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Halophiles and Halozymes from Tannery Effluent as well as Food Grade Table Salt Crystals

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Halophiles are extremophiles that grow in hypersaline niches. They are now considered as a source of valuable industrial enzymes besides novel genes and other bioactive biomolecules. Herein, we report the occurrence of halophilic bacteria in tannery effluent and in food grade salt crystals used as a food supplement. The bacterial strains were isolated using Zobell's agar medium with 15% NaCl and identified based on morphological, biochemical, physiological characteristics and partial gene sequences of 16S rRNA. Interestingly, species of *Oceanobacillus*, *Staphylococcus and Salimicrobium* were isolated from tannery effluent, whereas food grade salt crystals harbored species of *Halomonas elongata* and *Chromohalobacter salexigens.* It was observed that *Halomonas elongata* was dominant among the bacteria associated with the food grade salt crystals. The two samples under study showed diverse halophilic bacterial flora. The phylogentic tree constructed showed the inter-relationship among the halophiles. On further analysis *Oceanobacillus* sp not only showed ability to produce lipase and protease, but also could grow in Zobell's medium containing upto 30% NaCl. Lipase, amylase and protease were produced by *H. elongata* and *Chromohalobacter salexigens* sp., in lesser amounts.

Key words: Extremophiles, Halophiles, Halozymes, Tannery, Salt crystal.

Extremophiles are capable of growth and reproduction in harsh environments and the bacteria adapted to grow in hypersaline niches are termed halophiles [1]. These are salt loving bacteria capable of growing at high salt concentrations. The enzymes produced by extremophiles are endowed with molecular mechanisms of adaptation to extreme physical and chemical conditions[2]. Hence there is great interest in extremozymes as they can tolerate harsh conditions in a variety of industrial processes and have potential biotechnological applications [3]. Halozymes are enzymes produced by halophiles which have optimum specific activity at high salt concentrations [4] and can function under conditions in which most proteins get denatured or precipitated[5]. These enzymes have adapted to high concentration of NaCl in the surrounding environment by acquiring relatively large number of negatively charged amino acid residues on their surfaces to prevent precipitation [6]. An extracellular protease produced by *Halobacterium halobium* was exploited for efficient peptide synthesis in water/N'-N'-dimethylformamide[7]. High substrate solubility in presence of solvent is useful in synthetic reactions catalysed by enzymes from halophiles [7]. A β-galactosidase purified from a halotolerant psychrophile *Planococcus* sp. was reported to remain active at high salt

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concentrations, making it a possible reporter enzyme for halotolerant and halophilic organisms [9].

In tanneries, preservation of skin and hides uses salt at concentrations ranging from 40-50%. NaCl used during tanning acts as dehydrating agent and also has antibacterial activity. However, the tannery effluent was observed to selectively harbor halophiles which can tolerate high salinity [10]. Further bacteriorhodopsin from halophiles has found applications in optical switches and photocurrent generators in bioelectronics [11]. Thus halophiles and biological products derived from them have been recognized to have wide range of applications and hence investigations on harnessing halophiles have drawn the attention of scientists and technologists. In this context, the present communication reports on halophilic bacteria from tannery effluents as well as food grade table salt crystals and their haloenzyme profile.

MATERIALS AND METHODS

Samples

Tannery effluent and food grade table salt crystals were used for isolation of halophiles. The effluent was procured from tannery located in Erode district, Tamil Nadu, India. Food grade table salt crystals available in local market was purchased and used as samples.

Medium

Zobell's agar (ZA) medium (HI Media, Mumbai, India) was used for isolation and cultivation of halophilic bacteria. The ZA medium was supplemented such that the final NaCl concentration was 15% and suitable for isolation of halophilic bacteria.

Plating procedures

An aliquot of 100µl of the tannery effluent was spread plated on previously prepared Zobell's agar medium. In the case of table salt crystals, a solution of 1 gram of salt crystals in 1 ml of sterile distilled water was prepared and 100µl of the same was spread plated on the prepared Zobell's agar medium. The inoculated plates were incubated at 42°C for 7 days and were observed for appearance of bacterial colonies. Colonies which appeared on the surface of agar medium were isolated based on their colony morphology and subcultured in fresh sterile Zobell's agar medium. All isolates obtained were purified by streak plate method and stock cultures were prepared and maintained as agar slope cultures under sterile liquid paraffin. Glycerol stocks were also prepared and stored at -70°C for future studies.

Identification of bacteria

Gram staining was performed according to the method described by Dussault [12]. Biochemical characterization of the isolates was done according to Cheesebrough [13]. All the biochemical media used were supplemented with 15% NaCl. The tests included IMViC, production of Hydrogen sulphide (H_2S), urease, oxidase, catalase, coagulase, fermentation of glucose, sucrose, mannitol, lactose, glycerol, sorbitol, starch hydrolysis (amylase), casein hydrolysis (protease) and tributyrin utilization (lipase). Amylase production was checked on ZA supplemented with starch agar plates. Addition of iodine after growth of bacteria produced a colourless halo around colonies that produced amylase. Lipase production was indicated by a zone around the colonies (grown in tributyrin agar medium (HI India) supplemented with 15% NaCl) which produced lipase. Protease production was indicated by zones around colonies grown in casein agar plates supplemented with 15% NaCl. Cultures which showed enzyme production were retested for confirmation in Zobell's broth supplemented with 15% NaCl and respective substrate at different pH and at 37°C and 42°C and at different NaCl concentrations.

The partial sequences of 16S rRNA gene of the bacterial isolates were amplified by colony PCR [14] on a thermocycler MJ Mini (Biorad, USA) using halophilic bacterial primers[15]. Sequencing of the amplified 16S rRNA gene was by ABI 3730XL, according to Sanger's dideoxy method. The identity of the isolates was confirmed by nucleotide BLAST of the obtained 16S rDNA sequences. Sequences were submitted to NCBI. A Phylogenetic tree was constructed using MEGA4 [16] software and the evolutionary history was inferred using Maximum Parsimony method.

RESULTS AND DISCUSSION

Six isolates obtained from tannery effluent were designated as BTMT01, BTMT02,

BTMT03, BTMT04, BTMT08 & BTMT10. All the isolates were Gram positive. Among the isolates, BTMT01 and BTMT03 were observed as bacilli. Isolates BMT02 and BTMT04 were found to be cocci arranged in grape like cluster, while isolates BTMT08 and BTMT10 were noted as cocci. Isolates BTMT01 and BTMT03 were observed to produce large dry colonies on agar, compared to isolates BMT02 and BTMT04 which produced small circular cream coloured colonies. Whereas, isolates BTMT08 and BTMT10 produced colonies with an orange hue. Only five isolates could be obtained from food grade table salt crystals, all of them were Gram negative bacilli. They were designated as BTMT05, BTMT06, BTMT11, BTMT12 and BTMT13. Isolates BTMT05, BTMT06, BTMT12 and BTMT13 formed large opaque, convex, and mucoid colonies while isolate BTMT11 produced small translucent colonies. Biochemical tests were also performed before molecular identification of bacteria , results are shown in Table 1.

Among the isolates tested for different hydrolytic enzyme production, isolates *Oceanobacillus* sp. BTMT01 and *Oceanobacillus* sp., BTMT03 produced large quantities of lipase and protease. Further reconfirmation experiments also indicated considerable production of lipase and protease by both the isolates of *Oceanobacillus* sp. at 37°C and 42°C; and at pH 7.2. *Oceanobacillus* sp was found to grow in Zobell's medium containing upto 30% NaCl. All the isolates of *H. elongata* (BTMT05, BTMT06, BTMT12, BTMT13) and *Chromohalobacter salexigens* (BTMT11) isolated from food grade table salts were also observed to produce lipase, amylase and protease. However, the enzyme production was at a very low level.

The 16S rRNA gene of the isolates was amplified by PCR and identity was confirmed by sequencing and conducting NCBI nucleotide blast of the sequences. From the results obtained for identification it was inferred that species of *Oceanobacillus*, *Staphylococcus and Salimicrobium* were associated with tannery effluent while food grade table salt crystals harbored species of *Halomonas* and *Chromohalobacter salexigens*. The details of these bacteria along with their NCBI accession numbers are presented in Table 2. Further it was observed that *Halomonas elongata* was dominant among the bacteria associated with the food grade table salt crystals. It was noted that both the samples of effluent and food grade table salt crystals harbor different halophilic bacterial flora indicating influence of the their source.

The evolutionary history was inferred using the Maximum Parsimony (MP) method [17]. Tree #12 out of 15 most parsimonious trees (length = 375) is shown. The consistency index is (0.955556), the retention index is (0.986051), and the composite index is 0.943979 (0.942226) for all sites and parsimony-informative sites (in parentheses). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown

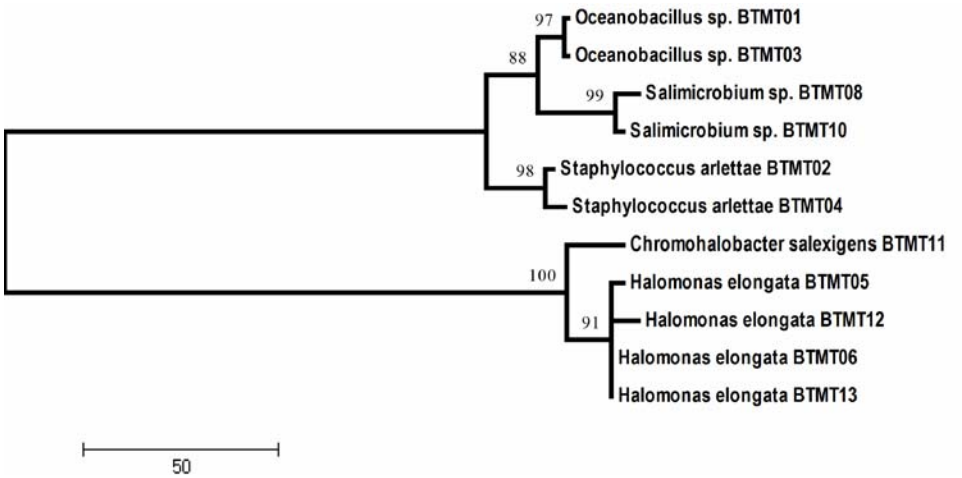

Fig. 1. Phylogenetic tree of the halophiles isolated from tannery effluent and table salt crystals

Table 1. Biochemical reactions of bacterial isolates from tannery effluent and table salt crystals

Characteristics	BTMT01	BTMT02	BTMT03	BTMT04	BTMT05	BTMT06	BTMT08	BTMT10	BTMT11	BTMT12	BTMT13
Gram staining	+	+	+	+	-	-	+	+	-	-	-
Morphology	bacilli	cocci	bacilli	cocci	bacilli	bacilli	cocci	cocci	bacilli	Bacilli	bacilli
starch	+	+	+	+	+	+	+	+	+	+	+
tributyrin	+	+	+	+	+	+	-	-	+	+	+
casein	+	+	+	+	+	+	+	+	+	+	+
sucrose	-	-	-	-	+	+	-	-	+	+	+
mannitol	-	-	-	-	+	+	-	-	+	+	+
glycerol	-	-	-	-	+	+	-	-	+	+	+
sorbitol	-	-	-	-	+	+	-	-	+	+	+
lactose	-	-	-	-	+	+	+	+	+	+	+
glucose	-	+	-	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-
MR	+	+	+	+	-	-	+	+	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	+	+	-	-	+	+	+
H_2S	+	-	+	-	+	+	-	-	+	+	+
Urease	+	-	+	-	+	+	-	-	-	+	+
catalase	+	+	+	+	-	-	+	+	+	-	-
oxidase	+	-	+	-	-	-	+	+	-	-	-
coagulase	-	+	-	+	-	-	-	-	-	-	-

(+ indicates positive result and – indicates negative result)

above the branches [18]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm [19] with search level 3 [18, 19] in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale; with branch lengths calculated using the average pathway method [19] and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 525 positions in the final dataset, out of which 311 were parsimony informative.

Although all the isolated bacteria are halotolerant, the phylogenetic tree (Fig 1) shows that the bacteria isolated from tannery effluent and those isolated from food grade table salt crystals are seen in different clusters. Similar organisms are paired on the same branch. Whereas, the *Halomonas elongata* and *Chromohalobacter salexigens* isolated from salt crystals are seen in two branches, where *Halomonas elongata* are clustered in a single branch reflecting their similarity.

A combination of salinity level exceeding the saturation level and high temperature provide a conducive environment for the growth and survival of halophiles. Halophiles adopt either of the two strategies for osmoadaptation; the salt-in- cytoplasm mechanism and the organic osmolyte mechanism. The salt-in cytoplasm is the strategy adopted by most archaea, wherein K^+ ions accumulate in the cell through an energy dependent potassium intake system. Thus the cytoplasm is exposed to increased ionic strength, consequently the proteins of these organisms have an excess of acidic amino acids which stabilizes the hydration shell of the molecule [20]. The organic osmolyte mechanism is seen in methanogenic Archaea, Bacteria and Eukarya. These organisms accumulate sugars, polyols, aminoacids or its derivatives by de novo synthesis or uptake from surroundings. The compatible solutes are water soluble, non-ionic and they do not interfere with the cellular metabolism [21]. Halophiles using organic osmolyte mechanism are more flexible than organisms using salt- in strategy and display wide salt tolerance. Probably these mechanisms explain the survival of halophiles entrapped in rock salt. The role of halobacteria in the formation of sodium chloride crystals has been studied. It is believed that they may be entrapped in the fluid inclusions within the crystal, retaining their viability, and contamination of salt preserved food could be attributed to the presence of these bacteria [22]. Birbir *et al*.,(2003) conducted a microbial survey of the salt crystal and brine samples collected from ^ereflikoçhisar salt lake in Turkey [23]. Most of the strains isolated were Gram negative rods producing enzymes such as gelatinase, lipase, cellulose and β-galactosidase. The high content of total salt and organic substances could enhance the growth of extreme halophiles.

Almost 10 % of moderately halophilic bacteria isolated from solar salterns and saline soils in Isla Cristina (Spain) were found to secrete proteases; other isolates could secrete enzymes like lipase amylase etc [24]. In fact Jie Lu *et al*.,(2001)

Table 2. NCBI Accession Numbers for halophilic bacteria isolated from Tannery effluent and table salt crystals

Source	Strain No.	Bacteria	NCBI Accession Number
Tannery effluent	BTMT01	*Oceanobacillus* sp.	JX975066
	BTMT02	*Staphylococcus arlettae*	JN228200
	BTMT03	*Oceanobacillus* sp.	JN228197
	BTMT04	*Staphylococcus arlettae*	JN228201
	BTMT08	*Salimicrobium* sp.	JN228199
	BTMT10	*Salimicrobium* sp.	JN228198
Commercial salt crystals	BTMT05	*Halomonas elongata*	KC019171
	BTMT06	*Halomonas elongata*	KC019170
	BTMT11	*Chromohalobacter salexigens*	JX975064
	BTMT12	*Halomonas elongata*	JX975065
	BTMT13	*Halomonas elongata*	JX975062

reported the isolation of an extremely halotolerant *Oceanobacillus ilheyensis* from Ilheya Ridge at a depth of 1050m below sea level [25] and *Oceanobacillus* sp. isolated from Howz Saltern, Lake in Iran have been reported earlier to produce hydrolytic enzyme [26]. In the present study also species of *Oceanobacillus* was observed to produce lipase and protease. It may be noted that proteases from halophiles have been reported to be used for processing of fish and meat based products and for the production of soy sauce [27]. A halotolerant intracellular protease was purified from *Bacillus subtilis* strain FP -133 isolated from fish paste [28]. Halotolerant and halophilic organisms isolated from Laguna Verde, Bolivia have been reported to produce lipases. Halophilic lipases are highly selective and efficient and have an added advantage that they have low energy requirements and this explains the growing interest of the scientific community in studying the properties of these enzymes [3]. A halothermophilic serine protease was purified from *Chromohalobacter* sp. isolated from solar saltern samples [15] which could retain 100% stability in the absence of NaCl. A serine metalloprotease isolated from *Pseudoalteromonas* sp was characterized [24]. Members of the genus *Halomonas* were reported to form a large proportion of the bacterial population isolated from solar saltern in Tunisia and most of the isolates exhibited enzymatic activities [29].

Halozymes have the ability to tolerate high salt concentration, it would bear unique structure and properties which could have industrial applications in food industry, leather industry detergents etc. The present results on potentials of halobacteria *Oceanobacillus* sp. isolated from tannery effluents to produce lipase and protease indicated the need to harness these halophiles for further exploitation. However, further studies are warranted to delve deep into the intricacies of the properties and functioning of these enzymes for potential biotechnological applications.

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