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Isolation and Molecular Characterization of a Marine Halophile *Pseudoalteromonas piscicida* JCR18 from Marina Coast, Chennai

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Halophiles are extremophiles that survive in environments with very high concentrations of salt. They transcend all domains of life namely archaea, eubacteria and eukaryotes. They are a good source of bioproducts, enzymes, heat shock proteins and antibodies which are well adapted to extreme conditions. A moderate halophilic bacterium *Pseudoalteromonas piscicida* strain JCR18 was isolated from off the Marina coast, Chennai and was characterized by 16S rRNA gene amplification for further studies on its proteolytic activity.

Key words: *Pseudoalteromonas*, marine, 16S rRNA.

Halophiles are microorganisms requiring salt for growth and those that are capable of growth under saline conditions occur in all three categories: Archaea, Bacteria and Eukarya. The term 'halotolerant' is used for non-halophilic microorganisms that are able to grow both in the presence as well as absence of salt. Kushner (1978) classified the variants as slight halophiles for those which live in seawater conditions of 3% (w/v) NaCl, moderate halophiles as those that can grow between 3-15% (w/v) NaCl and extreme halophiles for those that can grow upto 25 % (w/v) NaCl and borderline halophiles for those that grow upto 12% (w/v) NaCl (Margesin and Schinner, 2001). Most of the halophilic species are pigmented red due to the presence of the photosynthetic pigment, bacteriorhodopsin (Patel *et al.,* 2010). Halophiles being extremophiles are a good source of various bioproducts like enzymes, heat shock proteins (HSPs), saline-tolerant proteins, antifreeze proteins and antibodies which are unique in their behavior and activity which was acquired due to their adaptation to these extreme conditions of environment (Van den Burg, 2003). Numerous reports are available on the isolation and characterization of extreme and moderate halophiles for biotechnological applications (Gomes and Steiner, 2004; Schiraldi *et al.,* 2002; Margesin and Schiner, 2001; Van den Burg, 2003).

Of the halophiles, the group gammaproteobacteria comprise highly beneficial and resilient species of marine bacteria capable of surviving in saline environments (Eilers *et al.,* 2000). Among the γ-proteobacteria, the genus *Pseudoalteromonas* is worth noticing. This genus has several marine species which are able to produce a variety of bioactive compounds including extracellular enzymes. This ability enables them to compete for nutrients and colonize surfaces, live in association with other organisms and assimilate complex organic compounds (Holmström and Kjelleberg, 1999). *Pseudoalteromonas* sp is normally grouped under extremophiles, namely as psychrophilic and/or psychrotrophic, from which extracellular cold active serine- proteases have been identified (Wang *et al.,* 2008; Violot *et al.,* 2003; Collins *et al.,* 2002; Sanchez – Porro *et al.,* 2003). The present study was undertaken with the objective of isolating a novel alkaline protease from a halophilic strain from the marine environment .

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MATERIALS AND METHODS

Collection of samples

Seawater was collected in sterile plastic containers from different sampling points off the Marina Coast, Chennai. The sample was transported to the lab and processed immediately.

Screening of isolates

The samples were subjected to serial dilution using sterile seawater and the dilutions from 10^{-1} to 10^{-4} were spread plated on casein agar plates. The plates were incubated at 37°C for 48 h.

Isolation of a proteolytic strain

The colonies that showed different morphological characteristics were picked and quadrant streaked onto a marine-specific medium, namely YTSS agar(Yeast extract, 0.5%; Peptone, 0.25%; Seasalts, 2%; pH 7.5) to get single isolated colonies. The streaked plates were incubated at 37° C for 48 h.

Molecular Characterization of the organism

Isolation of genomic DNA

1.5 ml of the bacterial culture was transferred to a micro centrifuge tube and spun at 10,000 rpm for 2 min at 4°C. The supernatant was discarded and the pellet was resuspended in 467 µl of TE buffer by repeated pipetting. 30 µl of 10 % SDS and 3 µl of 20 mg/ml of Proteinase K was added to the sample and incubated for 1 h at 37°C. Equal volumes of Phenol: Chloroform (24:1) was added and mixed gently by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 min at 4°C. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 min at 4° C. The upper aqueous phase was transferred to a new tube and $1/10^{th}$ volume of 3 M sodium acetate was added. Double the volume of 95% ice-cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4°C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. The 70 % ethanol was discarded and the pellet was air-dried. The DNA was then suspended in TE buffer and run on 0.8% agarose gel.

DNA quantification

The isolated DNA was quantified using UV-spectrophotometer and the ratio of absorbance at 260/280 nm was calculated.The concentration of DNA was obtained using the formula: A_{260} x 50µg/ml x dilution factor.

16S rRNA gene amplification and DNA sequencing

The amplification of the 16S rRNA was carried out using the PCR universal primers Bac 16S-F: 5'AGAGTTGATCATGGCTCAG3' and Bac 16S-R: 5'TACGGCTACCTTGTTACGACTT3'. The PCR was carried out in a thermocycler of 25 µl reaction volume with final concentration of 1X 2mM $MgCl_2$, 0.275 mM dNTPs, 0.355µM of each primer, 100 ng/µl of template DNA, and 2.5 U of Taq polymerase. Negative controls without template were run simultaneously to rule out contamination. The amplified products were resolved by electrophoresis in a 1.5 % agarose gel. Sequencing of PCR products were done using a multi-capillary DNA sequencer. A comparison of the test strain with the non-redundant collection (GenBank, DDBJ, EMBL& PDB) was performed using BLAST. The best match to the query sequence was obtained and based on the maximum score a phylogenetic tree was constructed.

RESULTS AND DISCUSSION

Isolation and characterization of the organism

A total of 14 isolates were obtained following the initial screening by serial dilution. The colonies were detected only in plates with dilutions from 10^{-1} to 10^{-2}. The isolates thus obtained were quadrant streaked onto YTSS media to get isolated colonies. A single isolate showing an orange colour was chosen based on its proteolytic activity for further characterization (Fig 1).

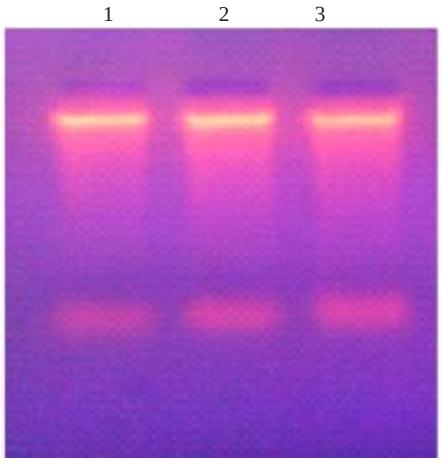
Isolation and quantification of genomic DNA

The genomic DNA was isolated (Fig 2) and the purity of the DNA was assessed using spectrophotometric method and was found to be 1.81. This value was consistent with the allowed values of A_{260}:A_{280} = 1.8 for pure DNA; 1.7 – 1.9 for fairly pure DNA (acceptable ratio for PCR) and less than 1.8 indicating the presence of proteins and greater than 1.8 indicating the presence of organic solvent.

16S rRNA gene amplification

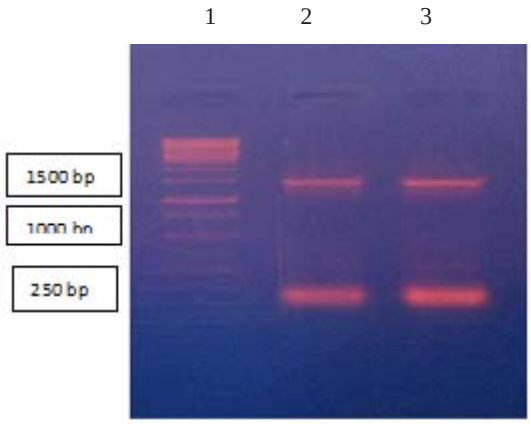
The amplification of the 16 S rDNA using

Fig. 1. *Pseudoalteromonas piscicida* on YTSS agar plate



Lanes 1, 2, 3: Genomic DNA of *P.piscicida*

Fig. 2. Genomic DNA isolation of 16S rRNA gene amplification



LANE 1: 1 kb DNA LADDER
LANE 2, 3: Amplified 16S rRNA gene

Fig. 3. PCR Amplification of 16S rRNA gene

PCR and the sequencing of the amplified product was carried out. The PCR product obtained was nearly 1500 bp (Fig 3). The sequencing carried out read a total of 481 bp (Fig 4). The sequence obtained was subjected to multiple sequence alignments using the 16S rDNA sequences of homologous strains. The 16S rDNA sequences of *Pseudoalteromonas piscicida* was obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and the hypervariable multiple sequence alignments were done with the tool, CLUSTAL W version 1.82. Using the obtained results, a phylogenetic tree was constructed by the neighbor-joining method. The multiple sequence alignments and the resulting phylogenetic tree drafted showed that the JCR 18 strain was closely related to

SEQUENCE:

>OciSeq_JB16_Bac16sf_014.ab1

AAAATGGCGGGGGGCACTAACCCTGGCCAAGTTCGAGCGGTAACATTTTTTAGCTTGCTAGAAGATGACGAGCGGCGGAC
GGGTGATTAATGCTTGGGAACTGCCTTGAGGTGGGGGACAACCATTGGAAACGATGGCTAATACCGCATAATGTCTACGG
ACCAAAGGGGGCTTCGGCTCTCGCCTTTAGATTGGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGC
GACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT
TTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCTAGCTGTGACGTTACTGACAAAAGAAGCACCGGCTAACTCCGTG
CCAGC

Fig. 4. Sequence and Alignment results for the Isolate

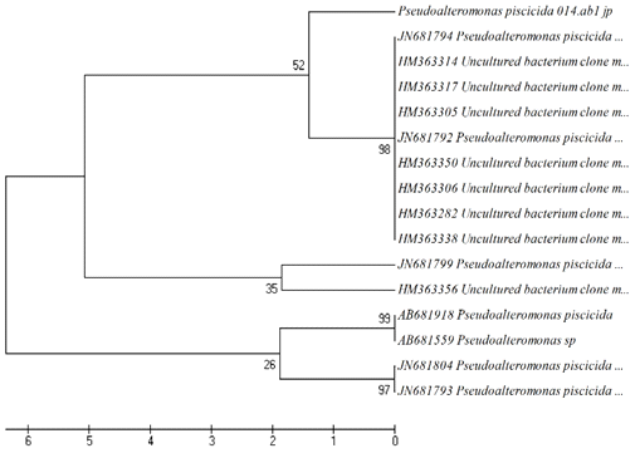


Fig. 5. Phylogenetic maximum-likelihood tree based on 16S rRNA gene sequences showing the relationship between *P.piscicida* active strain and related members of the genus *Pseudoalteromonas*

Pseudoalteromonas piscicida with 97 % homology categorizing it as a new strain of that genus.

From a biotechnological perspective, moderately halophilic bacteria pose an excellent model on the extent of adaptation to the ever-changing extracellular osmolality that exists around them. The trademark characteristic of most *Pseudoalteromonas* species is the production of characteristic biofilms. This may not be a welcome feature in industrial applications when done in huge bioreactors as this may present serious cleaning problems. The formation of these biofilms can be minimized by targeted knocking out of the genes concerned or by performing random or targeted mutagenesis. Alternatively, a simpler option would be to isolate and clone the gene of interest into suitable heterologous systems.

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