate found to be *Pseudomonas stutzeri*. The isolate is submitted in Gen bank under the accession no FR848954 *Pseudomonas stutzeri* VITNR-1. The accession no of other isolates are KM047486 *Bacillus subtilis* VITNJ1, KM047487 *Bacillus sp* VITNJ2, KM047488 *Bacillus licheniformis* VITNJ3, KM047489 *Bacillus sp* VITNJ4, KM047490 *Bacillus pseudomycoides* VITNJ5, KM047491 *Arthrobacter nicotianae* VITNJ6, KM047492 *Rummeliibacillus stabekisii* VITNJ7, KM04749 *Enterobacter sp*. VITNJ8, KM047494 *Bacillus safensis* VITNJ9. Further *Pseudomonas stutzeri* VIT NR-1 isolate was checked for antibiotic sensitivity test and *in vitro* antimicrobial activity for pathogens like *E.coli, Bacillus subtilis* ,*Klebsiella, Proteus*, *P.aeruginosa,* and *Staphylococcus.*

Key words: Antibiotic resistance, *in silico* analysis, Nutrient agar, *Pseudomonas stutzeri,* Rhizosphere , 16 S rRNA.

Microbial diversity in forest ecosystem plays a major role in maintaining the biological activity of soil. Forest soil microorganisms that are involved in soil energy cycling processes assume a vital part in producing available supplements for the forest trees [1]. The narrow zone of soil directly surrounding the root system is alluded to as rhizosphere. Rhizobacteria are rhizosphere competent bacteria that aggressively colonize plant roots and they are able to multiply and colonize all the ecological niches found on the roots at all stages of plant growth in the presence of a competing micro flora.

Plant growth promoting bacteria are a group of microscopic organisms that can effectively colonize plant roots and expand plant development and yield [2]. A group of beneficial rhizobacterial strains belong to genera of *Pseudomonas, Azospirillum Azotobacter, Bacillus*, *Burkholdaria* [3]. In the rhizosphere, bacteria are the most abundant microorganisms.

Members of the Pseudomonas genus are found in large numbers in all the major natural environments like terrestrial, freshwater and marine and they also form intimate associations with plants and animals. This universal distribution recommends a striking level of physiological and

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genetic flexibility[4]. *Pseudomonas stutzeri* is a non-fluorescent widely distributed species of the genus Pseudomonas belonging to the gamma subclass of Proteobacteria[5]. Members of *P.stutzeri* are strong denitirifiers[6].

Amirthi forest from where the soil sample was collected is situated under the Javadi Hills of Tellai which is 25 km away from Vellore. Latitude of the forest is 12° 432 56.513 N, and longitude 79° 32 24.023 E. The soil is highly rich in organic matter and suitable for the growth of microorganisms[7]. Although several research articles are documented on isolation & characterisation of various microorganisms from Amirthi forest there is no research paper published on isolation of *Pseudomonas stutzeri* from Amirthi forest till date [8, 9, 10].

The present study aims at isolation and characterization of novel bacteria from forest soil sample. The antimicrobial activity of *Pseudomonas stutzeri* was studied by preforming test against few pathogens.

MATERIALS AND METHODS

Sampling area and sample collection

Amirthi forest is situated in javadi hills. Soil samples were collected from this forest at a depth of 8-10 cm from the rhizosphere region. The collected soil samples were taken in a sterile container and were transferred to the laboratory for further microbiological analysis.

Analysis of physicochemical properties

The test soil sample was analysed for pH, electrical conductivity, organic matter, nitrate, nitrogen, available Phosphorus, exchangeable K, calcium exchangeable Ca, magnesium exchangeable Mg, sulphur available S and sodium exchangeable Na.

Isolation and identification of microorganism

Bacterial population was enumerated using 10-fold serial dilutions [11] .The bacteria were isolated by serially diluting the sample. The diluted samples were plated on nutrient agar. Morphologically distinct colonies were selected for further studies. All strains were characterized by the classical tests according to Bergey's Manual of Systematic Bacteriology[12]. Then the distinct colonies were sub cultured continuously on the suitable medium for the isolation of pure culture. *Pseudomonas stutzeri* was further sub cultured on various media to note down the colony morphology. The molecular characterization of the bacteria was done by 16s rRNA sequencing[13]. The purified PCR products of approximately 1,400bp were sequenced by using two primers 518F5'CCAGCAGCCGGGGTAATACG3 and 800R5' TACCAGGGTATGTAATCC3. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). 16S rRNA sequences of bacterial isolates have been deposited in the Gen Bank database under the accession no FR848954Pseudomonas stutzeri VITNR-1, KM047486 *Bacillus subtilis* VITNJ1 , KM047487 *Bacillus sp* VITNJ2, KM047488 *Bacillus licheniformis* VITNJ3, KM047489 *Bacillus sp* VITNJ4, KM047490 *Bacillus pseudomycoides* VITNJ5, KM047491 *Arthrobacter nicotianae* VITNJ6, KM047492 *Rummeliibacillus stabekisii* VITNJ7, KM04749 *Enterobacter sp*. VITNJ8, KM047494 *Bacillus safensis* VITNJ9. The partial 16S rRNA bacterial sequences isolated from Amirthi soil samples were analysed using BioEdit software[14]. The sequences were compared with the available sequences against 16S rRNA sequences (Bacteria and Archaea) database using NCBI's BlastN. Sequences were aligned using CLUSTALW program in Mega 6.0. A Neighbour-joining tree assessing the phylogenetic diversity of the sequences was constructed using Jukes and Cantor method. Bootstrap analysis was performed on 1000 random samples taken from the multiple sequence alignment. (Fig. 1)

Identification / Classification of the organism

Ribosomal Database Project II (RDP) – classifier was used to confirm the taxonomical/ hierarchical details of the source organism. The classifier tool assigned the hierarchy with the 95% threshold in the following way: Root [100%] Bacteria [100%] "Proteobacteria"[100%] Azomonas[44%] .

Annotation of 16S rRNA gene sequence

Oligo Calculator version 3.26 [15] was used to find the molecular weight and GC content of the 16S rRNA (FR8489541). The query (FR8489541) was subjected to restriction site analysis to the Restriction Mapper web server [http://

www.restrictionmapper.org/] and the minimum site length was fixed to five to avoid unwanted signals. With this parameter, the sequence was found to have 66 restriction sites. Further, ARNold server [16] was used to predict the srho- independent transcriptional terminators in the nucleic acid sequence. The search algorithm uses two complementary programs, Erpin and RNA motif. Erpin by Gautheret & Lambert [17] uses a structure-annotated alignment of 1200 terminator sequences from *Bacillus subtilis* and *Escherichia coli* as a training set. It builds a lod-score profile from this alignment and seeks high scoring instances of the profile in the user's sequence. RNA motif by Macke *et al*., [18]uses a descriptor developed by Lesnik *et al*.[19] to recognize *E. coli* terminators; however it can be applied to find terminators from any species. Essentially, the descriptor consists of a 4-18 bp helix, a 0-2 nt spacer and a 12 nt T-rich region. RNAmotif matches are scored using the sequence contents of the T-rich region and stability of the stem loop region and an empirical score cut off is defined to accept or reject matches.

Secondary Structure Prediction

The gene sequence was transcribed to RNA sequence to identify the rRNA secondary structure using the RNAfold server from Vienna RNA web suite [20] to predict the minimum free energy (MFE) using the dynamic programming algorithm originally proposed by Zuker and Stiegler [21]. Also, equilibrium base-pairing probabilities were calculated using John McCaskill's [22] partition function (PF) algorithm. Using RNAfold server MFE structure, centroid structure and pair probabilities were predicted.

Antibiotic resistance test

The isolates were screened for antibiotic sensitivity according to the Kirby-Bauer disc diffusion method [23] with eight antibiotics namely ampicillin ,bacitracin,ceftazidime, erythromycin, gentamycin,nitrofurantoin, ofloxacin,rifampicin 0.1 ml of bacterial culture was uniformly spread on sterile Muller Hinton agar plate. The culture was allowed to dry on the plate for 5-10 min at room temperature, antibiotic impregnated disc were placed on the plates aseptically on the bacterial colonies on Muller Hinton agar plates. The plates were incubated for 18-24 h at 37 °C. The diameter of the inhibition zone was measured and the isolates were classified as resistant (R) and Susceptible (S) following the standard method.

Antibacterial activity

The Mueller Hinton agar plates were prepared, sterilized and allowed to solidify in sterilized pertriplates. The various pathogenic microorganisms like *E.coli, Klebsiella, Proteus, P.aeruginosa , S.aureus* and *Bacillus subtilis* were inoculated on the sterilized plates using sterile cotton swabs. 5mm disc was dipped in the supernanet of the isolate *Pseudomonas stutzeri* VIT NR-1 and placed in the plates. Three discs were placed in every plate. The plates were incubated at room temperature for 24-48 h. If the zone of inhibition present it was noted down.

RESULTS AND DISCUSSION

Pseudomonas stutzeri was isolated by serial dilution and plating on selective media. The colonies were wrinkled and dry. The organism was found to gram negative bacillus and motile. Various biochemical tests were performed (Table 1) and the 16 S rRNA sequencing was done and the organism was found to be *Pseudomonas stutzeri*. The biochemical test for other microbial isolates showed that most of the isolates belonged to Genus Bacillus.

Table 1. Morphological and physical characteristics of *Pseudomonas stutzeri* VIT NR-1

S.No	Characteristics	Present/Absent
1.	Gram reaction	-
2.	Shape	Rod
3.	Indole	+
4.	Methyl Red	-
5.	Voges Proskauer	-
6.	Citrate Utilization	+
7.	Catalase test	+
8.	Oxidase Activity	+
9.	Urease Activity	+
10.	H_2S Production (SIM agar)	-
11.	Gelatin Liquefaction	+
12.	H_2S Production (KIA)	-
13.	Mannitol motility test	+
14.	Esculin hydrolysis	-
15.	Starch hydrolysis	+
16.	Casein hydrolysis	+
17.	Sucrose	+
18.	Lactose	_

Table 2. Characteristic of other Bacterial isolates from Amirthi forest

Biochemical Properties	VITNJ1	VITNJ2	VITNJ3	VITNJ4	VITNJ5	VITNJ6	VITNJ7	VITNJ8	VITNJ9
Gram Staining	+	+	+	+	+	+	+	-	+
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rods	Rods	Rods
Indole test	-	-	-	-	-	-	+	-	-
Methyl red test	-	-	+	+	-	-	-	-	+
Voges Proskauer	+	+	+	+	-	-	-	+	+
Citrate Utilization	+	+	+	+	+	-	-	+	-
Oxidase test	-	-	+	+	-	-	-	-	+
Catalase Test	-	-	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-

Table 3. Soil Analysis

Nutrient	Unit	Result
Nitrate	ppm	13.8
Phosphorus		
Bray P1	ppm	18.1
Bray P2	ppm	96.9
Parameters	Unit	Result
pH		7.01
Electrical conductivity	mS/cm	0.221
Organic matter	%	2.15
CEC	Meq/100gm	12.65
K saturation	%	1.18
Ca saturation	%	68.36
Mg saturation	%	24.70
Na saturation	%	5.77

Annotation of 16S rRNA gene sequence (FR8489541)

The molecular weight of the sequence was found to be 321.08 KD with 52.65% GC content by the analysis carried out using OligoCalc server [15]. Further, the strain was found to possess one terminator at sequence region between 926 and 946 by Erpin and RNA motif algorithm of ARNold server [16]. The secondary structure features lying within terminators correspond to 926-932 in stem region, 931-937 in loop region and 938-946 in stem region. The free energy of the predicted terminator stem-loop structure was -6.60 (kcal/mol) by RNAfold. Restriction apper server has identified

Table 4. Colony Characteristics of *Pseudomonas stutzeri* VIT NR1on various Media

Media	Colony characteristics
Citrate agar	Red brown, rust coloured colonies, rough, round, flat
Nutrient agar	Small, round, cream, flat, entire margin, off-white, smooth
MacConkey agar	Small, round, white, entire margin, flat, shiny
King's B	Small, round, cream, flat, entire margin, smooth
Pseudomonas agar	Small, round, flat, cream, entire margin, smooth
Pseudomonas F agar	Small, cream, round, flat, entire margin, smooth
Pseudomonas P agar	Small, round, regular, flat, cream, entire margin, shiny

66 restriction sites with maximum number (8 sites) for Hin4I restriction enzyme.

Secondary Structure Prediction

The secondary structure was predicted using RNAfold server (using MFE) [19] with a minimum free energy of -379.77 kcal/mol. The centroid secondary structure was predicted with a minimum free energy of -272.03 kcal/mol. Based on the energy of the predicted models, the secondary structure predicted by MFE gives the more stable structure with a minimum energy of -379.77 kcal/mol. Hence MFE model can be considered as the optimal secondary structure of the rRNA (Fig. 2). A mountain plot is also derived from RNAfold server which represents a secondary structure in a plot of height versus position, where

the height is given by the number of base pairs enclosing the base at position i.e. loops correspond to plateaus (hairpin loops are peaks), helices to slopes (Fig. 3).

Table 3 elaborates the various nutrients, pH present in the soil sample from which the organism was isolated . *Pseudomonas stutzeri* was sub cultured on various media to know the characteristics of colony morphology. The isolate showed small, round, cream flat, smooth colony characteristics except on citrate agar it showed red brown, rust coloured colonies (Table 4). Further when the isolate was checked for the antibiotic sensitivity test it showed resistance only to Nitrofurantoin and ampicillin (Table 5). *Pseudomonas stutzeri* exhibited antimicrobial activity against *Bacillus*, *E.coli* and *Proteus* (Table 6)

Table 5. Antibiotic sensitivity test

S.No	Antibiotic	Interpretation
1.	Nitrofurantoin	R
2.	Ampicillin	R
3.	Gentamycin	S
4.	Ofloxacin	S
5.	Rifampicin	S
6.	Bacitracin	S
7.	Erythromycin	S
8.	Ceftadizine	S

R- Resistance, S-Sensitive

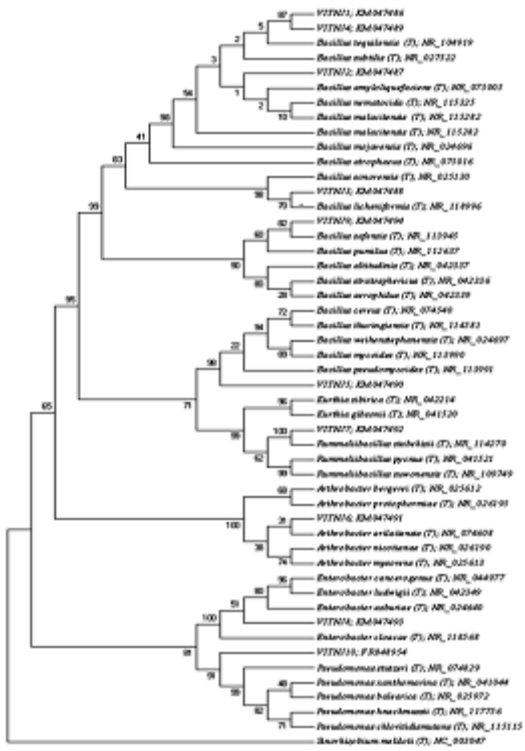

Fig. 1. Phylogenetic relationships of 16S rRNA sequences of the isolates from Amirthi forest to closely related sequences from Gen Bank

Table 6. Antimicrobial activity of *Pseudomonas stutzeri* VIT NR-1

Pathogen	Zone in mm
B.subtilis	7 mm
E.coli	10 mm
Klebsiella sp.	0 mm
Proteus sp.	3 mm
P.aeruginosa	0 mm
Staphylococcus sp.	0 mm

CONCLUSION

The forest ecosystem various depending upon temperature, pH, climatic conditions, soil types, relative humidity and various environmental factors. Generally forest soil is rich in organic matter content due to litter fall and the degradation of organic matter which increase the soil fertility and

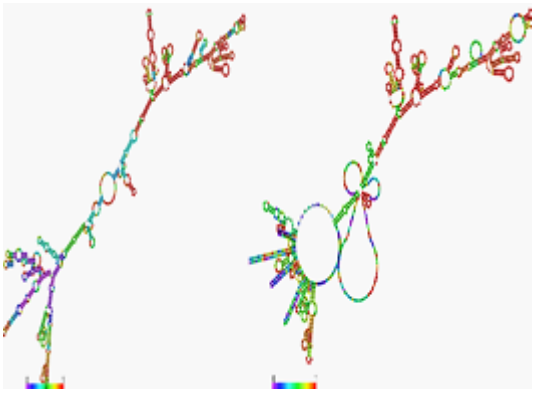

Fig. 2. (a) The MFE structure with of -379.77 kcal/mol of free energy (b) The centroid structure with -272.03 kcal/mol of free energy. The structure is colored by base-pairing probabilities, where the color - probability relation is shown below the secondary structures. For unpaired regions the color denotes the probability of being unpaired.

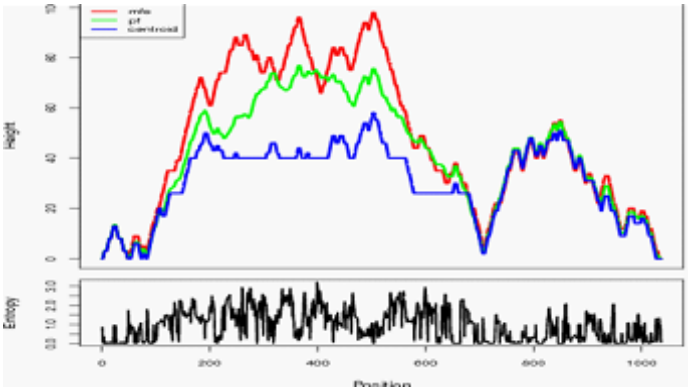

Fig. 3. Mountain plot (height versus position) for the secondary structure derived from RNAfold server. Red plot represents MFE structure, green plot represents partition function and blue plot represents centroid structure

maintains healthy soil which plays a major role in nutrition cycle. The Amirthi forest soil is rich in micro and macro nutrients, minerals, vitamins, enzymes and growth factors which support the growth of plants and the symbiotic associations between the microorganisms and plants. In fertile (humus) soil consist of beneficial micro flora which maintains a good soil quality, pH, texture, moisture, oxygen and the microbial load.

Pseudomonas stutzeri was isolated from Amirthi forest and the molecular characterization of the organism was carried out. Further Ribosomal Database Projecct II classifier was used to confirm the taxonomical and hierarchical details of the isolate which showed it was bacteria 100%. The isolate was subjected to Oligo calculator version 3.2615 to know the molecular weight and GC content of the isolate. The restriction site analysis showed the isolate has 66 restriction sites. Further, the isolate was subjected to ARNold server 16 to predict the srho- independent transcriptional terminators in the nucleic acid sequence and was found to have one terminator at sequence region between 926 and 946 by Erpin and RNA motif algorithm. The isolate gene sequence was transcribed to RNA sequence to identify the secondary structure and was found to have 19 with a minimum free energy of -379.77 kcal/mol. The centroid secondary structure was predicted with a minimum free energy of -272.03 kcal/mol. Further Antibiotic sensitivity test and antimicrobial activity of the organism was done.

Pseudomonas stutzeri strains have been reported to be involved in environmentally important metabolic activities like metal cycling, degradation of xenobiotic compounds. Role of this strain in the environment can be undertaken in future research studies.

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