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Isolation, Screening and Characterization of Halotolerant Microorganisms from Three Different Saltern Sites of Tamil Nadu, India

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Abstract: ***Extremozyme is one of the major products derived from the extremophile organisms in the biotechnology industry. Halophilic microorganisms have high salt tolerance capacity and they survive at extreme conditions. Enzymes like amylases, proteases, lipases and nucleases were produced from these halophiles. The isolation, screening and characterization of hydrolytic enzymes producing haloterant strains from three different sites in Tamil Nadu (Marina beach-Chennai, Salt pans-Marakanam and Tuticorin) were carried out. Eighteen halotolerant bacteria strains were isolated and identified by 16S rRNA gene sequencing analysis. Bacillus sp. was found to be dominant in the saltern sediments. From the hydrolytic screening results, six isolates were capable of producing protease, nine were able to produce amylase, and only one isolate produces cellulase and three isolates able to produce pectinase. In the antibiotic susceptibility test, most of the isolates were found to be sensitive to most of the antibiotics. Only two strains such as Cellulosimicrobium funkei (SW3) and Enterobacter hormaechei (WW2) were resistant to ten antibiotics.***

Keywords: Extremozyme, Halophiles, Amylase, Antibiotics, Pectinase

I. INTRODUCTION

Advent of enzymes from extremophiles signifies an essential breakthrough in biotechnology industry with an increase in the extermozyme usage in numerous industries. Halotolerant microorganisms which are able to existing in saline environments and it have valuable application in different fields of biotechnology. Recent past, halotolerant organisms are regarded as with great deal of significance as of its biotechnological potential, particularly for harboring genes responsible for salt/osmotic tolerance and enzymes such as amylases, proteases, lipases and nucleases of industrial capabilities [1]. The present investigation aims in isolation, screening and characterization of hydrolytic enzymes producing haloterant strains from three different sites in Tamil Nadu (Marina beach-Chennai, Salt pans-Marakanam and Tuticorin).

II. MATERIALS AND METHODS

A. Sample Collection and Culture Conditions

Samples were collected from saltern sediments (Figure.1) of Marina beach (Latitude: 13.0542° N, Longitude: 80.2837° E), salt pans of Marakanam (Latitude: 12.2° N Longitude: 79.95° E) and salt pans of Tuticorin (Latitude: 8.8100° N, Longitude: 78.1400° E). Temperature and pH of the sampling sites was 38 ºC and 7.5. Samples were collected in a sterile container and processed within 15 hours after collection.

B. Isolation of Microorganisms

Collected samples were serially diluted with saline water and plated in a nutrient agar plates with 5% sodium chloride, kept for overnight incubation. After seven days incubation different morphological isolates were picked, and sub cultured on the same medium. Purified strains were maintained in 80% glycerol stocks for further investigation.

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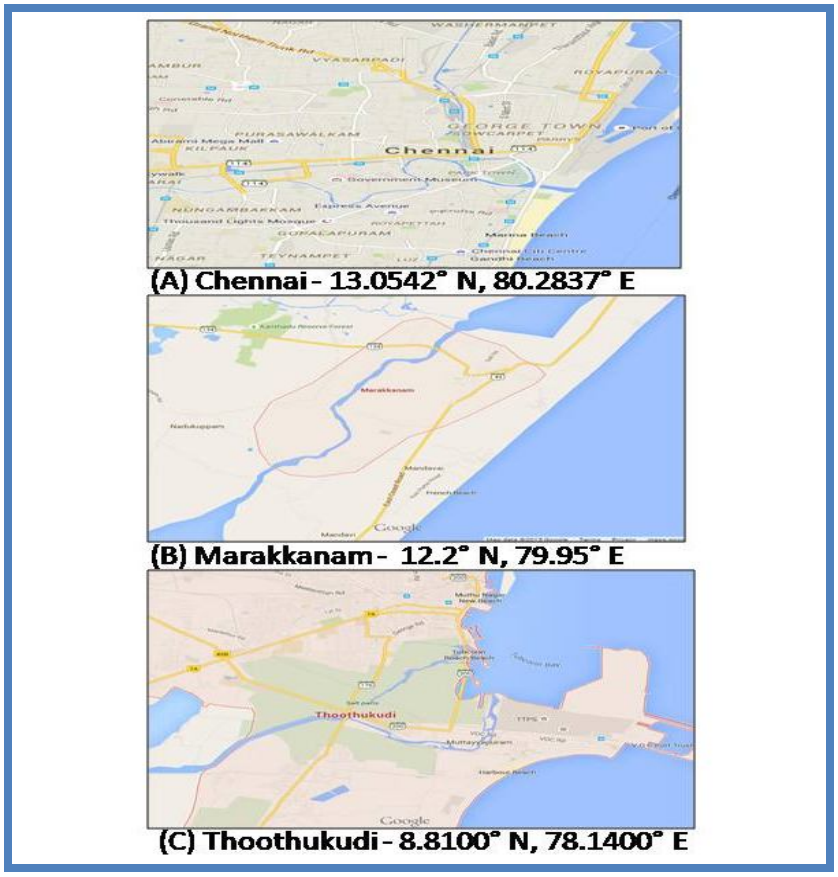

Figure 1: Sites used for sample collections (Source : google map).

C. Screening of Microorganisms for Extracellular Hydrolytic Activities

To investigate the production of extracellular hydrolases, different enzymatic agar plate assays were performed. The pH of media was adjusted to 7.3–7.6 and appropriate amount of salt were added for detecting hydrolytic activities from halotolerant bacteria. The presence of proteolytic activity was screened in nutrient agar containing 5% NaCl supplemented with casein as substrate. Plates were incubated for 7 days at 36 °C. Amylase activity was determined by using nutrient agar with 5% NaCl supplemented with starch as substrate. Plates were kept for 1 week incubation at 36 °C. After incubation for 1 week, the plates were flooded with 0.3% iodine solution. Carboxy Methyl Cellulase activity of the cultures was screened in a solid medium containing carboxy methyl cellulose (CMC) 5 g/L; K_2HPO_4 2 g/ L; $NaNO_3$ 1 g/ L; $MgSO_4$ 0.5 g/ L; KCl 1 g/ L; yeast extract 0.5 g/ L; glucose 1 g/ L; agar 17 g/ L, and 5% NaCl. After incubation at 36 °C for 7 days, the plates were flooded with 0.1% Congo red solution. To determine the pectinolytic activity on the plates medium containing pectin 10 g/ L, K_2HPO_4 2 g/ L, $(NH_4)_2SO_4$ 1.4 g/ L, $MgSO_4 \cdot 7H_2O$ 0.02%, nutrient solution 1 g/ L; $MnSO_4 \cdot H_2O$, 1.6 mg/ L; ($FeSO_4 \cdot 7H_2O$, 5 mg/ L $ZnSO_4 \cdot 7H_2O$, 1.4 mg/ L; $CaCl_2$, 2 mg/ L), agar 20 g/ L and 5% NaCl. After incubation at 36 °C for 7 days, the plates were flooded with 0.3% iodine solution [2].

D. Identification of Microorganisms

Morphological characteristics and preliminary gram's staining were studied for the isolated microbes as per the standard protocols. The biochemical characterization such as catalase, oxidase, nitrate reduction and sugars viz, glucose, mannose, sucrose and mannitol tests were investigated for all the isolated halotolerant bacteria according to Bergy's manual. Then all the isolates were subjected to DNA extraction. Cells were grown in nutrient broth with 5% Nacl and DNA was extracted as per the standard protocol [3]. The genomic DNA isolated from the bacteria was amplified using the following universal 16S rRNA primers: forward primer 5' GAG TTT GAT CCT GGC TCA G 3' (E.coli positions 8–27) and reverse primer 5'ACG GCT ACC TTG TTA CGA CTT 3' (E.coli positions1494–1513). Polymerase chain reaction was carrying out as described previously [4] with slight modifications. The initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 sec, primer annealing at 60 °C for 1 min, and extension at 72 °C for 1.5 min. The final elongation step was at 72 °C for 10 min. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel in 1X TAE. The amplified PCR products were sequenced (Macrogen, Seoul,

Korea). The obtained sequence was compared with NCBI database using BLAST search tool and the phylogenetic tree was constructed with the MEGA v5.04 [5] using neighbor joining method with a bootstrap value of 1000.

E. Antibiotic Susceptibility Test

All the isolated strains were tested for antibiotic susceptibility test to determine the resistant pattern of the isolated strains. Overnight cultures were taken and swabbed on Mueller Hinton Agar (MHA) supplemented with 5% NaCl and the following antibiotics disc were placed on the MHA plates : Bacitracin (10 U), Vancomycin (30 µg), Gentamycin (10 µg), Tetracyclin (30 µg), Ofloxacin (5 µg), Penicillin G (10 U), Streptomycin (10 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Ampicillin (10 µg), Methicillin (30 µg), Novobiocin (5 µg), Rifimpicin (5 µg), Cefoxitin (30 µg), Amikacin (30 µg) and Erythromycin (15 µg). Zone of inhibition was measured after 48 h of incubation.

III. RESULTS AND DISCUSSION

A. Isolation of Halotolerant Bacteria

In the present study, totally eighteen halotolerant bacteria were obtained from three different sites of Tamil Nadu, Marina beach, saltern sediment of Marakanam and Tuticorin. Gram's staining indicated that out of eighteen strains twelve were gram positive and six were gram negative rods, in which few of the isolates were pigmented (Figure 2 and 3). Most of the isolates were rods, circular and ellipsoidal in shape. The strains were able to grow in 5-10% NaCl concentration and showed the halotolerant property.

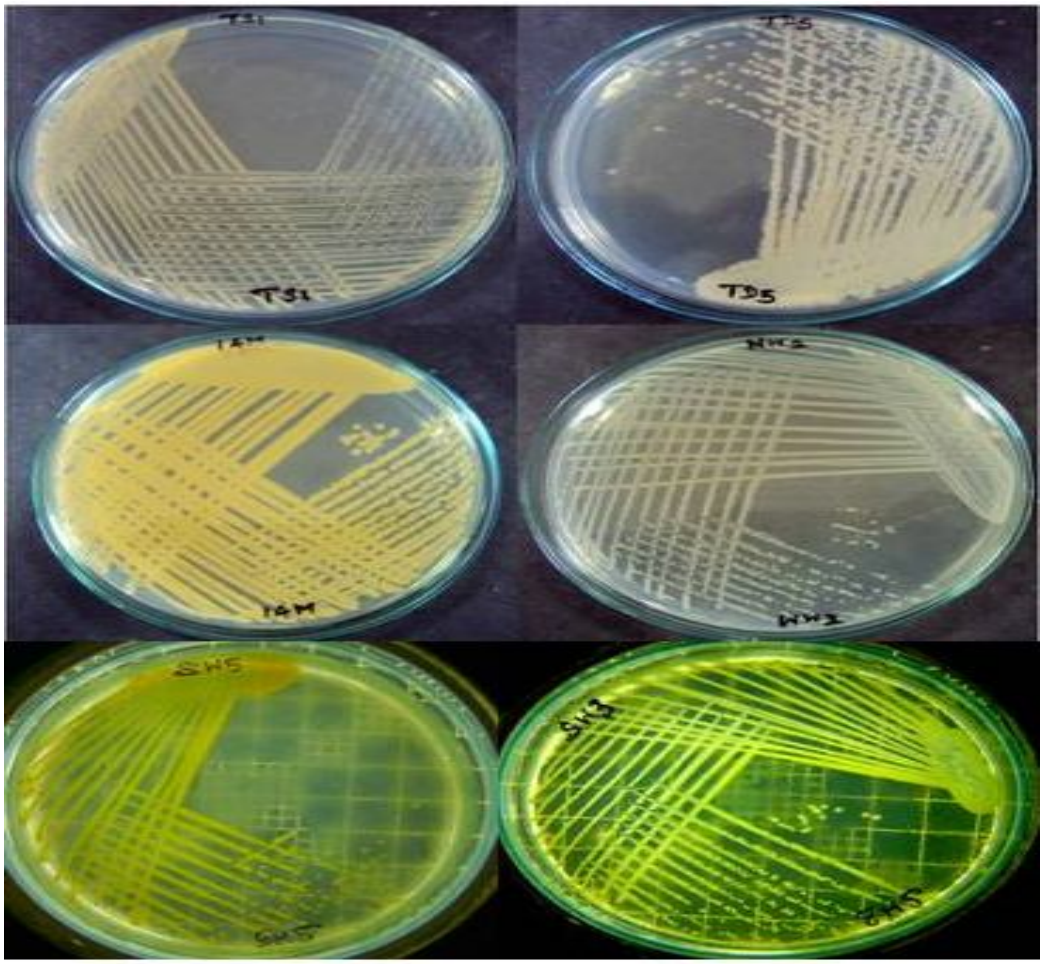

Figure 2: Pure culture of isolated halotolerant bacteria. TS1-Bervibacterium tationis, TD5-Bacillus licheniformis, 14M-Halobacillus trueperi, WW2-Enterobacter hormaechei, SW5-Arenibacter latericius and SW3-Celluloimicrobium funkeii.

Moderate halophilic bacteria have been reported, that the bacteria could grow in media containing NaCl from 3-5% and they were widely scattered in different marine environments like crystallizer ponds, salt marshes, saline soil and solar salterns [6- 8]. Hongyu *et al.*, [9] reported halophilic organisms isolated from salt ponds of China and those organisms were able to grow in 20% of NaCl.

Most of the halophilic bacteria require huge amount of NaCl and most of them necessitate magnesium ions for their growth whereas halotolerant bacteria does not need magnesium ions for their growth [10]. Biochemical characterizations of all the isolated strains were tabulated in Table 1 and 2. The strains showed positive for catalase, oxidase and nitrate reductase. Most of the strains were capable of fermenting glucose, xylose, mannitol, sorbitol and sucrose.

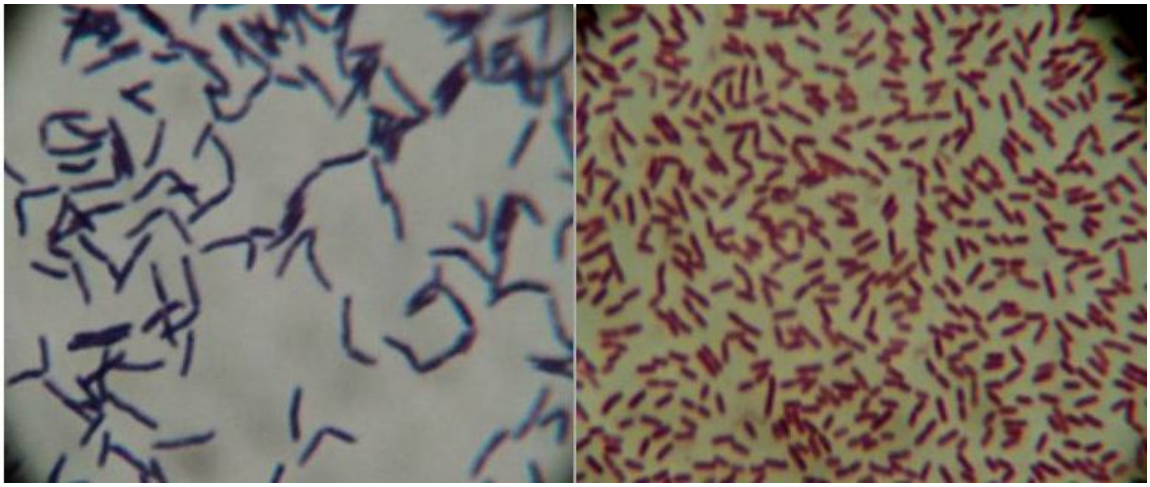

Figure 3: Gram's staining of TD6- *Bacillus licheniformis* and WW2- *Enterobacter hormaechei*.

Table 1: Biochemical Profile of the Isolated Halotolerant Bacteria

Characteristics	SW3	SW5	SW6	SS3	WW2	WW5	2M	3M	4M
Grams staining	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
Motility	+	-	+	+	+	+	+	+	+
Pigmentation	Pale yellow	Yellow	Pale pink	NP	NP	NP	-	Cream	Light orange
Shape	C	E	E, S	C	C	C	E, S	C	E
Growth in NaCl	3-8%	3-8%	3-12%	2-5%	2-5%	2-5%	2-10%	3-15%	3-12%
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	-	-	-	-	+	+
Glucose	+	+	-	+	+	+	+	-	+
Mannose	+	-	-	+	+	+	+	+	-
Sucrose	+	+	-	-	-	-	+	+	+
Sorbitol	-	+	-	-	-	-	-	+	-
Mannitol	+	-	-	-	-	-	+	-	-
Nitrate reduction	+	+	-	+	+	+	_	-	-
Starch Hydrolysis	-	-	-	+	+	+	+	+	-
Casein Hydrolysis	-	-	-	+	+	+	+	-	-

Positive (+), Negative (-), C- Circular, E- Ellipsoidal, S- Spheric

Table 2: Morphological and Biochemical Characterization of the Isolated Halotolerant Bacteria

Characteristics	15M	16M	TY	TS1	CW	TD3	TD4	TD5	TD6
Grams staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Motility	+	+	+	+	+	+	+	+	+
Pigmentation	Dirty white	White	Pale yellow	Creamy yellow	White	White	Dirty white	White	White
Shape	C	E	C	C	C	E	C	E	E
Growth in NaCl	3-5%	3-10%	3-5%	3-8%	3-7%	3-10%	3-5%	3-8%	3-10%
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	+	-	+	+	+	+
Glucose	-	+	-	-	+	+	+	+	+
Mannose	-	+	+	+	-	+	-	+	+
Sucrose	+	+	-	-	-	+	+	+	+
Sorbitol	+	+	-	+	+	+	+	+	+
Mannitol	+	+	-	+	-	+	+	+	+
Nitrate reduction	-	+	+	-	+	+	-	+	+
Starch Hydrolysis	-	+	-	-	-	+	+	+	+
Casein Hydrolysis	+	+	-	-	-	+	+	+	+

Positive (+), Negative (-), C- Circular, E- Ellipsoidal, S- Spherical

B. Screening of Hydrolytic Enzymes

Among the eighteen isolated halotolerant bacteria a wider range of hydrolytic enzymes was detected (Table 3 and 4). Seven organisms were able to produce protease, nine organisms were able to produce amylase, three organisms were able to produce pectinase and one organism was able to produce cellulase. In which, four organisms were able to produce multiple hydrolytic enzyme such as protease, amylase and pectinase. Majority of the organisms showed positive for amylase enzyme (Figure.4). Khunt *et al*., [11] has been reported that majority of halophiles contains amylase activity. Similarly, Sanchez-Porro *et al*., [12] and Moreno *et al*., [13] were reported the occurrence of DNase, followed by amylase and lipase in the halophiles.

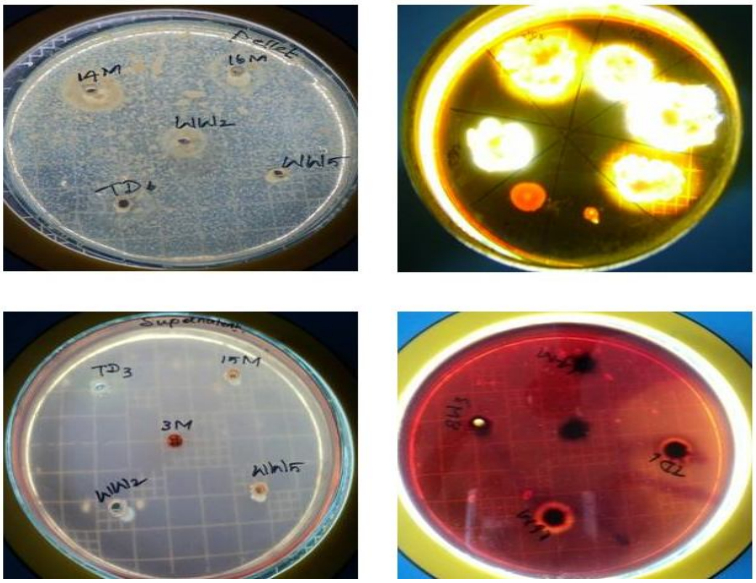

Figure 4: Screening of hydrolytic enzymes from isolated halotolerant bacteria in casein, starch, carboxymethyl cellulose and pectin plates at 37 ºC and zone of clearance was determined by spot and well diffusion method.

Table 3: Qualitative Screening of Hydrolytic Enzyme Activity From the Isolated Halotolerant Bacteria

Isolated strains		Protease	Amylase	Cellulase	Pectinase
Cellulosimicrobium funkei		-	-	+	-
Arenibacter latericius		-	-	-	-
Pseudidiomarina donghaiensis		-	-	-	-
Kangiella spongicola		-	-	-	-
Enterobacter hormaechei		+	+	-	-
Cronobacter malonaticus		-	+	-	-
Virgibacillus proomii		-	+	-	-
Halomonas ventosae		-	+	-	-
Halobacillus trueperi		-	-	-	-
Bacillus altitudinis		+	-	-	-
Bacillus vallismortis		+	+	-	+
Kocuria palustris		-	-	-	-
Brevibacterium stationis		-	-	-	-
Kocuria salsicia		-	-	-	-
Bacillus vallismortis		+	+	-	+
Bacillus licheniformis		+	+	-	-
Bacillus licheniformis		+	+	-	-
Bacillus vallismortis		+	+	-	+

C. Identification of Isolated Halotolerant Bacteria

Genomic DNA was extracted from the eighteen isolated halotolerant bacteria and the PCR products were obtained by the 16S rRNA gene amplification using universal primers. The obtained 16S rRNA gene sequence confirmed 97%-99% similarity with their closest phylogentic relatives (Figure 5). The isolated type strains and its accession number were tabulated in Table 4. The Phylogenetic characteristics of eighteen halotolerant bacteria were found closely related to Virgibacillus proomii 2M (HQ992819), Halomonas ventosae 3M (HQ992820), Halobacillus trueperi 14M (JN993994), Bacillus attitudnis 15M (JF769748), Bacillus vallismortis 16M (HQ992821), Cellulosimicrobium funkeii SW3 (JN993996), Arenibacter latericius SW5 (JN993997), Pseddidomarina donghaiensis SW6 (HQ992822), Enterobacter hormaechei WW2 (JN993998), Cronobacter malonaticus WW5 (JN993999), Bacillus vallismortis TD3 (HQ992817), Bacillus licheniformis TD6 (JF769746), Bacillus licheniformis TD5 (JF769749), Bacillus vallismortis TD4 (HQ992818), Bervibacterium stationis TS1 (JF769747), Kocuria palustris TY (JN993993), Kocuria sp CW (JN993998), Kangiella spongicola SS3 (JN993995). Bacillus vallismortis was previously isolated from soil and sea water samples [14, 15]. Our results of morphological and biochemical characteristics were also reported [16]. Among the eighteen isolated strains Bacillus sp. was the most predominant in the saltern sediments. Similarly in the previous studies Ventosa et al., [17] has been reported that the gram positive bacteria consigned to Bacillus were widely represented in saline soils.

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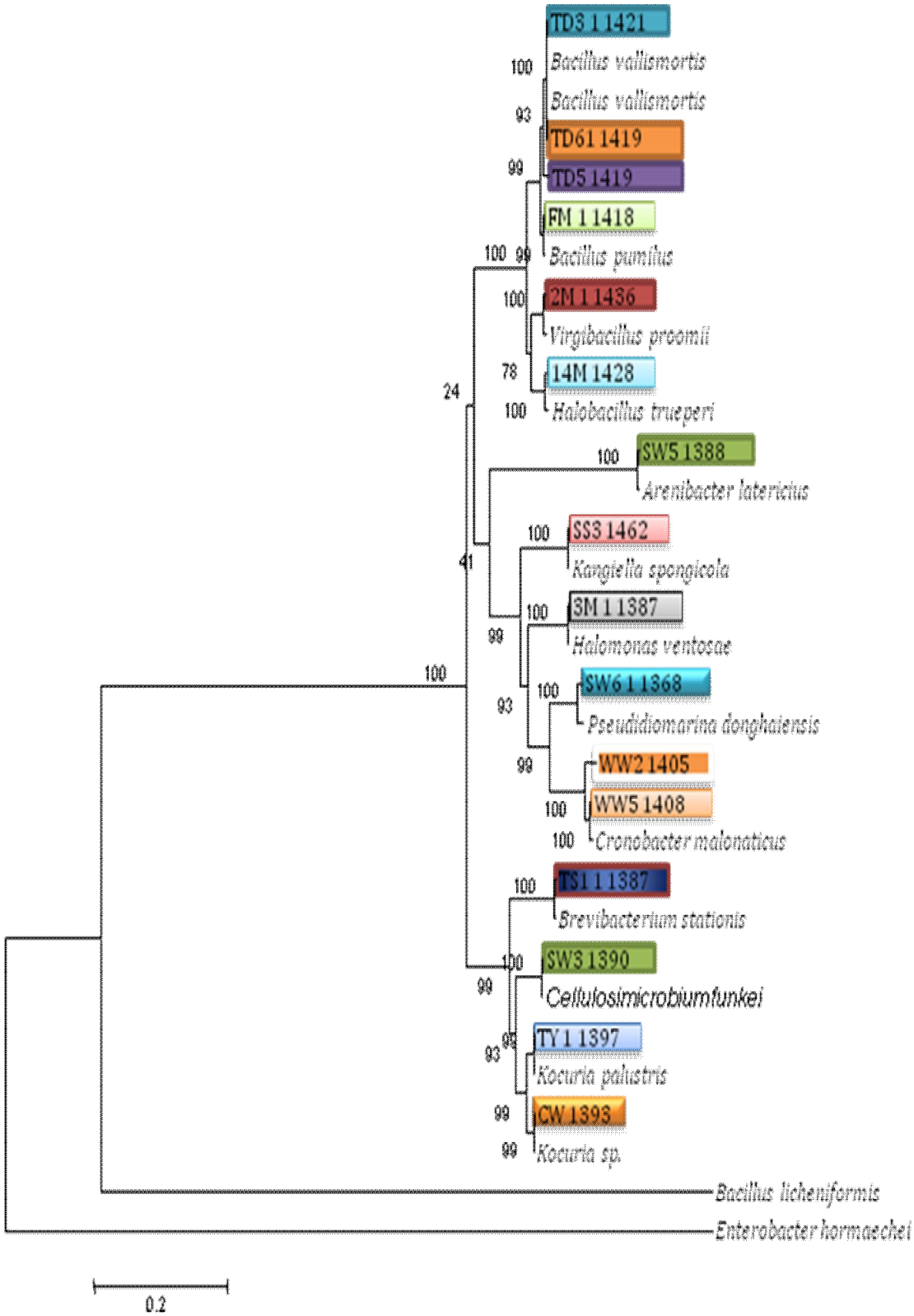

Figure 5: Phylogenetic tree showing the position of the halotolerant isolates, based on the partial 16S rRNA sequence comparison, obtained by the neighbour-joining method. Bootstrap values are indicated on the branches.

Table 4: Phylogenetic Identification of Isolated Halotolerant Bacteria of Sequence Similarity With its Type Strains and Their Accession Numbers

Strain	Type strain	Sequence similarity	Accession number
SW3	Cellulosimicrobium funkei	99%	JN993996
SW5	Arenibacter latericius	99%	JN993997
SW6	Pseudidiomarina donghaiensis	99%	HQ992822
SS3	Kangiella spongicola	99%	JN993995
WW2	Enterobacter hormaechei	97%	JN993998
WW5	Cronobacter malonaticus	99%	JN993999
2M	Virgibacillus proomii	97%	HQ992819
3M	Halomonas ventosae	98%	HQ992820
14M	Halobacillus trueperi	98%	JN993994
15M	Bacillus altitudinis	98%	JF769748
16M	Bacillus vallismortis	99%	HQ992821
TY	Kocuria palustris	99%	JF769747
TS1	Brevibacterium stationis	98%	JF769750
CW	Kocuria salsicia	99%	JN993993
TD3	Bacillus vallismortis	99%	HQ992817
TD6	Bacillus licheniformis	99%	JF769746
TD5	Bacillus licheniformis	97%	JF769749
TD4	Bacillus vallismortis	99%	HQ992818

D. Antibiotic Susceptibility Test

Results of antibiotic susceptibility test (Figure 6) of all the isolates were presented in Table 5. Most of the isolates were sensitive to most of all the antibiotics whereas Cellulosimicrobium funkeii SW3 was resistant to ten antibiotics such as Gentamycin (10 µg), Penicillin G (10 U), Streptomycin (10 µg), Ampicillin (10 µg), Nalidixic acid (30 µg), Methicillin (30 µg), Novobiocin (5 µg), Rifimpicin (5 µg), Cefoxitin (30 µg), Amikacin (30 µg) and Cronobacter molanticus WW2 was sensitive to ten abtibiotics such as Bacitracin (10 U), Vancomycin (30 µg), Penicillin G (10 U), Streptomycin (10 µg), Ampicillin (10 µg), Methicillin (30 µg),

Novobiocin (5 µg), Rifimpicin (5 µg), Cefoxitin (30 µg), Erythromycin (15 µg). All the isolates were showed sensitive to ofloxacin, chloramphenicol and tetracycline whereas streptomycin was resistant to eleven isolated strains.

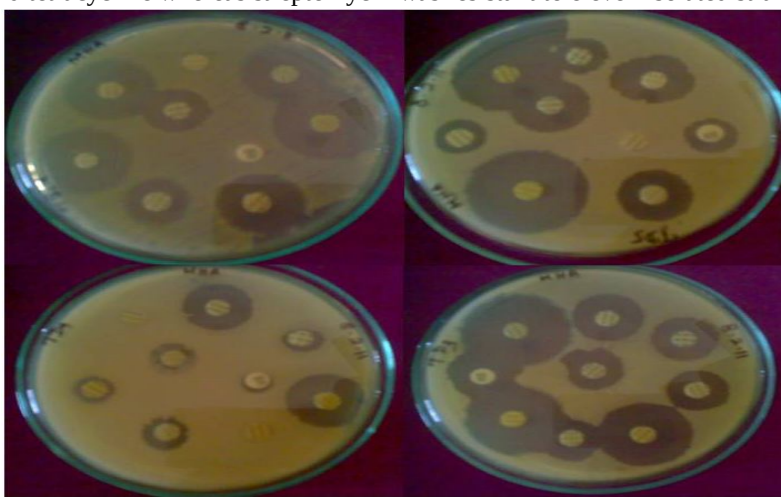

Figure 6: Antibiogram (zone of inhibition) TS1-Bervibacterium stationis, TD5-B. licheniformis, TD6-B. licheniformis and TD4-B. vallismortis.

Table 5: Antibiotic Susceptibility Test for the Identified Halotolerant Strains

Strain	B	VA	G	T	OF	P	S	C	NA	A	M	NV	R	CF	AK	E
SW3	S	S	R	I	S	R	R	S	R	R	R	R	R	R	R	I
SW5	I	S	R	S	S	I	R	S	I	I	S	S	S	S	R	S
SW6	S	S	S	S	S	R	S	S	S	R	S	S	S	S	R	S
SS3	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	S
WW2	R	R	S	S	S	R	R	S	S	R	R	R	R	R	S	R
WW5	S	S	S	S	S	I	R	S	S	S	S	S	S	S	R	S
2M	S	S	S	S	S	S	I	S	S	I	S	S	S	S	S	S
3M	S	R	S	S	S	S	I	I	S	S	S	R	S	S	S	S
14M	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
15M	R	S	S	S	S	R	R	S	I	S	S	R	I	S	S	S
16M	R	S	S	S	S	S	I	S	S	S	S	I	S	S	S	S
TY	S	S	S	S	S	S	I	S	R	S	S	S	S	S	S	S
TS1	S	S	S	S	S	R	S	S	R	S	S	R	S	S	I	S
CW	S	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S
TD3	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
TD4	R	S	S	S	S	R	R	S	I	I	I	I	I	S	R	R
TD5	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
TD6	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S

B, bacitracin (10 U); VA, vancomycin (30 µg); G, gentamycin (10 µg); T, tetracyclin (30 µg); OF, ofloxacin (5 µg); P, penicillin G (10 U); S, streptomycin (10 µg); C, chloramphenicol (30 µg); NA, nalidixic acid (30 µg); A, ampicillin (10 µg); M, methicillin (30

µg); NV, novobiocin (5 µg); R, rifimpicin (5 µg); CF, cefoxitin (30 µg); AK, amikacin (30 µg) and E, erythromycin. Resistant - R, Sensitive - S and Intermediate - I.

IV. CONCLUSION

In the current study, eighteen halotolerant bacteria were isolated from different sites. All of them were identified by 16S rRNA gene sequencing analysis. Bacillus sp. was found to be dominant in the saltern sediments. From the hydrolytic screening results, six isolates were capable of producing protease, nine were able to produce amylase, and only one isolate produces cellulase and three isolates able to produce pectinase. In the antibiotic susceptibility test, most of the isolates were found to be sensitive to most of the antibiotics. Only two strains such as Cellulosimicrobium funkei (SW3) and Enterobacter hormaechei (WW2) were resistant to ten antibiotics. As there was more number of amylase producing strains in the present study, the amylase producing strains were chosen for further studies.

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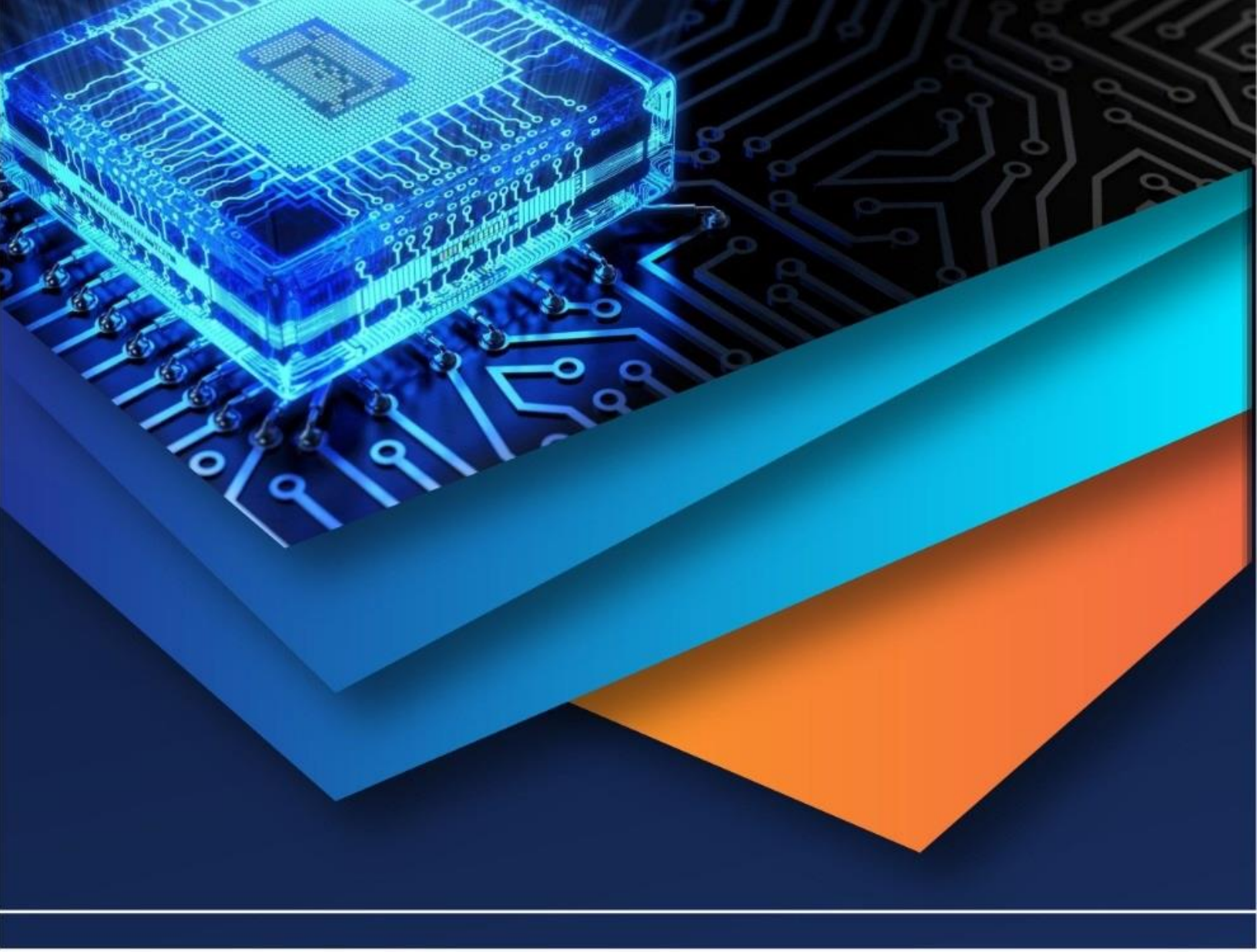

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