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ISOLATION AND MOLECULAR CHARACTERIZATION OF FEW BACTERIAL STRAINS FOR THEIR ANTIMICROBIAL POTENTIAL FROM NAGAVALI RIVER BASIN SRIKAKULAM, ANDHRA PRADESH, INDIA

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ABSTRACT

The present investigation encompasses molecular characterization of few antimicrobial agent producing microbes from Nagavali river basin, Andhra Pradesh. Three antimicrobial agent producing bacterial strains were isolated and identified with the help of ribotyping as *Pseudomonas sp.* (BP-1 and BP-2) and *Lysinibacillus sphaericus* (BP-3). The 16s rRNA gene sequences were submitted to NCBI GenBank with accession numbers HM359121, HM359122 and HM359123. The antimicrobial activity of the whole cell protein extracts of all the isolated strains was examined and it was found that *Lysinibacillus sphaericus* (BP-3) exhibited maximum inhibitory effect on the test strains viz: *Eschereshia coli* (MTCC 40), *Staphylococcus aureus* (MTCC87), *Proteus vulgaris* (MTCC426) and *Pseudomonas aeruginosa* (MTCC 424). The present investigation indicated that Nagavali River basin of Srikakulam, Andhra Pradesh is a potential source of antibiotic producing bacteria and can be commercially explored.

INTRODUCTION

The top cultivable antimicrobial agent producers present in soil are the *Actinomycetes* (Pandey *et al.,* 2002) of the genera *Streptomyces* and *Nocardia* (Osborne *et al.,*2000).The Actinomycetes in particular *Streptomyces sp.* are responsible for the production of over 70% of the reported antibiotics and antimicrobial agents like amphotericin, erythromycin, streptomycin, tetracycline, and rifampicin (Omura *et al.,* 2001, Dairi *et al.,*1999 and Lo *et al.,*2002). Majority of these antibiotics are broad spectrum and have different modes of action, e.g. inhibition of protein synthesis by streptomycin and tetracycline and cell membrane damage by amphotericin. These microbes also exhibit vast metabolic versatility. They produce many important enzymes and secondary metabolites with antibacterial, antifungal and antiviral properties as byproducts of various physiological pathways. *Lactococcus lactis* PSY2 is one such bacteria isolated from marine environment which produces an antibacterial agent namely Bacteriocin (Lactococcin PSY2) (Sarika *et al.,* 2011). Recent studies reveal that members of *Bacillus* genus e.g. *Bacillus subtilis* 168 produce non ribosomal oligopeptides with antifungal and antimicrobial properties such as surfactins, inturinics and bacilysin (Oskay *et al.*,2004 and Thomashow *et al.*,1997). The genus *Pseudomonas* is another potent source of antimicrobial agents in soil like pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1- carboxylic acid (PCA), and 2, 4-diacetylphloroglucinol (Phl) (Raaijmakers *et al.,*1997 and Gardener *et al.,* 2000). Even though research is going on but the full potential of these microbes still remain unexplored.

Application of modern molecular methods like high throughput sequencing in the recent years has been providing an insight into the patterns of microbial diversity in different habitats (Roesch *et al.*, 2007, Sahu *et al.,* 2014). Study of these antibiotic producing microbes and standardization of isolation procedure i.e. media composition and downstream processing for human applications is a major thrust area of research in industrial microbiology (Oparina *et al.,*1984). The present investigation is an attempt to screen for such antimicrobial agent producing microbial strains from the Nagavali river basin, Srikakulam district of Andhra Pradesh.

MATERIALS AND METHODS

Soil samples were collected from Nagavali River basin (18º 10′ to 19º 44′ ºN Latitude and 82º 53′ to 84º05′ ºE longitude) of Srikakulam district, Andhra Pradesh, India in sterile bottles and transported to the laboratory. Isolation of bacterial samples was done using the serial dilution plate method of Garthright (1998). After the incubation period colonies that presented antagonism were designated as Antimicrobial Agent Producing Microbes (AAPM's). These AAPMs were sub cultured and purified by repeated streaking on nutrient agar

plates. Three pure bacterial strains BP-1, BP-2 and BP-3 were isolated and were maintained on nutrient agar and stored at 4ºC and at -20ºC as glycerol stocks. All isolates were initially screened by conventional tests *i.*e. Gram staining (Hucker and Conn 1923, 1927), growth and morphologic characteristics on nutrient agar (Cappuccino and Sherman 2007). Biochemical analysis viz. catalase (Smibert and Krieg, 1994), oxidase (Tarrand and Groschel, 1982), motility, indole production, gelatin liquefaction (Conn *et al.,* 1957), oxidative fermentative carbohydrate utilization, decarboxylation of lysine, urease activity were performed by KB002 HiAsssorted Biochemical test kit of Himedia Laboratories. Additional tests included phenylalanine deamination, nitrate reduction, citrate utilization and H_2S production.

The whole cell protein isolation was done as per (Korkoca and Boynukara, 2003) with minor modifications. The antimicrobial ability of the isolates was tested by a modification of the Kirby-Bauer method as described by Boyle *et al*. (1979). The test microorganisms used were *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 424), *Proteus vulgaris* (MTCC 426) and *Staphylococcus aureus* (MTCC 87). A sterile tetracycline antibiotic disc was used as a positive control. Anti biograms were incubated 24-48 hours at 37ºC. Zone of inhibition was measured with a ruler using a cm scale.

Genomic DNA was extracted from the isolates using Chromous Genomic DNA isolation kit (RKT09). Each genomic DNA was used as template for amplification by PCR on Eppendorf Thermal cycler with the aid of 16SrRNA primers (16S Forward Primer: 5'- AGAGTRTGATCMTYGCTWAC-3' 16S Reverse Primer: 5'-GYTAMCTTWTTACGRCT-3') according to (Panda *et al.,* 2013) with minor modifications. Sequence analysis was performed on 1500 bp PCR product using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The three 16S rRNA sequences were aligned and compared with other 16S rRNA genes available in the GenBank by using the NCBI Basic Local alignment search tools BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Three antimicrobial agent producing microbes (AAPMs) were isolated from the Nagavali river basin namely BP-1, BP-2 and BP-3. It was found that BP-3 exhibited comparatively better inhibitory activity than strains BP-1and BP-2 as depicted from Fig. 1. To further identify these strains their biochemical properties were studied followed by ribotyping. Cellular morphology studies and gram staining revealed that BP-1 and BP-2 were rod shaped, gram negative while BP-3 was short rod shaped gram positive bacteria (Table 1). The biochemical test results revealed that the strains BP-1, BP-2 and BP-3 were negative for glucose, adonitol, lactose and sorbitol utilization, nitrate reduction, phenylalanine deamination, H_2S production, Indole and MR-VP test. All the three strains showed positive results for lysine utilization while only BP-2 was positive for arabinose utilization and BP-1 and BP-3 were negative. BP-3 was negative for citrate utilization while BP-1 and BP-2 were positive. BP-3 was positive for ornithine utilization and BP-1 and BP-2 were negative. BP-1 was negative for Urease production while BP-2 and BP-3 were positive (Table 2).

Phylogenetic trees for all the three strains were constructed using Weighbor (Weighted Neighbor Joining: Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) as given in Figures-2, 3 and 4.The genomic DNA of the three strains was isolated followed by amplification of their 16s rRNA gene. Amplicons having band size 1500bp of strains BP-1, BP-2 and BP-3 were sequenced. Nucleotide blast lead to the identification of strains: strain BP-1 shared homology with *Pseudomonas sp*19-28; (GenBank entry: EU167964) (99%), strain BP-2 with *Pseudomonas* sp. c-1-2; (GenBank entry: FJ593900) (99%) and the strain BP-3 with *Lysinibacillus sphaericus* 2317-2; (GenBank entry: DQ286297)(100%).

Table 1: **Colony morphology and Gram reaction of the strains.**

Strain	Colony size(mm)	Colony morphology	Gram reaction	Cellular morphology
BP-1	1	Circular, translucent,Flat with entire margin.	-ve	Rods
BP-2	1	Circular, flat, translucent with entire margin.	-ve	Rods
BP-3	1	Circular, opaque, raisedrhizoid margin	+ve	Short Rods

Table 2: Biochemical tests for the candidate strains

Biochemical tests	BP-1	BP-2	BP-3
Glucose	-ve	-ve	-ve
Adonitol	-ve	-ve	-ve
Lactose	-ve	-ve	-ve
Arabinose	-ve	+ve	-ve
Sorbitol	-ve	-ve	-ve
Citrate utilization	+ve	+ve	-ve
Nitrate reduction	-ve	-ve	-ve
Lysine utilization	+ve	+ve	+ve
Ornithine utilization	-ve	-ve	+ve
Phenylalanine deamination	-ve	-ve	-ve
Urease production	-ve	+ve	+ve
H_2S production	-ve	-ve	-ve
Indole Test	-ve	-ve	-ve
MR-VP Test	-ve	-ve	-ve

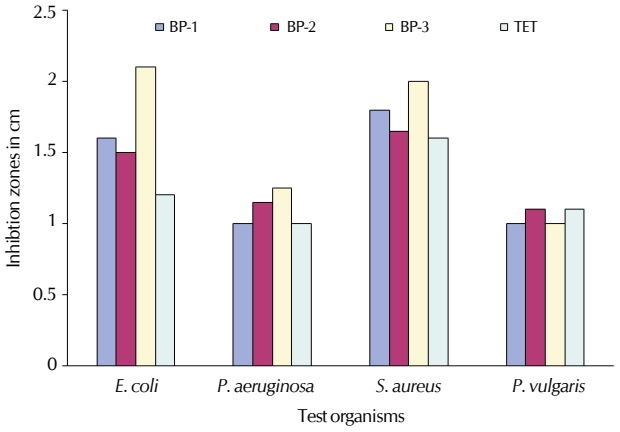

Figure 1: Antimicrobial activity of the candidate strains isolated from Nagavali river basin. Data presented were the mean of three samples

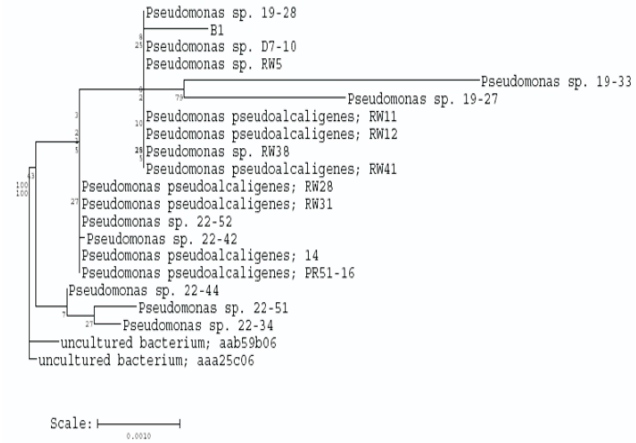

Figure 2: Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-1

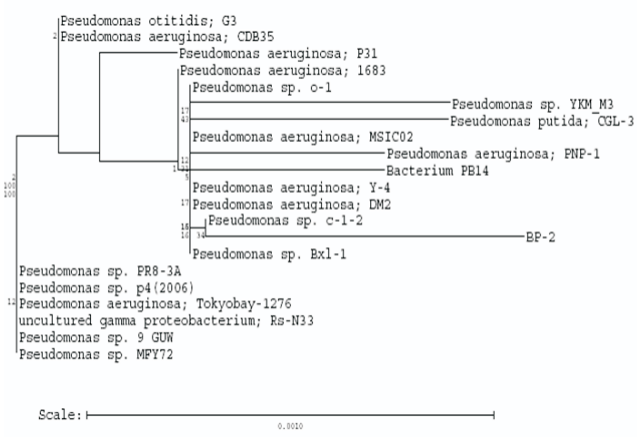

Figure 3: Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-2

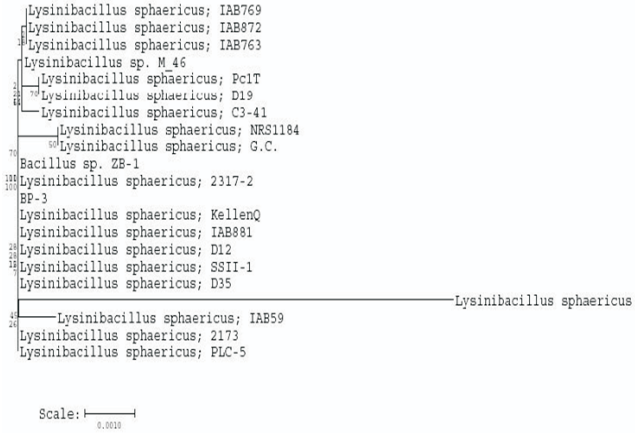

Figure 4: Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-3

The 16SrRNA gene sequences obtained have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI accession numbers (HM359121, HM359122, and HM359123).

DISCUSSION

In the 19th century various disease causing pathogenic microbes were elucidated. In order to fight these microbes antimicrobial chemotherapy was proposed as the possible solution. However with time it became evident that these pathogenic microbes were beginning to develop resistance against the antibiotics. It was imminent that new antimicrobial agents must be characterized so as to continue our fight against infectious diseases and the overall betterment of human health (Saga and Yamaguchi, 2009). Thus researchers are always in search for potential antimicrobial agent producing microbes from various habitats like soil, river bed, hot springs etc. (Kumari *et al.,* 2014, Mashoria *et al.,* 2014).

The present study involved molecular characterization of three antimicrobial agent producing bacterial strains from Nagavali River basin. This particular river is highly polluted as different industrial discharge and agro wastes are continuously dumped in it. Studies confer that such industrially polluted areas are home to various antimicrobial agent producing bacteria (Singh and Mishra, 2013, Kaur *et al.,*2014). Two antimicrobial agent producing *Pseudomonas sp.* were isolated from this site. Investigations by many other researchers show that *Pseudomonas sp.* isolated from different ecological regions are potential antimicrobial agent producers which needs to characterized in detail (Jennifer and Loper, 1995, Tawiah *et al.,* 2012 and Thomashow *et al.,*1990). However the antimicrobial agent production by *Lysinibacillus sp.* isolated from diverse habitats like sea coasts, marine fishes like *Triacanthus strigilifer* and spoiled food samples was a discovery made in the recent years (Ahmad *et al.*, 2014, Abideen and Babuselvam, 2014, Shanmugaraju *et al.*, 2013, and Ahmad and Khan, 2015). The results of antimicrobial activity analysis done by Shanmugaraju *et al.*, (2013) are in agreement with the results obtained in the present investigation that *Lysinibacillus sphaericus* possess higher antimicrobial activity potential as compared to *Pseudomonas sp.* Thus, *Lysinibacillus* sp. isolated from different habitats can be used as a candidate strain for production of potential antibiotics

against food borne bacterial and fungal pathogens at a commercial scale (Xiaomin *et al.,* 2008, Praveen *et al.,* 2012). It is evident that the microbial diversity of Nagavali river basin remains largely unexplored and should be studied using advanced molecular techniques like metagenomics to facilitate isolation and characterization of more anitimicrobial agents for future use in chemotherapeutic purposes.

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