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In vitro Antibacterial Potential of Halophilic Bacterial Secondary Metabolites from Salted Fish

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ABSTRACT

Living organisms can be found over a wide range of extreme conditions. Most of the organisms living in extreme environments (*i.e,* extremophiles) belong to the prokaryotes. Halophiles are interesting class of extremophilic organisms that have adapted to harsh, hypersaline conditions. They are able to compete successfully for water and resist the denaturing effects of salts. The present study was an investigation on the *in vitro* antibacterial effect of secondary metabolites from halophilic bacteria isolated from salted fish samples. The cured salted fish samples were collected and enumerated using halophilic Nutrient Agar supplemented with 4% NaCl. The isolated and purified bacterial cultures are numbered as SF1, SF2, SF3, SF4 and SF5 are further identified using VITEK 2 system as *Bacillus vallismortis, Ralstonia mannitolytica, Bacillus subtilis, Rhizoboum radiobacter* and *Kocuria kristina.* Growth kinetics of halobacterial isolates were determined by spectrophotometric assay. The antibiotic resistance pattern of tested pathogenic microorganisms using the commercial antibiotics was screened and almost all the tested microorganisms are resistant to Penicillin. The antimicrobial activity of secondary metabolites of halophilic bacteria against drug resistant microbes was assessed using the Agar well diffusion assay. Among the different extracts of the halophilic bacteria, the chloroform extracts of *R. mannitolytica* showed maximum antibacterial activity against *Bacillus subtilis* MTCC 441 and *Xanthomonas campestris* MTCC 2286. The results of antimicrobial activity are considerable because it enables the identification of potential secondary metabolites present in marine halophilic bacteria, which act as source of innumerable therapeutic agents. Further research is highly warranted to find out the active principle responsible for the antibacterial property and to elucidate the structure of particular compound.

Keywords: Antimicrobial activity, drug resistant microbes, halophilic bacteria, *Ralstonia mannitolytica,* secondary metabolites.

INTRODUCTION

Extremophiles are organisms adapted to live physically or geochemically in extreme conditions. The extremophiles are present in numerous and diverse genetic lineages of both bacteria and archaea. Halophiles are interesting class of extremophilic organisms that have adapted to harsh, hypersaline conditions. They are able to compete successfully for water and resist the denaturing effects of salts. [1]

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Halophiles inhabit hypersaline environments all over the world and there are currently 15 recognized genera in the family. [2] Halophilic microorganisms are found in all three domains like *Archea, Bacteria* and *Eukarya*. They are heterotrophs that normally respire by aerobic means. Most halophiles are unable to survive outside their high-salt native environment. Heterotrophic bacteria acquire at least some of their carbon from organic molecules like glucose. Based on their cell wall composition they may be either gram-positive or gram-negative type. Most of them are facultative anaerobes *i.e*. they are capable of surviving in presence or absence of oxygen and some are strictly anaerobes. Most of these bacterial species can tolerate a wide range of environmental conditions like high and low temperature, different pH levels, wide range of salinity *etc.* [3] Despite the requirement for high salinity, there are instances where these strains have also been isolated from sources such as seawater [4] and sea sand. [5] According to Oren [6], the antibacterial property of the halophilic bacteria is much promising. In this study, the salted cured fish samples were analysed for the halophilic bacterial diversity and the metabolites from the isolates were screened for antimicrobial activity against drug resistant pathogens *in vitro*.

MATERIALS AND METHODS

Collection of the sample

The naturally sun dried salted fish samples were collected from local fish drying centres of Kayamkulam coast, Alapuzha District (Lat. 9° 10' N;Long. 76° 30' E) during the period of January, 2015, in sterile polythene bags and brought to the laboratory in iced-chest. Bacteriological analyses were made within 4 - 6 h of sampling.

Media preparation

The halophilic bacteria were estimated using halophilic agar medium containing NaCl-40.0 g/l; Peptone-5.0 g/l; Beef Extract- 3.0 g/l; Agar-15.0 g/l; Distilled water-1000 ml; pH (at 25°C)7.2 – 7.4 (Hi Media, Mumbai) supplemented with 4% NaCl. All the chemicals used were of analytical grade. The media was sterilized at 121°C for 15 min by autoclaving at 15 lbs $inch^{-2}$.

Isolation of halophilic bacteria

One gram of serially diluted salted fish samples was plated with sterile Halophilic Nutrient Agar with 4% of NaCl and incubated at 30°C for 24 hrs. Halophilic bacteria from the sample has been isolated by pour plate, purified by straking and incubated at room temperature at 48 hours. For enrichment, the broth inoculated with one gram of sample was kept on rotary shaker at room temperature at 150 rpm for 48 hours. After attaining visible growth, the bacterial colonies were enumerated. All the determinations were carried out in triplicates and the results were expressed as counts per gram dry weight. Different morphological colonies were picked up from the petriplates and restreaked thrice in an appropriate agar plates before a pure culture was established in agar slants. Isolated cultures were maintained in respective agar slants at 4°C for short periods (3 - 6 months). For longer periods, agar slants are stored in liquid nitrogen at –6° to –80°C under low oxygen tension or in 5% DMSO (w/v).

Identification of bacterial strains

The isolated species were identified based on morphological and biochemical characteristics by following the methods of Bergey's manual of Determinative Bacteriology. [7] The cured salted fish samples were collected and enumerated using halophilic Nutrient Agar supplemented with 4% NaCl. The isolated and purified bacterial cultures *viz*., SF1, SF2, SF3, SF4 and SF5 (salted fish). Then the culture was further purified by quadrant streaking and axenic cultures are made and further characterized up to species level by VITEK system.

Identification of bacterial strains using VITEK 2 systems: version 0.6

In this study, morpologically different halobacterial strains were identified using the API 20NE system (BioMerieux Vitek, Inc. Hazelwood, Mo., USA). The features of VITEK® 2 specifically include 21 CFR Part 11 compliance (for electronic records and signatures) and a colorimetric reagent card (BCL) used to identify the spore-forming Gram-positive bacilli (*i.e., Bacillus* and related genera). The other colorimetric reagent cards (GN, GP, and YST) apply to all system formats for both industrial and clinical laboratories. The culture requirements table that lists parameters for appropriate culture and inoculum preparation. These parameters include acceptable culture media, culture age, incubation conditions and inoculum turbidity.

Suspension preparation

A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 × 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted accordingly and measured using a turbidity meter called the DensiChek.

Inoculation

Identification cards are inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The cassette can accommodate up to 15 tests (VITEK 2). The filled cassette is transported automatically into a vaccum chamber station. After the vaccum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells. Using VITEK 2 system they are identified as *Bacillus vallismortis, Ralstonia mannitolytica, Bacillus subtilis, Rhizoboum radiobacter* and *Kocuria kristina.*

Growth kinetics of halophilic bacterial isolates

In order to optimize the growth of halophilic bacterial isolates, the bacterial cultures were inoculated at 30°C

for 24 hours in the halophilic Nutrient agar medium and kept for incubation at 30°C temperature. The Halophilic Nutrient broth (Hi Media, Mumbai) were prepared in a 500 ml conical flasks and the pH was maintained to 7 using 1N HCl and 1N NaOH. Then the media was sterilized, cooled and inoculated with 1ml of halophilic mother culture and kept for incubation at 30°C in a shaker cum incubator (REMI, India) for constant shaking at 120 rpm for five days. The growth of the halobacterial isolates was determined spectrophotometrically at 500 nm.

Selection of microorganisms for antibacterial testing

Eight pathogenic bacterial strains procured from Microbial Type Culture Collection (MTCC, Chandigarh, India) were employed in the present study. 24 hours fresh cultures of the Gram negative organisms such as *Escherichia coli* (MTCC 585), *Klebsiella pneumoniae* (MTCC 3040); *Mycobacterium smegmatis* (MTCC 994), *Pseudomonas aeruginosa* (MTCC 7925), *Shigella flexneri* (MTCC 1457), *Xanthomonas campestris* (MTCC 2286) and Gram positive organisms such as *Bacillus subtilis* (MTCC 428), *Staphylococcus aureus* (MTCC 3160) were used as the test pathogens. Nutrient agar and nutrient broth was used for storage and sub-culturing of the bacterial pathogens respectively. Muller Hinton Agar was used for antibacterial assay.

Antibiogram study of pathogens

Antibiogram studies of test pathogens are essential for determination of antimicrobial effectiveness since some of the pathogens are resistant against potential antibiotics. Here, all the test organisms were checked for antibiotic susceptibility by employing commercial antibiotic discs (Hi-Media, Mumbai). The antibiotics such as Chloramphenicol (30µg /disc), Gentamycin (30µg /disc), Penicillin (10µg /disc), Streptomycin (25µg /disc), Tetracycline (30µg /disc) were employed for the study. The antibiotic discs were impregnated on Muller-Hinton Agar plates which were pre-seeded with test organisms and incubated at 37 ± 0.5°C for 12 to 14 hours. The antibiotic sensitivity pattern of the test pathogens were deduced from the standard interpretation chart.

Isolation of secondary metabolites

The identified halophilic bacterial species were cultured in 500 ml shake flask batch fermenters with halophilc Nutrient broth for 72 hours in an orbital shaker cum incubator at 30°C for the production of secondary matabolites at 10% inoculum concentration in an orbital shaker at 120 rpm. After 72 hours, samples were withdrawn, centrifuged to get cell free extracts. The metabolites may produce in the stationary phase or idiophase' of the culture growth. The components needed for the antimicrobial activity was obtained from the crude filtrate by solvent extraction method.

Extraction

About 20 ml of the cell free supernatant each, add 20 ml chloroform and Distilled water using separating funnels in the ratio 1:1 (v/v) and shaked vigorously for one hour for complete extraction of bioactive compounds. Then it was filtered by using Whatmann filter paper. After filtration the extract was separated and stored in separate tubes. Then it was evaporated in a water bath at 60°C and the residue obtained was used for further analysis.

Antibacterial Activity of halophilic bacteria

All bacterial cultures were plated out on Nutrient agar and incubated for 24 h at 30 ± 0.5°C and colonies from this fresh culture were used for making suspension. The antimicrobial activity was assessed using the Agar well diffusion assay. [8-9] In fresh inoculum of approximately 10^6 CFU /ml- McFarland turbidity range of tested drug resistant microorganisms was used for the study. 100µl of the bacterial suspension was uniformly spread on sterile Muller Hinton Agar plates. After solidification of the agar, wells were made with a 6 mm sterile cork borer. Different concentrations of the halophilic bacterial secondary metabolite extracts were made with 99% (v/v) DMSO (Dimethyl sulfoxide) and 100µl of the extract were poured in the wells. The plates were incubated for 24 h at 37 ± 0.5°C. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. [10] The antibiotic, Streptomycin (25µg /disc) was served as a positive control (PC) and negative controls (NC) were made by DMSO alone. The experimental data were expressed as mean ± SD of triplicates using Microsoft Excel Software Programme.

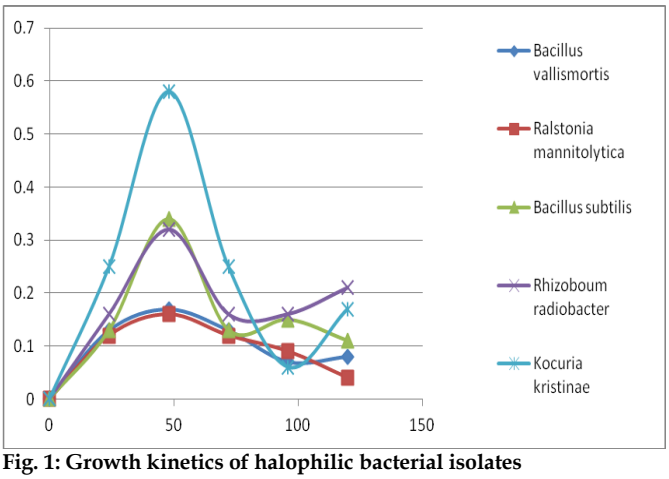

Fig. 1: Growth kinetics of halophilic bacterial isolates

RESULTS AND DISCUSIION

The antibacterial properties of the halobacterial isolates have shown that the halophiles are potential sources of new antimicrobial agents. The occurrence and distribution of halophilic bacteria in salted cured fish samples was determined and it was found that they are in the order of 32×10^5 CFU/g. Earlier results in literature viewed that, halophilic bacterial diversity of several environments. [11-12] The present finding also supports the view of earlier findings and it may be due to the fact that they need salt for their metabolism and growth. [4, 13-14] The isolated species were identified based on their morphology and biochemical characteristics. The colony characteristics of the

halophilic bacteria collected from dried salted fish was given in the Table 1. Based on morphological and biochemical differences, the species was isolated and denoted as SF1-SF5 (salted fish).

Table 1: Colony characteristics of Halophilic bacteria collected from dried salted fish

Characteristics	*Halophilic bacteria*				
	SF1	***SF2***	***SF3***	***SF4***	***SF5***
Size	Moderate	Small	Large	Small	Small
Shape	Rhizoidal Floral arrangement, Frost like appearance	Round	Round	Round	Round
Colour	Cream	Cream, Transparent	Cream	Yellow	Light Red
Elevation	Flat	Flat	Raised	Raised	Raised
Margin	Rhizoidal	Circular	Circular	Circular	Circular

Table 2: Characterization of Halophilic bacteria using Vitek 2 systems

Characteristics	*Halophilic bacteria*				
	SF1	***SF2***	***SF3***	***SF4***	***SF5***
Sample Media used with 4% Na Cl	Salted fish Halophilic Nutrient Agar	Salted fish Halophilic Nutrient Agar	Salted fish Halophilic Nutrient Agar	Salted fish Halophilic Nutrient Agar	Salted fish Halophilic Nutrient Agar
Colourimetric Reagent Card	BCL	GN	BCL	GN	GP
Probablity (%)	88%	87%	94%	99%	89%
Halophilic bacteria identification	*Bacillus vallismortis*	*Ralstonia mannitolytica*	*Bacillus subtilis*	*Rhizoboum radiobacter*	*Kocuria Kristinae*

BCL- Gram-positive spore-forming bacilli; GN- Gram Negative fermenting and non-fermenting bacilli; GP- Gram Positive cocci and non-spore-forming bacilli

Table 3: Antibiogram studies of test pathogens against commercial antibiotics

Microorganisms	**Zone of inhibition (mm in dia.) against antibiotics**				
	Chloramphenicol (30µg /disc)	**Gentamicin (30µg /disc)**	**Penicillin (10 µg /disc)**	**Streptomycin (25 µg /disc)**	**Tetracycline (30 µg /disc)**
E.coli MTCC 585	32 ± 0.1 (S)	28 ± 0.5 (S)	7 ± 0.1 (R)	27 ± 0.1(S)	27 ± 0.1 (S)
Klebsiella pneumoniae MTCC 3040	29 ± 0.1 (S)	25 ± 0.1 (S)	8 ± 0.1 (R)	22 ± 0.5 (S)	2 ± 0.1 (R)
Mycobacterium smegmatis MTCC 994	33 ± 0.5 (S)	30 ± 0.1 (S)	11 (R)	25 ± 0.1 (S)	28 ± 0.1 (S)
Pseudomonas aeruginosa MTCC 424	32 ± 0.5 (S)	27 ± 0.1 (S)	8 ± 0.1 (R)	23 ± 0.1(S)	25 ± 0.1 (S)
Shigella flexneri MTCC 1457	23 ± 0.1 (S)	30 ± 0.1 (S)	-	24 ± 0.5 (S)	27 ± 0.1 (S)
Streptococcus aerogens MTCC3108	33 ± 0.1 (S)	26 ± 0.1 (S)	-	23 ± 0.1 (S)	26 ± 0.1 (S)
Xanthomonas campestris MTCC 2286	32 ± 0.1 (S)	28 ± 0.1 (S)	10 (R)	25 ± 0.5 (S)	26 ± 0.1 (S)
Bacillus subtilis MTCC 441	28 ± 0.1 (S)	23 ± 0.1 (S)	-	21 ± 0.1 (S)	26 ± 0.1 (S)
Staphylococcus aureus MTCC 3160	32 ± 0.5 (S)	28 ± 0.1 (S)	13 ± 0.5 (I)	22 ± 0.1 S)	28 ± 0.1 (S)

Values are the average of experiments in triplicates. R=Resistant (≤11mm); I = Intermediate (12-14 mm); S =Sensitive (≥15mm)

Table 4: Antibacterial activity of halophilic bacteria against pathogenic microorganisms

Micro organisms	**Zone of inhibition (mm in dia.)**											
	Bacillus vallismortis		***Ralstonia mannitolytica***		***Bacillus subtilis***		***Rhizoboum radiobacter***		***Kocuria kristinae***		**-ve control**	**+ve control**
	Chl	DW	Chl	DW	Chl	DW	Chl	DW	Chl	DW	DMSO	Streptomycin (25µg)
E. coli MTCC 585	T	T	T	-	T	-	T	T	-	T	-	27 ± 0.1
Klebsiella Pneumoniae MTCC 3040	T	-	-	-	T	-	-	T	-	-	-	22 ± 0.5
Mycobacterium smegmatis MTCC 994	12 ± 0.1	T	T	-	11 ± 0.1	-	12 ± 0.1	T	T	T	-	25 ± 0.1
Pseudomonas aeroginosa MTCC 424	10 ± 1.0	T	9 ± 0.5	-	12 ± 0.5	-	19 ± 0.1	T	-	-	-	23 ± 0.1
Shigella flexneri MTCC 1457	-	T	T	-	T	-	-	T	-	T	-	24 ± 0.5
Streptococcus aerogens MTCC3108	-	-	T	-	-	-	T	T	-T	-	-	23 ± 0.1
Xanthomonas compestris MTCC 2286	11 ± 0.5	10 ± 0.5	18 ± 0.5	-	12 ± 0.5	T	12 ± 0.1	T	-	T	-	25 ± 0.5
Bacillus subtilis MTCC 441	9 ± 0.5	-	19 ± 0.1	-	-	-	-	-	10 ± 0.5	-	-	21 ± 0.1
Staphylococcus aureus MTCC 3160	9 ± 1.0	-	T	-	T	10 ± 1.0	T	11 ± 0.1	-	-	-	22 ± 0.1

Chl- Chloroform, DW- Distilled water, DMSO- Dimethyl Sulfoxide (Control), T=Trace (≤ 7mm), - =No activity Values are the average of experiments in triplicates

The isolated and purified bacterial cultures were further identified using VITEK 2system (Table 2). They are identified as as *Bacillus vallismortis, Ralstonia mannitolytica, Bacillus subtilis, Rhizoboum radiobacter* and *Kocuria kristina.* The results from the Vitek GNI+ test card analysis reveals that positive identifications have a confidence level at least 85% and must be corroborated by morphological characters. The Vitek GNI+ represents an automated, rapid system for identification of Gram negative bacteria (bioMerieux). Growth kinetics of halophilic bacterial isolates *viz*., as *Bacillus vallismortis, Ralstonia mannitolytica, Bacillus subtilis, Rhizoboum radiobacter* and *Kocuria kristinae* was determined by spectrophotometric assay at 500 nm and the results are given in the Fig. 1. From the results, it is clear that the isolate SF5 (*Kocuria kristinae*) showed maximum growth. From the results, it is clear that the isolate showed sationary growth on the third day. Growth kinetics of halophilic bacterial isolates were determined by spectrophotometric assay and the results reveals that the idiophase of their growth.

The resistance pattern of tested microorganisms using commercial antibiotics such as Chloramphenicol (30µg /disc), Gentamycin (30µg/disc), Penicillin (10µg /disc), Streptomycin (25µg /disc), Tetramycin (30µg /disc) reveals that almost all the tested microorganisms are resistant to Penicillin (Table 3). Table 4 shows the results of antibacterial activity of halophilic bacteria against drug resistant pathogenic microorganisms. Among the different extracts of the halophilic bacterial species, the chloroform extracts of *R. mannitolytica* showed maximum antibacterial activity against *Bacillus subtilis* MTCC 441 (19 ± 0.1 mm) followed by *Xanthomonas campestris* MTCC 2286 (18 ± 0.5 mm).

The antibacterial properties of the halobacterial isolate *R. mannitolytica* have shown that the halophiles are potential sources of new antimicrobial agents. This study reveals that *R. mannitolytica* is one of the most promising haophilic bacteria which able to produce the maximum of antibacterial activity. In a study by Kustiariyah and Jarman [15] and Prasanth Williams [16], the ethyl acetate extracts exhibited microbicidal activity against the test organisms. Likewise, in our study, the chloroform extracts showed the maximum antibacterial effect, which may be due to their difference in polarity. The results of this research highlight that the organic solvent extracts exhibits greater antimicrobial principles. [17]

This study reveals the importance of halophilic bacteria in the control of drug resistant pathogeinc diseases. Therefore, the microbes from marine environments especually the halophilic bacteria represent a potential source of new antimicrobial agents. The results of antimicrobial activity are considerable because it enables the identification of potential antimicrobials and other secondary metabolites present in marine halophilic bacteria, which are act as source of innumerable therapeutic agents. Further research is highly warranted to find out the active principle responsible for antimicrobial activity and to elucidate the structure of particular compound.

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