Studies on the microbial diversity of salted fishes under aerobic conditions

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Abstract

A total of 93 isolates of halophilic and moderately halophilic bacteria were isolated from salted fishes, *Scoliodon* sp. and *Thrissina thryssa* obtained from retail fish outlets in Cochin, Kerala, India. The viable count of the bacteria in *Scoliodon* sp. and *Thrissina thryssa* ranged from 10^3-10^6 per g. The morphological, biochemical, and physiological investigations were done to characterize the isolates, which showed good growth in medium containing 3-10% (w/v) NaCl at 37°C. In *Scoliodon* sp. the maximum of 87.5% isolates were Gram-positive cocci and in *Thrissina thryssa* 95.5% of the isolates were Gram-positive rods. The optimum conditions such as temperature, pH, and salts (NaCl) were determined. The utilization of organic compounds such as fructose, lactose, glucose, sucrose, arabinose, trehalose, maltose, salicin, mannose, cellobiose, rhamnose, dulcitol, xylose, raffinose, sorbitol, adonitol, inulin, galactose, inositol, mannitol, hydrogen sulfide, nitrate, citrate utilization, MR-VP, triple sugar iron, as well as the hydrolysis of organic compounds such as casein, gelatin, aesculin, starch, Tween 20, and Tween 80 were also investigated. Moreover, an alkalophilic bacterial strain *Bacillus halodurans (B. halodurans)* was isolated from *Thrissina thryssa* and confirmed by 16S rDNA analysis. These studies revealed that salted fishes offer an optimal environment for the viable, diverse, potentially and industrially important bacterial community.

Introduction

Salting and drying has been used for a long time as a method of fish preservation.[1] Production of canned salted fishes is a traditional activity in the fishing industry in the countries around the Mediterranean Sea. Salting is used for the preservation of fishes from spoilage owing to tissue autolysis and microbial action.[2] Microbial ecology of salted fish products is influenced markedly by the water activity of the product. The high salt and low moisture contents of the salted fishes have contributed to the extreme halophilic bacteria, which have been the most studied.[3,4] A broad range of bacteria inhabit salted fishes; among these moderate halophilic bacteria are the dominating flora. Moderately halophilic bacteria are a heterogeneous group of microorganisms characterized by growth over a wide range of salt concentrations (0.5-2.5 M NaCl).[5] Moderate halophilic bacteria were also recovered from seafoods.[6,7] To date, several studies have reported the microbial ecology of hypersaline waters.[8,9] Few studies have been done regarding the moderately halophilic and halotolerant bacteria in salted fish products.[10] While the microbiology of salted fish has not been studied extensively in the recent past, many different species of moderately halophilic bacteria have been isolated from various habitats such as saline lakes,[11] hypersaline soils,[12] and solar salterns.[13] To our knowledge no studies relating to the bacterial profile of salted *Scoliodon* sp. and *Thrissina thryssa* have been done. The aim of this work was to characterize the microbial diversity with the main emphasis on the *Bacillus halodurans (B. halodurans)* strain.

Materials and Methods

Sampling and culture conditions

Six samples each of dried salted fishes like *Scoliodon* sp. and *Thrissina thryssa* were obtained from retail fish shops in Cochin, Kerala, India. Salinity of the samples was checked using a salinometer. Ten-gram samples were excised aseptically from each fish and homogenized in 90 mL of sterile saline water with a Mortar and Pestle and serially diluted up to 10^{-4}. Then 0.5 mL of each dilution was used to spread in the growth media. Enumeration and isolation of the bacteria were done on salt agar, high-salt casein agar, and nutrient agar with 3% NaCl. The plates were incubated at room temperature for 3-7 days in sealed polythene bags.

Characterization of isolates

All of the 93 isolates were observed for their colony morphology, size, and pigmentation production pattern on the respective media after 2-7 days' incubation and Gram staining was performed according to.[14] Indole, sulfide production, catalase, oxidase, nitrate reductase activity tests and starch hydrolysis were carried out.[15] Gelatin hydrolysis and casein hydrolysis were performed as previously described.[16,17] Motility was observed with the wet mount technique.[18] Optimal growth temperature, pH, and salt concentration were determined in the nutrient medium with 10% (w/v) marine salts containing: NaCl, 8.1 g; $MgCl_2$, 0.7 g; $MgSO_4$, 0.96 g; $CaCl_2$, 0.036 g; KCl, 0.2 g; $NaHCO_3$, 0.006 g; and NaBr, 0.0026 g.[16] The pH range for growth was determined in similar media, with the pH range of 8.0-10.0. The growth rate was determined in 0.5, 2, 5, 10, and 20% NaCl and cultivated at 15, 25, 30, 35 and 45°C. The following compounds were tested as sole carbon sources: glucose, fructose, lactose, mannitol, trehalose, maltose, sucrose, salicin, mannose, cellobiose, rhamnose, dulcitol, xylose, raffinose, sorbitol, adonitol, inulin, galactose, inositol, and arabinose in basal medium with 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.001% (w/v) phenol red, and 10% (w/v) marine salts. These isolates will be further studied for their salt tolerance, growth at different pH range and temperature.

Identification of *Bacillus halodurans*

Identification and biochemical characterization of *B. halodurans* using the conventional method was carried out as described by.[19]

Genomic DNA isolation for 16S rDNA analysis

Genomic DNA extraction from *B. halodurans* was performed as described by.[20] 1.5 mL of the late exponential phase bacterial cells were transferred into a microfuge tube and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was discarded. The clean cells were suspended in 200 µL of lysis buffer and incubated at 37°C for 30 min. Then, 30 µL of 10% SDS and 10 µL of proteinase K (20 mg/mL) were added. The tube contents were gently mixed and incubated at 37°C for 1 h. An equal volume of phenol:chloroform was added and the tube was centrifuged at 12,000 rpm for

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Table 1. Oligonucleotide primers used for PCR amplification of 16S rDNA.

Primer designation	Sequence (5'–3')	Reference
27f	AGAGTTTGATCMTGGCTCAG	37
1525r	AAGGAGGTGWTCCARCC	37

Table 2. Growth of microorganisms isolated from *Scoliodon* sp. and *Thrissina thryssa*.

Sample	No. of sample	Average count range(CFU/gm)		
		Salted agar media (5% NACl)	Nutrient agar media (3% NACl)	High salt casein agar media (25% NACl)
Shark	6	3.3×10^4-5.5×10^4	4.2×10^4-9.7×10^4	1.1×10^4-1.2×10^4
Anchovies	6	1.1×10^4-1.7×10^4	1.9×10^4-2.7×10^4	-

Table 3. Number of isolates from *Scoiodon* sp. and *Thrissina thryssa*.

Sample	Total	No. of isolates		
		Salted agar (5% NaCl)	Nutrient agar with (3% NaCl)	High salt casein agar (25% NaCl)
Shark	38	17	21	-
Anchovies	55	15	30	10

5 min at 4°C. The supernatant was transferred to a new microfuge tube. Twice the volume of cold absolute ethanol was added to the supernatant and gently mixed to precipitate the DNA. The tube was then centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was discarded. The precipitated DNA was washed with 500 µL of 70% ethanol, and dried at 37°C for 30 min. The DNA pellet was resuspended with 50 µL of TE buffer and kept overnight at 4°C to dissolve the precipitated DNA.

Polymerase chain reaction amplification of 16S rDNA

The 16S rDNA of halophilic bacterium isolate was amplified by PCR using primers 27f and 1525r (Table 1). The PCR was performed using a PTC-150 Mini cycler (MJ Research, Waltham, MA, USA) with a primary heating step for 2 min at 95°C, followed by 30 cycles of denaturation for 20 sec at 95°C, annealing for 60 sec at 55°C, and extension for 2 min at 72°C, then followed by a final extension step for 7 min at 72°C. Each 25 µL reaction mixture contained 2 µL of genomic DNA, 14.25 µL of MilliQ water, 2.5 µL of 10× buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTP's mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.0 µL of each primer (20.0 pmoles/µL), and 0.25 µL of *Taq* DNA polymerase (MBI Fermentas, USA). The PCR-amplified product was analyzed on 1% agarose gel containing ethidium bromide (0.5 µg/mL) and 1 kb DNA molecular weight marker (MBI Fermentas), and documented using a gel documentation system (Alpha Imager 1220, Alpha Innotech Corporation, San Leandro, CA, USA).

Sequencing and *in silico* analysis of polymerase chain reaction amplicon

The PCR amplicon was purified by the MinElute Gel purification Kit (Qiagen, Hilden, Germany) and was sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The nucleotide sequences obtained were compared against database sequences using BLAST[21] provided by NCBI (http://www.ncbi.nlm.nih.gov) and were aligned and clustered using the CLUSTAL-X version 1.81 program.[22]

Results

Isolation of microorganisms

About 75% of the isolates obtained from both the *Scoliodon* sp. and *Thrissina thryssa* samples were grown optimally in a 3-10% NaCl media and were thus considered as moderate halophiles.[23] *Scoliodon* sp. and *Thrissina thryssa* were analyzed for the growth of bacteria by inoculating the sample on salted agar media, (5% NaCl), nutrient agar (3% NaCl), and high-salt casein media (25% NaCl). The counts observed for the samples in two different media are given in Table 2. Nutrient agar with 3% NaCl had an elevated count in both the samples analyzed and *Scoliodon* sp. had a higher count than *Thrissina thryssa*. A total number of 93 isolates were randomly selected for characterization. The source of the isolates are given in Table 3.

Morphological observations

Of the 48 isolates from dried shark, 42 (87.5%) were Gram-positive cocci and the remaining 6 (12.5%) were Gram-positive rods.

Table 4. Characteristic features of the bacterial isolates.

Morphological and phenotypic characters	No. of isolates
Morphology	
Rods	45
Cocci	48
Colony pigment	
White	20
Yellow	26
Cream	32
Pink	13
Orange	1
Salts: growth at	
0.5%	70
2.0%	72
5.0%	78
10.0%	75
20.0%	10
pH: growth at	
pH 8	89
pH 9	85
pH 10	56
Temperature: growth at	
15°C	39
25°C	63
30°C	89
35°C	93
40°C	79
Utilization of carbohydrates	
Glucose	13
Fructose	9
Arabinose	5
Mannitol	13
Trehalose	9
Lactose	2
Maltose	12
Sucrose	15
Salicin	9
Mannose	10
Cellobiose	8
Rhamnose	5
Dulcitol	10
Xylose	14
Raffinose	11
Sorbitol	13
Adonitol	7
Inulin	11
Galactose	6
Inositol	8
Cytochrome oxidase	50
Catalase	93
Gelatin hydrolysis	12
Casein hydrolysis	17
Starch hydrolysis	27
Indole production	Nil
Motility	20
Hydrogen sulfide production	18
Nitrate reduction	27
Citrate utilization	11
Methyl red	17
Voges Praskeur	21
Triple sugar iron agar test	21

In the case of isolates from dried anchovies, of the 45 isolates, 43 (95.5%) were Gram-positive rods and 2 (4.4%) were Gram-positive cocci (Table 4).

Pigmentation pattern

All isolates were observed for their colony color. Of the 93 isolates, 21.5% were whitish, 27.9% were yellowish, 34.4% were creamy, 13.9% were pinkish, and 1% were orange in color.

Phenotypic characterization of isolates

Of 93 total isolates, the following sugars were utilized: 5.3% arabinose, 13.9% glucose, 9.6% fructose, 2.1% lactose, 9.6% trehalose, 13.9% mannitol, 13% maltose, 16% sucrose, 9.6% salicin, 10.7% mannose, 8.6% cellobiose, 5.3% rhamnose, 10.7% dulcitol, 15% xylose, 11.8% raffinose, 14% sorbitol, 7.5% adonitol, 11.8% inulin, 6.4% galactose, and 8.6% inositol. Moreover, 21.5% of the isolates were found to be motile. 19.3% isolates revealed positive result for H2S. About 29% of the isolates were found to reduce nitrate. 11.8% of the isolates were found to utilize citrate. 18.2% isolates revealed positive result for Methyl Red test. 22.5% isolates revealed positive result for Voges Praskeur. 22.5% isolates exhibited positive result for triple sugar iron agar test.About 29, 18.2, and 12.9% of the isolates exhibited a positive result for starch, casein and gelatin hydrolysis, respectively. Out of 48 isolates from the shark samples, all were found to be catalase positive, 56.25% were oxidase positive, and the remaining 43.75% were oxidase negative. Of the 45 isolates from the anchovy samples, all were catalase positive, and in the case of the cytochrome oxidase test, 51.1% were positive and 48.8% were negative. No isolates produced indole from tryptophan.

Growth range and optimal growth conditions

Of the 93 isolates, 80.6% were grown in 10% NaCl, 77.4% in 2% NaCl, 75.2% in 0.5% NaCl, 83.8% in 5% NaCl, and 10.75% in 20% NaCl. The optimum NaCl range for growth was 2-10% among the 93 isolates. pH range for growth also varied among the isolates; 95.6% of the 93 isolates were found to have good growth at pH 8, 91.3% at pH 9, and 60.21% at pH 10. The optimum range of pH was found to be 7-9. The optimal growth temperature for the 93 isolates was determined to be 30°C. The isolates thus have a wide growth range, both in salt concentration and pH.

Isolation and identification of *Bacillus halodurans*

Morphological, physiological, and biochemical characterization of *B. halodurans* were found to be: a Gram-positive, spore-forming rod; motile; oxidase positive; catalase negative; and utilized glucose, fructose, galactose, sucrose, lactose, and arabinose. Hydrolysis of Tween 40, 60, casein, gelatin, and starch is obtained but the hydrolysis of Tween 20 and reduction of nitrate were not observed. The isolate also exhibited good growth on nutrient agar plates with 15% NaCl and at pH10.0. Moreover, good growth was observed in the temperature range of 15-55°C. These properties support the previous report for the identification of *B. halodurans*.[19] Furthermore, the *B. halodurans* isolate was confirmed by 16S rDNA analysis. The sequence of the 16S rDNA gene 1502 bp (Figure 1) of our isolate showed 100% identity to that of the previously reported *B. halodurans* C-125 (BA000004)[24] and *B. halodurans* MS-2-5 (AB359904).[25] Thus, the bacterial strain was identified and confirmed as *B. halodurans*, on the basis of these results. The 16S rDNA sequence generated in this study was submitted to GenBank and has been given the accession no. GU367604.

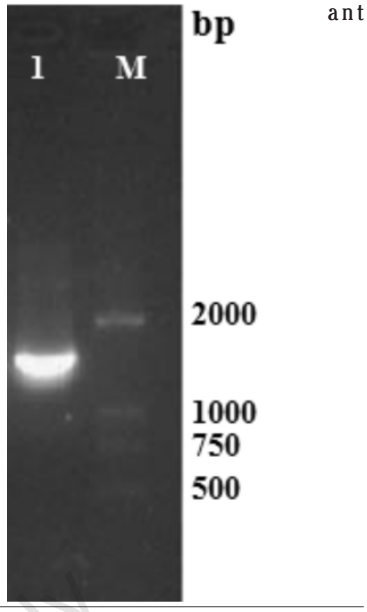

Figure 1. 16S rDNA amplicon of *Bacillus halodurans*. Lane M: -1 kb DNA Ladder. Lane 1: Amplicon of 16S rDNA (1,502 bp).

Discussion

Several species of moderately halophilic eubacteria, obtained from diverse natural saline habitats, have been isolated and described in recent years.[4] Microbial diversity of salted *Scoliodon* sp. and *Thrissina thryssa* in this study was the first in saline ecosystems to be studied in detail from a microbiological standpoint. In view of the great commercial importance of the halophilic and moderate halophilic bacteria, it is surprising how little research has been done to study the microbiology of salted fishes. Our primary interest was in characterizing the microorganisms with the potential value of biotechnological interest. Presently there are only nominal reports of the isolation of bacteria from salted foods.[10] In our study, we isolated 147 bacterial strains, of which 93 of both halophilic and moderately halophilic bacteria were characterized in detail. The majority of the Gram-positive cocci (87.5%) were found in *Scoliodon* sp. whereas the maximum numbers of Gram-positive rods (95.5%) were observed in *Thrissina thryssa*. These results agree with those reported by other authors in salted cod[10] and other cured food commodities such as smoked catfish,[26] fermented sausages,[27] and cured meats.[28] Gram-positive cocci are the dominant bacterial survivors in salted fishes.[29] *Staphylococcus aureus* and *Staphylococcus xylosus* have been found in green salted and dried salted products, respectively.[30] All the isolates in this study were found to use various organic compounds including sugars as substrates and should be considered chemoorganotrophs. For the first time, we have isolated an industrially important alkalophilic *B. halodurans* strain from *Thrissina thryssa*. Previous studies reported the isolation and identification of *Marinococcus* and *Halobacillus* strains from other saline environments, including athalassohaline and thalassohaline lakes and marine waters.[31,32] In our study, about 75% of the isolates from both salted fish samples revealed optimal growth in 3-10% NaCl media and were thus considered as moderate halophiles.[33] Recent research on different hypersaline habitats focused on the screening of new bacteria with potential applications in biotechnology.[34] The potential industrial use of these microorganisms has been highlighted for the production of compatible solutes, biopolymers, and bioremediation processes,[33,34] prompting us to screen our collection of halophiles for molecules of industrial interest. Moreover, extensive studies on these bacteria will certainly offer different potential applications in various pharmacochemical industries.[35,36]

Conclusions

Based on the results, it can be concluded that the identification and characterization of halophilic and moderately halophilic bacteria presenting in salted *Scoliodon* sp. and *Thrissina thryssa* have opened several possibilities for future research in the field of industrial biotechnology and microbial spoilage aspects.

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