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Research Article

HALOPHILIC ABILITIES OF BACTERIA IN SELECTED LOCATIONS OF TAMIL NADU

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Abstract- Halophiles are salt loving organisms, which can grow in extreme environments of differential salt concentrations. They extend from prokaryote, archae to eukaryotic microorganisms including algae. Halophiles are classified based on their halotolerance abilities into slight, moderate and extreme halophiles. In this present study, the isolation of halophiles was carried out by enrichment culture method using halophilic medium Tryptic Soya Agar (TSA) as a selective media. Screening of extreme halophiles was done by increasing the NaCl concentration up to 4.31 M. Morphological observations and biochemical tests were performed. Out of 90 isolates, 13 isolates were extreme halophiles and survived up to 4.31 M NaCl and remaining 77 isolates were moderate halophiles.

Keywords- *Halophiles, Tindivanam, NaCl, Morphological, Biochemical*

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Introduction

Halophiles are salt loving organisms, which can survive under high salt concentrations. They belong to extremophiles. Halophiles are classified based on their halotolerance capacity into slight halophiles - 0.3 to 0.8 M (1.8 to 4.7 % NaCl), moderate halophiles - 0.8 to 3.4 M (4.7 to 20 % NaCl) and extreme halophiles - 3.4 to 5.1 M (20 to 30 % NaCl) [1, 2] . They extend from prokaryote, archae to eukaryotic microorganisms such as algae. They are normally found in hypersaline lakes, coastal dunes, saline deserts, salt marches and inland salt seas and springs [3,4]. Halophiles use variety of energy sources, they can be aerobic or anaerobic. Anaerobic halophiles include phototrophic, fermentative, sulfate-reducing, homoacetogenic and methanogenic species [5]. High salinity resembles harsh environment for organism to acclimatize. Most halophilic organism utilize energy to exclude salt from their cytoplasm to survive. Basically, two different strategies prevent desiccation through osmotic movement of water out of their cytoplasm. In the first, accumulation of osmoprotectants which are known as compatible solutes synthesized by halophiles or accumulated from environment. The second, involves influx of potassium (K+) ions into cytoplasm [6]. Halophiles have vivid industrial and biotechnological applications like β-carotene and ectoine used in production of fermented foods, a stabilizer for enzymes and also used in cosmetic industry, treatment of saline/hypersaline wastewaters, production of exopolysaccharides, poly-beta-hydroxyakanoate bioplastics and biofuel [7]. Aim of this research was to reveal halophiles and screen out extreme halophiles from the isolated one. To examine their cell morphology, biochemical characters and asses the halotolerant bacterial diversity in Tindivanam Taluk of Vilupuram District of Tamil Nadu, India.

Materials and Methods

Enumeration and isolation of halotolerant bacteria

Enumeration of halotolerant bacteria from soil samples collected from 4 villages of Thindivanam Taluk was carried out by adopting serial dilution. Tryptic Soya Agar medium containing (10%, w/v) of NaCl was poured into one milliltre of appropriate soil dilutions, which were added into sterile petri plates. The plates were incubated at 28º±2ºC for 48 h, colonies differing in morphology were repeatedly streaked to obtain pure cultures. The pure culture of extreme halophiles was maintained on Tryptic soya agar slants and also stored in 60 % glycerol stocks at – 80ºC for further studies [8].

Screening of Halotolerant bacterial strains against NaCl concentration

Tryptic soya agar (TSA) media with different NaCl concentrations was prepared by adding (10 to 25%, w/v) NaCl [8]. The colonies which could proliferate under varying concentrations of NaCl in TSA medium was considered as halotolerant bacteria. The colonies which survived were subjected to increasing NaCl concentration to obtain extreme halophiles and were repeatedly streaked to obtain pure cultures. Extreme halophiles were rechecked in different ratio of sterile water and sea water [9]. Extreme halophiles were inoculated in Tryptic soya broth containing (10-25%, w/v) NaCl, after incubating at 28ºC for 48 h, growth was measured in terms of optical density values at 600 nm in spectrophotometer (m/s. Shimadzu, Japan).

Characterization of Halotolerant bacterial isolates

Morphological characterization

The morphological characters of Halotolerant bacterial isolates were identified by following the methods [10]. The colony characters *viz*., margin, elevation, colour, shape, surface of the isolates was observed as described [11].

Gram staining

The Gram's reaction of Halotolerant bacterial isolates was performed by following the method of [12]. Resulted Gram staining was confirmed through KOH string test. About 10 per cent of the KOH was prepared and one to two drops of KOH was placed on the slide followed by loop of culture was suspended in it. In case of Gram negative bacteria, a string will be formed in between glass slide and inoculation loop since the bacterial cell wall gets dissolved in 10 per cent KOH whereas in case of Gram positive bacteria no string will be formed as its cell wall won't dissolve in 10 percent KOH.

Table-1 *Enumeration of Halotolerant bacteria from rhizosphere and non- rhizosphere soil samples*

SN	Sample	Total bacterial population CFU× $10^4 g^{-1}$ of dry soil	Population of selected Halotolerant bacteria CFU× $10^4 g^{-1}$ of dry soil	Total bacterial population CFU× $10^5 g^{-1}$ of dry soil	Population of selected Halotolerant bacteria CFU× $10^5 g^{-1}$ of dry soil	Total bacterial population CFU× $10^6 g^{-1}$ of dry soil	Population of selected Halotolerant bacteria CFU× $10^6 g^{-1}$ of dry soil
1	1	21	3 (0.24)	14	2 (0.15)	8	4 (0.30)
2	2	24	2 (0.15)	13	3 (0.24)	10	2 (0.15)
3	3	23	3 (0.24)	16	1 (0.00)	9	3 (0.24)
4	4	21	3 (0.24)	12	4 (0.30)	7	1 (0.00)
5	5	19	4 (0.30)	11	3 (0.24)	10	3 (0.24)
6	6	20	2 (0.15)	16	2 (0.15)	12	3 (0.24)
7	7	17	3 (0.24)	13	4 (0.30)	13	4 (0.30)
8	8	18	1 (0.00)	15	3 (0.24)	12	1 (0.00)
9	9	19	1 (0.00)	12	2 (0.15)	7	2 (0.15)
10	10	26	1 (0.00)	12	1 (0.00)	11	-
11	11	23	1 (0.00)	14	1 (0.00)	10	1 (0.00)
12	12	20	-	12	1 (0.00)	7	1 (0.00)
13	13	26	2 (0.15)	13	-	6	-
14	14	21	2 (0.15)	14	1 (0.00)	12	4 (0.30)
15	15	20	2 (0.15)	12	2 (0.15)	10	1 (0.00)

Values in the parenthesis are $\log_{10}$ transformed values

Table-2 *Growth at various levels of NaCl concentration*

SN	Isolates	Growth at various levels of NaCl concentration					
		0 M Control	0.86 M 5 % NaCl	1.71 M 10 % NaCl	2.57 M 15 % NaCl	3.43 M 20 % NaCl	4.31 M 25 % NaCl
1	S1	++	++	++	++	+	+
2	S2	+++	+++	+++	+++	++	++
3	S3	+++	+++	+++	+++	++	++
4	S4	++	++	++	++	+	+
5	S5	+++	+++	+++	+++	++	+
6	S6	+++	+++	+++	+++	++	+
7	S7	++	++	++	++	+	+
8	S8	+++	+++	+++	+++	++	++
9	S9	+++	+++	+++	+++	++	++
10	S10	++	++	++	++	+	+
11	S11	+++	+++	+++	+++	++	+
12	S12	++	++	++	++	+	+
13	S13	++	++	++	++	+	+

+++ Fast growers ++ Moderate growers + Slow growers

Table-3 *Morphological characters of Halotolerant bacterial isolates*

Morphological observations						
SN	Isolates	Colony colour	Topography	Margin	Shape	Gram's reaction
1	S1	Creamy white	Small circular	Erose	Rod	+ve
2	S2	Creamy white	Small circular	Entire	Rod	+ve
3	S3	Creamy yellow	Small circular	Entire	Rod	-ve
4	S4	Creamy yellow	Large circular	Erose	Rod	+ve
5	S5	Creamy white	Small circular	Entire	Rod	-ve
6	S6	White	Small circular	Entire	Rod	+ve
7	S7	Creamy yellow	Small circular	Erose	Rod	+ve
8	S8	White	Small circular	Entire	Rod	+ve
9	S9	Creamy white	Small circular	Entire	Rod	+ve
10	S10	Creamy white	Large circular	Entire	Rod	+ve
11	S11	White	Small circular	Entire	Rod	+ve
12	S12	Creamy yellow	Small circular	Entire	Rod	-ve
13	S13	Creamy yellow	Small circular	Entire	Rod	-ve

Table-4 *Biochemical characters of Halotolerant bacterial isolates*

Biochemical test													
Isolates	IMViC test				Urease test	Nitrate reductase test	Carbohydrate fermentation test			Starch hydrolysis	Gelatin Hydrolysis	Motility test	Catalase test
	Indole	Methyl red	Voges Proskauer	Citrate utilization			Glucose	Sucrose	Fructose				
S1	+	+	-	+	-	+	A	A	A	-	-	-	+
S2	+	+	-	+	+	+	AG	AG	AG	+	+	+	+
S3	+	+	-	-	-	+	A	A	A	-	-	-	+
S4	+	+	-	+	-	+	AG	AG	AG	+	+	+	+
S5	+	-	+	-	-	+	AG	AG	AG	-	-	-	+
S6	+	+	-	-	-	-	AG	AG	AG	-	-	-	+
S7	+	+	-	-	+	+	AG	AG	AG	-	+	-	+
S8	+	+	-	+	+	+	AG	AG	AG	+	+	+	+
S9	+	-	+	+	-	+	A	A	A	+	+	-	+
S10	+	-	+	+	-	-	A	A	A	+	-	-	+
S11	+	+	-	+	+	+	A	A	A	+	+	-	+
S12	+	+	-	+	-	+	AG	AG	AG	+	-	-	+
S13	+	-	+	+	-	-	AG	AG	AG	+	-	-	+

A-Acid G-Gas AG-Acid and gas + positive – negative

Biochemical characterization

Indole production test

The Halotolerant bacterial isolates were inoculated in peptone water broth and incubated at 28±2ºC for 48-96 h. After incubation 0.5 ml of Kovac's reagent was added and shaken. Development of pink or red colour in the alcohol layer indicated a positive reaction [13].

Methyl red test

The methyl red test was performed by inoculating the isolates in 5 ml glucose phosphate broth and incubating at 28±2ºC for 2 to 5 days. As growth occurs, five drops of 0.04 % solution of alcoholic methyl red was added and observed for bright red colour formation which indicated a positive result and yellow colour indicated negative result.

Voges- Proskaeur test

The test organisms were inoculated in 5 ml glucose phosphate broth and incubated at 28±2ºC for 48 h. As growth occurs about 1ml of potassium hydroxide containing 0.3 % creatine and 3ml of α-napthol solution was added. Positive reaction is indicated by the development of pink colour within 2-5 min.

Citrate utilization test

Citrate utilization test was performed by streaking the halotolerant bacterial isolates on the Simmon's citrate agar slants. The slants were incubated at 28±2ºC for 48 h and observed for the colour change in medium from green to blue which indicated the positive result [14].

Urease test

Halotolerant bacterial isolates were streaked on urea agar slants. Slants were incubated at 28±2ºC overnight and observed for the development of purple pink colour which indicated positive urease test [15].

Nitrate reduction test

Nitrate reductase activity of the isolates was tested by inoculating the organism in 5ml nitrate broth. Incubate the broth at 28±2ºC for 96 h and after incubation one ml of α-napthylamine reagent and one ml of sulphanilamide reagent was added. A red colour development within few minutes indicated the presence of nitrate, a positive reaction [16].

Catalase test

The Halotolerant bacterial isolates were streaked on the nutrient agar plates and a drop of three percent H_2O_2 was placed on colonies of nutrient agar. Effervescence indicates the catalase positive reaction [12].

Carbohydrate fermentation test

The Halotolerant bacterial isolates were inoculated in nutrient broth containing respective reducing sugar and pH indicator phenol red along with a Durham's tube to check gas production. Change of the medium from red color to yellow and gas bubble in Durham's tube indicates acid and gas production.

Starch hydrolysis

The Halotolerant bacterial isolates were streaked on starch Agar media and incubated at 28±2°C for 48h. 1% Gram's iodine reagent solution was prepared and poured on top of plates. Clear area after adding Gram's iodine reagent indicates starch hydrolysis by the activity of amylase.

Gelatin hydrolysis

The Halotolerant bacterial isolates were stab inoculated into tubes containing nutrient gelatin. Inoculated and uninoculated control tubes were incubated at 28±2°C for 7 days. Gelatin liquefies above 28°C, to confirm liquefication the tubes were immersed in ice bath for 15 to 30 minutes, tubes were tilted to observe gelatin hydrolysis. Hydrolysed gelatin will result in a liquid medium even after to cold temperature, while uninoculated control medium will remain solid [4].

Motility Test

Halotolerant bacterial isolates were stab inoculated into motility test medium along with Triphenyl tetrazolium chloride (TTC) in the centre to depth of 0.5 inch. Incubate the inoculated isolates at 28±2ºC for 48 h. Pink color diffusing zone of growth line indicates a positive result [13].

Results and Discussion

Enumeration of bacterial population

Soil sample was serially diluted and plated on tryptic soya agar (TSA) media petriplates. The colony forming unit per gram of soil in different dilution factor in TSA media was represented in [Table-1]. A total of 90 isolates were selected, pure cultured and stored for further analysis.

Screening for salt tolerance

In this present study, salt tolerance with various concentration of NaCl showed different levels of tolerance in bacterial isolates. Bacterial isolates were screened upto 25 % NaCl concentration. Out of 90 isolates which were screened on Trypticase soy broth (TSB), 13 isolates exhibited significant salt tolerance up to 25 % NaCl concentration. These 13 extreme halophiles growth rate at different NaCl concentration is represented in [Table-2].

Colony and Cell Morphology

Most of the isolated colonies were transparent, translucent, circular, smooth. Color pigmentation varied from creamy yellow, white to creamy white. Cells shape to be rod.

Biochemical Tests

IMViC Test

All isolates showed positive result for indole production. Isolates S1, S2, S3, S4, S6, S7, S8, S11 and S12 showed methyl red positive test. Isolates S5, S9, S10 and S13 showed Voges Proskauer test positive as pink color was observed. Isolates S1, S2, S4, S8, S9, S10, S11, S12 and S13 were found to be citrate utilization test positive as the color of media turned blue.

Fermentation of carbohydrates

Isolates S1, S3, S9, S10, S11 produced only acid, whereas isolates S2, S4, S5, S6, S7, S8, S12 and S13 produced both acid and gas in the presence of reducing sugar glucose, fructose and sucrose. Isolates S2, S7, S8, and S11 were found to be urea test positive. Isolates S1, S2, S3, S4, S5, S7, S8, S9, S11 and S12 showed positive and developed reddish color. Isolates S2, S4, S8, S9, S10, S11, S12 and S13 showed positive result for starch hydrolysis and formed clear area. Isolates S2, S4, S7, S8, S9 and S11 showed positive result for gelatin hydrolysis. Isolates S2, S4 and S8 showed positive result for motility test. All isolates showed positive result for catalase test. Halophilic bacteria were categorized based on salt tolerance capacity and growth rates of extreme halophiles were checked in different NaCl concentration [Table-2]. In our study the extreme halophiles ranged from fast, moderate and slow growers, these results were in accordance with those reported [8,9]. Halophiles isolated and enumerated had different phenotypic characters [Table-3]. Details of colony morphology and results were in accordance with those reported by [16] who reported their isolates to be Creamy white to yellow in pigmentation, rod to coccus in cell shape. In the present study Halophiles showed positive result for many of the biochemical test [Table-4] which was in agreement to result obtained [11,15].

Conclusion

In the present study, diversity analysis of halotolerant bacterial communities from soil samples of Tindivanam Taluk of Villupuram District of Tamil Nadu was analysed. Further diversity analysis from extreme environments of high salt concentrations should be focused to isolate better performing cultures. Only biochemical test and screening based on salt concentration was focused in this study. Future study based on plant growth promoting traits would prove beneficial for ameliorating salinity stress in crops.

Application of research: Studies related to amelioration of salinity stress in rice crop using halophilic bacterial strains.

Research Category: Agricultural Microbiology

Abbreviations: M- Molar, CFU- Colony Forming Unit.

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Study area / Sample Collection: Tindivanam Taluk of Vilupuram District of Tamil Nadu, India.

Cultivar / Variety name: Nil

Conflict of Interest: None declared

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