Plasmid profiling of Extremophilic Bacteria isolated from Salt Pan Ecosystem

Mythily S. Assistant Professor, Department of Bio-Sciences, Galgotias University

ABSTRACT

Soil sample was collected from the salt pan of Tuticorin and were isolated through enrichment plating technique. Five morphologically different colonies were selected from 5% salt concentration agar plate. Among the isolated bacteria, one bacterium produced pigmented colony. All the 5 isolates were subjected to gram staining and that most of the isolates were gram negative rod shaped except TSP3 which was gram positive and pleomorphic. Among all the isolates, TSP1, 2, 4 and 5 were gram negative rod shaped. The bacteria were identified as *Halobacterium* sp., *Pseudomonas* sp., *Bacillus* sp., *Escherichia coli, and Micrococcus* sp. Plasmid profile reveals that existence of a12 Kbp size plasmid in soil extract which is not isolated from cultured bacteria. The plasmid profiling among isolates exhibited the presence of different size of plasmid which showed significant difference of non cultrablebacteria.PCR amplicon of 16sr DNA isolated from pigmented bacterialphylogeny revealed that the culturable bacteria belong to *Halobacterium* sp. The present study concludes the salt pan contain some non culturable bacterial strain.

Keywords: Salt tolerance, Plasmid profiling, metagenome, Halobacteriumsp,

INTRODUCTION

Hypersaline habitats favor microbial species having complexity in their composition and nature. Microbes adapted to life at high salt concentrations are highly diverse. Halophiles have been discovered from diverse habitats including the most noxious environments on the planet. Halophilic microorganisms are used for enzyme production of valuable enzymes and bioactive compounds. Halophilic microbial products meet out the demand of biotechnological and pharmaceutical industry due to its diverse functionality (Ramganesh et al 2017). Halophilic bacteria also produce wide range of secondary metabolites and extracellular polysaccharides, enzymes (Enache and Kamekura 2010). Salt pans are large ponds filled with saltwater from the marine or another source and less explored among pharamceutical industries (Kamat and Kerkar2004). The salinity of the water gradually increases as water evaporates until it reaches saturation (26% at 20°C).Ninety-nine per cent of all micro-organisms in almost every environment on earth remain, as yet, uncultured.

Metagenomics is a new and increasingly sophisticated field which is concerned with the direct isolation of DNA from a defined habitat, followed by cloning of the complete genomes of the entire microbial population in that habitat and may provide insight into the isolation and characterization of the principal microbes in these habitats directly from the sample of hypersaline environments (Ventosa et al 2015). The metagenome sequencing has also started to reveal the functional potential of soil communities, including study on genes involved in biogeochemical cycling and tolerance. A current challenge is to go beyond predictive understanding of gene function based on the genome/metagenome to understanding of actual functions carried out by the soil microbiome in situ to construct a DNA library on sequences of interest. Complexity of metagenomic analysis the problems of assembly following shot-gun cloning from complex microbial environments are compounded when even more diverse ecosystems are targeted by this approach(Kowalchuk et al 2007). Soil borne micro-organisms are one of the earth's greatest sources of bio diversity with estimates ranging between 3000 and 11,000 microbial genomes per gram of soil. Moreover, nearly 140 mega bases of sequence taken from Minnesota farmland soil contained <1% of sequence with any overlaps and formed no contigs indicated highly diverse environments is virtually impossible with current technologies(Al-Amoudi et al 2016). The composition of soil directs the diversity of the contained microbiome and its potential to produce bioactive compounds, many studies has been focused on sediment types with unique features characteristic of extreme environments. The objective of this study is to isolate bacteria from salt pan and to perform plasmid profiling.

MATERIALS AND METHODS

Sample collection: The soil sample from salt pan was collected from keela arasadi salt pan, located in Tuticorin in the aseptic box and transported to the laboratory for the further process.

Isolation of bacteria from soil sample: The collecetd soil sample was plated by pour plate technique followed by serial dilution. One ml of $10^7$was plated on LB agar enriched with different salt concentration(1, 2, 3 and 5 %). The Plates were incubated at 35 ±2°C for 24-48 h and colony forming unit was recorded.

Biochemical characterization: Isolated salt tolerant bacterial colonies were subjected to Biochemical tests IMViC, Catalase and Oxidase.

Cell morphology by Gram staining: Thin smear were made in a clean slide and heat fixed before stain process. Staining was done by flooding the smears with crystal violet solution for 1 minute and the smear was washed in a gentle and direct stream of tap water and the slide was flooded with iodine mordant for 1 minute. After washing with distilled water, the smear is decolorized using 95% ethanol until no more colour flows from the smear, then it is rinsed in water and counter stainedfor30 seconds. It was again rinsed with water and air dried and then it was observed microscopically under oil-immersion objective.

Plasmid isolation: 1.5 ml of isolated culture was taken and cells are harvested by centrifugation for 10 minutes and 100 µl of GTE buffer was added to the cell pellet and the cells are suspended in the buffer by gentle mixing then 100µl of lysis solution was added to the content and gently mixed and 150µl of neutralization solution is added to the contents, mixed well and kept at ice cold condition for 10 minutes and then it was centrifuged for 7000 rpm for 10 minutes.The supernatent was transferred to a fresh tube and equal volume of isopropanal were added and it wascentrifuged at 1000 rpm for 10 minutes. The pellets was washed twice with 70% ethanol and suspended in 50µl TE buffer.The plasmid profile was studied by AGE.

Chromosome DNA: 2ml of culture and collected soil filtrate sample was taken and centrifuged at 6000 rpm for 15 minutes and the pellet was resuspended with 600µl of TE buffer. 30µl of 10% SDS, 20µl of lysosyme in the concentration of 10ml ,100µl of Nacl was added and then incubated at room temperature for an hour.Then the mixture was treated with 100µl of CTAB and incubated at 35˚c for 10 minutes. After incubation, the mixture was treated with chloroform and isoamyl alcohol in the ratio of 24:1 and centrifuged at 5000 rpm for 15 minutes. Then the supernatent was collected and mixed with chloroform phenol and isoamyl alcohol in the ratio of 24:25:1 and centrifuged.Anequal volume of isopropanal was added to the supernatent and centrifuged.10 ml of 75% ethanol and 1ml of 3m sodium acetate was added to the precipitate and centrifuged at 5000 rpm for 10 minutes and the pellet was resuspended with TE buffer.

PCR amplification: Inthetubes Hi-chrom PCR mix was added which is pre mixed ready-to-use solution containing Taq DNA polymerase, dnTPs, $MgCl_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Upstream and dowstream primer 12.5µl was(5′-ATYCCGGTTGATCCTAC −3′) and D56 (5′-GYTACCTTGTTACGAC-3′)added and downstream primer 12.5µl was added and DNA sample was added about 5µl and molecular biology grade water for PCR 25µl was added and sample mixed tubes were placed in thermocycler initial denaturation was 94˚c for 10 minutes, denaturation was 94˚C for 50 seconds, annealing was 60˚c for 35 seconds, extension was 75˚C for 35 seconds and final extension was 72˚C for 10 minutes and PCR started to run for about 30 cycles. Amplicon were elctrophoresed and the sequence were BLAST at NCBI.

RESULT AND DISCUSSION

Totally 23X 10^7 colonyforming units (CFU)bacterial colonies were recordedbyenriched isolation procedure. Different shades of red, yellow and white colored colonies were isolated. Data reveals no bacterial colonies were developed among 1 and 2%NaCl. Bacterial colonies areisolated on 3 and 5% NaCl concentration. The observed colonies were sub cultured individually on enriched Nutrient agar. Morphologically five distinct colonies were identified and designated as TSP1to TPS5. Sizes of colonies were irregular rhizoidal, small circular opaque, filamentous, smooth punctiform. Both opaque and translucent colonies were found and some are pigmented (TSP1) in nature. Most of the isolates (60%) were Gram negative rod shaped bacilli. TSP3 were

Gram positive and pleomorphic. Among all the isolates, TSP1, 2, 4 and 5 were Gram negative rod shaped. Among salt tolerant bacterial diversity it was frequently reported that the predominant isolates comes under of Gram negative bacteria among salt pan(Dutra Medeiros et al 2016;Sawale et al 2013).

The biochemical test reveal that most of the isolates showed negative for indole, Voges Proskauertest and few are positive on MR, catalase, oxidase tests (Table 1).Isolate TPS1 positive on catalase, methyl red and citrate where as TPS2 showed positive on catalase only. Isolate designated as TPS3 gave positive on citrate and oxidase only. TPS4 positive on MR and oxidase where as TPS5 is positive on citrate only. Based on biochemical features isolates are identified as *Halobacterium*sp, *Pseudomonas*sp, *Bacillus*sp, *E.coli* and *Micrococcus* sp. Isolates TSP1 and 4 were only MR positive. Methyl red test showed positive by isolated strain which indicates the bacterium undergoes carbohydrate metabolism leading to the formation of acidic end products like organic acid. Different genera of halophiles have evolved multiple mechanisms favoring their survival in the noxious environments. Some microorganisms when propagated in culture medium give distinctive colours of their colonies. Such pigmentation comes in a variety of hues, and often provides important diagnostic clues in laboratories for the identification of isolates. The red to pink pigment produced by .*S.marcescens* was prodigiosin isolated and reported as anticancer agent (Haferburg et al2017). A novel halophilic strain were isolated under aerobic conditions from the sample and characterized morphologically as well as bio chemically by Basaket al(2015). Hyper saline habitats favor microbial species having complexity in their composition and nature. Even with recent advancements in technology in molecular science, the fraction of discovered microorganisms is minor and exploration is still needed (Torsvik and Ovreas 2002). The novel halophilic bacteria, *Salinibacter* sp. isolated from most of the salt pans and most isolates were able to grow from 0.5 to 20% NaCl and at temperatures as low as 0 to 5°C reported by Dobson et al(1991).

Table 1**: Biochemical Results for Halophilic bacterialisolates**

Culture code	**MR**	**Citrate**	**Catalase**	**Oxidase**	**Possible Genus**
TPS1	Positive	Positive	Negative	Positive	*Halobacterium*sp
TSP2	Negative	Negative	Positive	Negative	*Pseudomonas* sp
TSP3	Negative	Positive	Negative	Positive	*Bacillus*sp
TSP4	Positive	Negative	Negative	Positive	*E.coli*
TSP5	Negative	Positive	Negative	Negative	*Micrococcus*sp

Molecular identification of the isolates:

In order to identify the halophilic bacteria, 16SrRNA was amplified using specific primers. A PCR product of around 1400bp was detected in the isolates TSP1.The amplified sequenced was subjected to BLAST analysis for sequence similarity. The similarity matrix for these comparisons is 98% and divergence is 0.006. Thephylogram reveals that the isolate is closely related to *Halobacteriumsalinarum*. The plasmid profiling among isolates and metaplasmidshows that the presence of different size of plasmid compared with 25kbp marker DNA. From direct sampling isolation one band with 12 kbp in size was isolated and characterized. The isolates TSP1 showed presence of threedifferent plasmid ranged between size of 1-5 kbp.TSP2-4 showed single plasmid band with molecular weight 5kbp. No plasmid was isolated in TSP5(Lane 8). The plasmid size of isolated bacterial colonies are1-8Kbp ranges only. Microorganisms from samples collected from the various sites of salt mining sites were highly diverse group of halotolerant and halophilic microorganisms with different morphological characteristics (Lo´pez-Garcıa and Moreira 2008).Recent studies based on 16S rRNA sequence analysis have permitted a determination of the phylogenetic position of most moderately halophilic bacteria and metagenomics a versatile tool permit to find out metabalomic of unculturable bacteria (Wilson and Piel2013).

Plate 1: PCR amplification and Plasmid profile

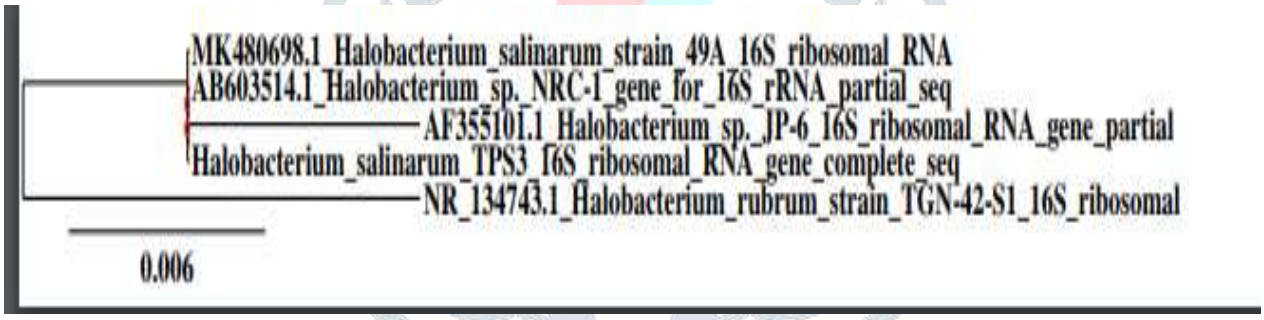

Figure 1. 16srDNArelatedness of pigmented halophile isolate

CONCLUSION

The plasmid profile confirms the presence of different bacterial population are unable to isolate and also shows ecologically important bacteria that survive on salt pan.

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