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MODERATELY HALOPHILIC BACTERIA FROM SOLAR SALT PANS OF RIBANDER, GOA: A COMPARATIVE STUDY

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ABSTRACT

Hypersaline environment of solar Salt pans are habitats of robust and diverse halophilic microorganisms .The present study makes an effort to isolate and compare the variation and species specific characteristic of the bacteria , isolated from the solar saltpans of Ribander, Goa.The moderate halophiles are studied considering their potential enzymatic applications. Comparative analysis yield valuable information of the extreme ecosystems .Besides, solar saltpans are endangered ecosystems, harbouring potential microbes. This study makes an effort to create awareness for conservation of this unique ecosystem. Solar saltpan water was used as a source for isolation after enrichment of the culture . Morphological , Biochemical, Antibiotic sensitivity, physiological and enzymatic(Protease)characteristics of the isolates were compared. The 16S rRNA sequencing and G+C analysis were used to identify and differentiate halophilic bacterial strains .The 2 moderately halophilic bacteria isolated were phylogenetically similar to *Alkalibacillus HSD20* and *Virgibacillus panthotheticus*, speceis .The isolated bacteria displayed promising potential for protease production and varied species specific characters .The Ribander solar saltpans are a treasure of halophilic bacteria with a sparkling future. The study encourages further exploration of these extremophiles, their haloenzymes and saline habitats.

Key words: Solar salterns, Moderately halophilic bacteria, Protease, species specificity

[1]INTRODUCTION

Hypersaline environments are widely distributed on the earth's continent where they exist either as natural water bodies such as permanent saline lakes, ephemeral saltpans and salt marshes,or as artificial solar salterns [1].Microorganisms that thrive in these environments have been broadly classified into halophilic microorganism (that is, require salt for their viability) and halotolerant

microorganisms which are able to grow in the absence as well as in the presence of NaCl. Halophiles can be further divided into slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles optimal growth at 3 - 15% (w/v) salt and extreme halophiles that grow optimally at 25% (w/v)salt [2]. Halophilic and extremely halotolerant microorganisms are present in each of the three domains of life: archaea, bacteria and eukarya .The domain bacteria typically contains many types of halophilic and halotolerant microorganisms that spread over a large number of phylogenetic subgroups. Most of these are moderate rather than extreme halophiles [3]. Scientific interest in extremophilic microorganisms, especially hyperthermophiles, thermoacidophiles, archaebacterial anaerobes, and hyperhalophiles, has recently increased .One reason for this interest is the need to understand the biochemical mechanisms involved under extreme conditions because of possible biotechnological use of enzymes and molecules from such organisms. Among extremophilic bacteria, thermophiles are the most intensively studied. In contrast, less attention has been paid to halophilic microorganisms [4]. Halophilic and halotolerant bacteria secrete a wide range of hydrolytic enzymes into their surrounding environment. Several of these enzymes which include amylases, proteases, xylanases and cellulases display polyextremophilic properties. They are generally haloalkaliphilic and thermotolerant which renders them amenable to an array of industrial processes, normally performed at extreme conditions of temperature and pH[5]. Multi-pond solar salterns, which are used worldwide for salt production along tropical and subtropical coastal areas, present an environment with increasing salt concentrations, from seawater to NaCl saturation. Characteristic salt-adapted microbial communities are found along the salinity gradient[6] . Solar saltpans are found worldwide. Because of the increasing salinity of the environment, solar salterns are considered extreme environments with very restricted biology [7]. It is surmised that marine environments like solarsaltern may yield newer strains which may prove to be a rich resource of newer bioactive metabolites [8]. However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are relatively few reports in the literature about their production and characterization [9].As industrial process conditions are harsh, there are demands for biocatalysts that can withstand the process conditions. The majority of the enzymes used to date originate from mesophilic organisms and, despite their many advantages, the application of these enzymes is restricted due to their limited stability at the extremes oftemperature, pH and ionic strength. On the other hand, extremophiles are a potent source of extremozymes,which show outmost stability under extreme conditions. Extremozymes have great economic potential in many industrial processes(*e.g.* agriculture, food, feed and drinks, detergents, textile,leather, pulp and paper[10]. Out of the vast pool of Extremozymes, halophilic proteases are the most widely exploited enzymes in the processing of food, leather and detergents [11].Proteases are hydrolytic enzymes, which can degrade different protein sources, so find potential application for waste treatment, bioremediation, wool quality improvement, meat tenderization, in food, leather, pharmaceutical and detergent industries[12].

Goa’s traditional salt industry is said to have been a major supplier of salt to the country and an exporter to some foreign countries

since the 10th century.Sparked by the availability of microbial diversity data in these salterns and the potentials of moderately halophilic bacteria , an effort is made to study and compare the species . Research aims at making an effort to conserve these unique microflora for biotechnology research and industrial use.

[II] MATERIALS AND METHODS

The traditional salt industry has been existing in Goa since 500 A.D. Goan salt was considered to be of the best quality and was exported to several African and Arabian countries during the post Medieval period. Goa has over 200 salt pans and produces 35,000 metric tones of salt annually[13].

2.1Sampling site : The study site was the Ribandar saltpans (15 ^{0} 30.166 N and 73 ^{0} 51.245 E) Goa, India and is situated along the Mandovi estuary in Tiswadi taluka. The total area of this saltpan is 12,329.12m^{2} (Figure 1) The site is subjected to heavy annual rains(125 cm) during the monsoons. The prevailing climate is characterized by three distinct seasonsviz. Pre-monsoon: from February to May; Monsoons: from June to September and PostMonsoon: from October to January. The climate on an average is generally warm and humid,fluctuating from a minimum of 20^{0}C in the month of December to 42 ^{0}C in May.

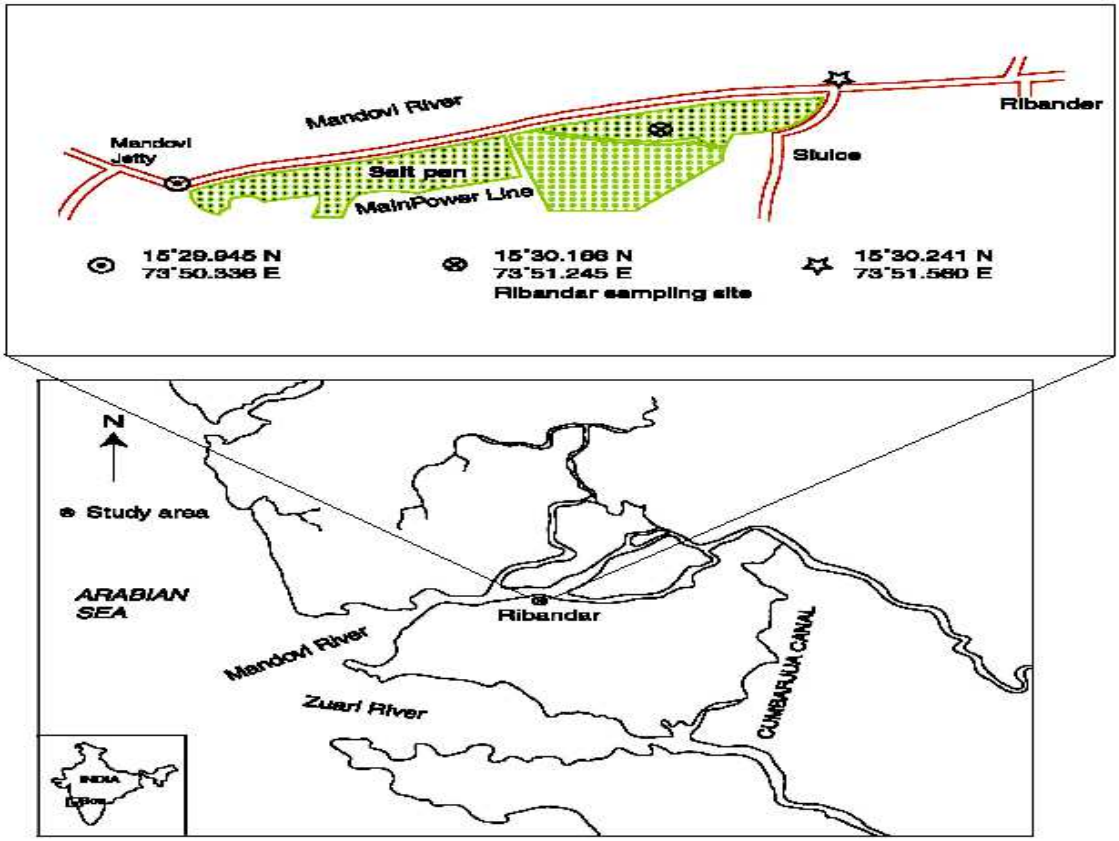

Figure-1 Study area map: Shaded area depicts the location of Ribander salterns

2.2Sample Collection -Overlaying saltpan water was collected in sterile glass bottles and stored at 4^{o}C. Samples were analysed within a 24 hours of collection.The sample collection was performed as specified in IS:162-1981[14].

2.3Enrichment and isolation of halophiles - Halophiles were enriched in halophilic broth(Abrram gibbons media) containing (gm/lit): Casein acid hydrolysate-5, Yeast extract- 10, Proteasepeptone-5, Trisodium citrate- 3, Potassiumchloride- 2, Magnesium sulfate- 20, Fe SO4-0.05 ,Sodiumchloride-150(15%), pH- 7.0 for 10-12 days [15]. From enriched 15 % NaCl (w/v) halophilic broth, cultures were streaked on respective agar media by four sector method for the purpose of isolation into pure culture[16].

2.4Characterization of organisms -Gram staining ,Cell morphology and motility were examined on freshly prepared wet mounts by light microscopy. Colony pigmentation was recorded on agar plates after 10 days of growth was performed [16]. The cultures were purified on the same salt concentration and medium from which they were isolated. All tests were performed at 15 % salt concentration . Cultures were tested for biochemical characteristics and carbohydrate utilization[17]. The isolates were identified according to [24,25].

2.5 Physiological characterization

Salt concentration optimum - The isolates were screened for their salt tolerance level by growing them on to nutrient broth tubes with concentrations of salt (NaCl) ranging from 0% to 25% and subsequently measuring their optical density[16].pH was maintained at 7.0 and temperature at 37^0C.

pH optimum - Nutrient broth was prepared at different pH range to detect the tolerance level of the strains and subsequently measuring their optical density at each pH **[**16].Salt concentration was maintained at 15% and temperature at 37^oC

Temperature optimum - All the isolates were tested for the temperature tolerance by subjecting them to different temperature(10. , 25 , 37, 40, 45, 50, 55, 60 ^{o} C) in nutrient broth and subsequently measuring their optical density[16].Salt concentration was maintained at 15% and pH at 7.0.

2.6 Antibiotic susceptibility test

Halophilic bacteria are suseptable to antibiotics unlike the halophilic archaea . This character also differentiates the halophilic bacteria and archaea.Test for antibiotic sensitivity was performed by the Kirby-Bauer disc method[16].The antibiotics tested were Penicillin, Gentamycin,Streptomycin and chloramphenicol.All in a concentration of 10mcg/disc(Himedia)

2.6 Genotypic analysis

A]16srRNA sequencing

The 16S rRNA genes were isolated from the cells PCR amplifications of the 16S rRNA gene, from the purified genomic DNAs, were carried out using the primer sets 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´).The purified PCR product was done using BigDye Chemistry, and performed as per the manufacturer's protocols (Applied Biosystems 3730xl DNA Analyzer) The closest known relatives of the sequenced organisms were determined by sequence database searches. The purified extension products were separated in the ABI 3730xl DNA Analyzer by capillary electrophoresis. Sequence data analysis was done using ChromasPro and Sequencing Analysis software.

B] G+C analysis:

The mol%GC content of DNA should be part of the description of the type strain of the type species of a new genus [18].1-2 g wet biomass (centrifugal pellet) are needed. The biomass can be sent either in frozen state on dry ice or suspended in isopropanol/water (1:1, v/v) at ambient temperature. Lyophilized cells in a corresponding amount can also be sent. However, the use of lyophilized cells may result in a lower DNA yield.Cells are

disrupted by French pressing and DNA is purified on hydroxyapatit according to Cashion et al. [19]. The DNA is hydrolyzed with P1 nuclease and the nucleotides are dephosphorylized with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleosides are analyzed by HPLC [20]. Lambda-DNA and three DNAs with published genome sequences representing a G+C range of 43-72 mol% are used as standards. G+C values are calculated from the ratio of deoxyguanosine and thymidine according to the method of Mesbah et al. [21].

2.7Screening of isolates for extracellular enzyme production :Standard bacteriological tests for extracellular enzyme production were performed for protease enzyme . Cultures were spotted on skimmed milk-agar plates and incubated at 37°C for 5-7 days. Presence of zone of clearance surrounding the culture spot was taken as a measure of protease production[22].

Protease production by the isolates:The isolates were further analysed for the amount of protease produced by them. The protease production medium was similar to the growth medium except that the yeast extract (10 g/l) was replaced by same quantity of soybean meal. The isolates were grown in the production medium at 37°Cfor 96 h under shaking conditions (200 rpm) and the enzyme activity was estimated at interval of 24 h.

Enzyme activity

The culture was centrifuged at 10,000 × g for 20 min (4ºC) and the culture supernatant was used as a source of protease. The caseinolytic activity was estimated using modified Anson's method. The assay was performed at 37°C using 1% casein as a substrate. The substrate was prepared in 50 mM Tris-HCl buffer (pH 7.2) containing 2M NaCl. The concentration of NaCl was set at 2 M in the assay system as casein is known to lose its original conformation at higher NaCl concentrations [23]. One millilitre of casein buffer solution was preincubated at 37°C for 5 min.The reaction was initiated by adding 1 ml of enzyme. After incubation for 10 min at37°C, the reaction was terminated by adding 3 ml of 5% (w/v) trichloroacetic acid(TCA). For blank tubes TCA was added before enzyme. The content was centrifuged and the absorbance of supernatant was measured at 280 nm. One unit of enzymeactivity was defined as 1 µg of tyrosine released per minute.

RESULT AND DISCUSSION

Two halophilic species belonging to the genus Alkalibacillus [A1] and Virgibacillus [V1] were isolated from the solar salterns of Ribander**.** Both the cultures belonged to phylumXIII firmicutes , class bacilli and family bacillaceae. A1 belonged to the genus II. *Alkalibacillus* and V1 belonged to the Genus XIX. *Virgibacillus* . The isolates displayed varied species specific characters. The characteristic features of the 2 species are compared in Table 1. The isolates displayed same morphology and presence of spores. Yellow pigment production was seen in A1 isolate, which appeared at salt concentration of 20% , however at 5%-15 % the colonies displayed creamish colour.This characteristic is similar to the species *Alkalibacillus haloalkaliphilus*.V1 isolate displayed creamish grey colour.A1 was an alkaliphilic halophile ,with an pH optimum of 9-10. Several biochemical tests were performed to differentiate the isolates. Certain biochemical properties of microorganisms also relate to their potential in Biotechnology. Both the isolates were sensitive to the antibiotics(Table 1) ,a criterion to differentiate the bacteria from archaea. The physiological characterization revealed

diverse values for both the species.The bacteria isolated had an optimum growth value in the range of 5-15% NaCl, and thus can be classified as moderately halophilic bacteria accoding to Ventosa et al 1998[2].

Fritze [2002] recommended that phenotypic characterization results should not be directly compared without full background knowledge of the precise conditions used for a particular test. This can be particularly true for the group of Gram-positive endospore-forming bacteria that were formerly classified as the genus *Bacillus* but have now been reclassified based upon phylogenetic diversity into separate lineages [26].Therefore, we also used 16S rDNA sequence analysis to ensure the accurate taxonomic position of the halophilic strains reported in this study[Table 2]. G+C analysis also revealed the diversity and ultimately the specificity of species at the genotypic level. On the basis of the phenotypic characteristics and the comparison of partial 16S rRNA gene sequences, the isolates were identified, and were phylogenetically similar to *Alkalibacillus HS20D*[A1] and *Virgibacillus Panthotheticus* [V1], which was well supported by the phenotypic, biochemical, and physiological characteristics. Alkaliphilic micro-organisms, mainly those belonging to the genus *Bacillus*, are of considerable industrial interest, particularly for the production of enzymes (proteases, xylanases, glycosidases,Alkaliphilic micro-organisms are also often halophilic. There are a large number of recognized species of the genus *Bacillus* and related genera that could be described as moderately halophilic or halotolerant, some of which are also alkaliphilic [30].Alkaliphilic halophilic, Gram-positive, spore-forming motile Bacillus-like strain YIM 012, was isolated from one of the hypersaline soil samples collected in Xin-jiang province, China. Its optimum growth occurred at pH 7.0-8.0[28]. Similarly a Gram-positive, strictly aerobic and moderately halophilic bacterial strain was obtained from the sea water sample collected from International Sea Water Bathing Place in Weihai, a city on the shore of the Yellow Sea. [29] *Alkalibacillus filiformis* sp.was., isolated from a mineral pool in Campania, Italy[31].Recently Virgibacillus albus sp. nov., a novel moderately halophilic bacterium isolated from Lop Nur salt lake in Xinjiang province, China.[32]*Virgibacillus salarius* sp. nov., a halophilic bacterium isolated from a Saharan salt lake in 2008[33], and shared similarity with our isolate V1.

Screening of isolates for extracellular protease production-

Most halophilic bacteria are known to secrete proteases into the external environment that can have unique properties for application to biotechnology. A1 showed significant zone of clearance , an indication of protease production. V1 showed comparatively less zone of clearance on skimmed milk agar plates. These results were indicative of*Alkalibacillus* species as potential protease producers. The results are supported byFrizte 1996[30]. Hence can be investigated for further quantification of protease production.Protease production by these organisms in soybean meal-based production medium was studied as a function of time Maximum protease production 14.24 ± 1.55 U/ml was observed from A1 at 72 h. Protease production by V1 was 12.76 ± 0.09 U/ml which was comparatively less and was also achieved at 72 h of growth. These results were suggestive of A1 as potential isolate for production of protease.

CONCLUSION

Comparative studies of micro flora in the extreme environments results in better

understanding of the ecosystem and can benefit in designing the applications.The Ribander saltern habitat supports diverse bacterial species . This diversity enriches the ecosystem and it is inducing to study its genetic complexity and correlate it with morphological complexity in the aspects of exploring biopotentials from these marine species. Hypersaline environments represent a valuable source of extracellular hydrolytic enzymes with potential in different economical fields. This literature survey indicates that both the moderately halophilic bacteria have a broader catabolic versatility and capability than previously thought. The research makes a sincere effort to create awareness for protecting this endangered ecosystem. As they are endangered coastal Salinas due to pollution and enchroacment. The microbial diversity can prove to be a valuable future resource in various industrial and biotechnological processes. Such microbes can also be used as a source of gene(s) that can increase salt tolerance indifferent crop species through genetic transformation.

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Tables:

Table 1- Morphological, Biochemical, Enzymatic and genotypic characteristics of the two halophilic species .

Tests	A1	V1
Gram's Stain	Positive	Positive
Endospore staining	+	+
Cell shape	Long Rods	Rod
Pigments	Yellow	Creamish grey
H2S Production	-	+[weak]
Nitrate reduction	_	+
Motility	+	+
Catalase	+	+
Oxidase	-	+
NaCl optimum	10-15%	5- 10%
pH optimum	8-9.0	7.0
Temperature optimum	30-37^{o}C	30-37^{o}C
L-Arabinose	_	+
D-Mannitol	_	-

D-Lactose	**_**	**+**
D-Trehalose	**_**	**+**
D-Glucose	**+**	**+**
D-Galactose	**_**	**_**
D-Fructose	**_**	**+**
G+C	**38 %**	**37%**
Antibioticsensitivity(Penicillin, Gentamycin,Strptomycin and chloramphenicol.)	**s**	**s**

Key-+[positive results/acid formation]
_[Negative results]
S[Sensitive]

Table 2. Identification of isolated halophilic strains based on 16S rRNA gene sequence and their accession numbers (BLAST similarity search results)

Strain ID	Strain name / Genus	Number of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related taxa	Sequence similarity (%) of 16S rRNA gene
A1	Alkalibacillus sp	1463	EF517966.1	Alkalibacillus sp HS20D	98%
V1	Virgibacillus sp	1533	JN791392.1	Virgibacillus pantothenticus	88%