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Thermophilic Bacteria as a Source of Novel Polymers for Biotechnological Applications

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Authors' contributions

This work was carried out in collaboration between both authors. Author PR designed the study, managed the analyses of the results, performed the statistical analysis and wrote the first draft of the manuscript. Author AS performed literature survey carried out sample collection and laboratory experiments. Both authors have read and approved the final manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: We analyzed thermotolerant bacterial isolates of thermophiles from various regions in South East coasts of India.
Study Design: A total of forty three (9.6%) thermotolerant bacterial isolates of thermophiles were secluded out of 444 isolates obtained from water samples from the Adyar River, Marina coast, Muttukadu Backwaters, Kovalam coast (India) and a prawn hatchery. In addition a novel thermotolerant Gram positive *Bacillus* type bacterium from the pelletized feed of fish was examined.
Results: Amongst 134 isolates that grew on *E. coli* FAGI agar, 23 (17%) were thermotolerant when tested at 15 psi for 30 min. Of the 72 isolates that grew on *Staphylococcus* Baird Parker agar 10 (13%) were thermotolerant, whilst among the 128 isolates that grew on *Salmonella* agar ÖNÖZ 10 (8%) were thermotolerant. In contrast, none of the 110 isolates that grew on TCBS agar (*Vibrio* sp.)

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exhibited thermotolerance, although they were isolated from the same regions as the other bacteria. The optimal temperature for the growth of most thermophiles was 45ºC, except for 9 isolates. Of the latter, 7 grew at 50ºC, one at 52ºC and one at 55ºC, respectively. There were some extremely thermotolerant bacterial isolates that exhibited consistency in profuse growth at 15 psi (121°C) for 30 min. Other isolates showed inconsistency in growth after autoclaving the cultures at 15 psi (121°C) for 30 min, and these bacteria showed development of endospores. Isolates of thermotolerant bacteria that grew on *Staphylococcus* Baird Parker agar and *Salmonella* agar ÖNÖZ were predominantly antibiotic resistant when compared with those bacterial isolates that grew on *E. coli* FAGI agar. A major 43 kDa toxin-protein as well as 15 other minor protein subunits was detected in the thermotolerant *Bacillus* sp. isolate. The proteins were toxic and caused mortality to gold fish *Cyprin macrophthalmus.*

Conclusions: This study documents 43 thermotolerant bacterial isolates from water samples and a *Bacillus*-type bacteria from fish feed in Chennai region, India and these bacterial isolates offer the potential for exploitation in biotechnological applications.

Keywords: Thermophiles; bacterial isolates; growth; antibiotic resistance; pathogenicity.

1. INTRODUCTION

Bacteria successfully thrive in every part of the earth's surface, ranging from boiling hot waters of thermal springs, ice, air, and soils of backyard gardens to the inter- and intracellular environments of living organisms. Some of the bacteria infect higher organisms causing disease, and there are many methods and chemotherapeutic tools available to control the detrimental effects of bacterial infections. Thanks to their short life spans and ability to multiply exponentially, these microorganisms have been exploited for the benefit of humankind for thousands of years in the manufacture of cheese, wine, bread, antibiotics and pharmaceuticals, to control pests, treat sewage, and to degrade pollutants, plastics and oil spills. In recent years, biotechnologists have continued to search the surface of the earth/water for microbes that might prove to be valuable in the development of new drugs, innovative processes and products for improving existing industrial processes. Normally, thermotolerant bacteria are collected from environments with temperatures up to 75ºC and from depths extending to 2.8 kilometers below the earth's surface. However, biologists have found thermotolerant bacteria capable of growing at 110ºC in deep-sea hot springs and volcanic vents, while the subsurface microorganisms survive and multiply at temperatures as high as 140ºC. A thermophile is an organism that thrives at temperatures between 41 and 122ºC [1-4]. Thermophilic bacteria are classified as psychrophiles -10°C to 25°C; mesophiles 10°C to 45°C; thermophiles (40°C to 80°C) having temperature optimum between 40 and 70ºC, whereas the extreme thermophiles exhibit growth beyond 80ºC. Thermotolerant (facultative thermophiles/ moderate thermophiles) bacteria are known to exist in nature at elevated temperatures and exhibit growth at both 37 and 55ºC [1-5]. Such thermophiles have a high potential in biotechnology and are candidates for investigation regarding possible roles in industrial and biotechnological processes. It is well recognized that the temperature stability of enzymes from thermophiles, which is often accompanied by a wide pH range and solvent resistance, can prove useful in biotechnological applications in, for example, the food, chemical (detergent), and pulp and paper industries [2,3]. Such enzymes include DNA polymerases, cellulases, hemicellulases, amylases, proteases, lipases etc. In the current study, attempts were made to collect, isolate, characterize and identify thermotolerant bacteria from various sources such as fish feed, infected fish/shrimp, and sea/river waters. We further evaluated their growth characteristics for various industrial and biotechnological applications and their potential utility in prevention and management of diseases. We have also developed a suitable growth media for the isolation of thermophiles from various sources and for their cultivation in laboratory environments.

2. MATERIALS AND METHODS

2.1 General

A total of 444 bacterial isolates were obtained from water samples from various sources: Adyar River, Marina coast, Muttukadu Backwater, Kovalam coast, pelletized prawn feed and prawn hatchery in and around Chennai, Tamil Nadu, India. The bacterial isolates were grown on

Salmonella agar ÖNÖZ, *Staphylococcus* Baird Parker agar, *E. coli* FAGI agar, and *Vibrio* TCBS agar, and were characterized and identified [6,7]. The isolated thermophilic bacteria were further cultured in LB broth and plated on nutrient agar plates. Gram staining [6] was used to differentiate the bacteria amongst the microbial flora and to study morphology. Regular biochemical tests viz. Indole test, MRVP test, Citrate Utilization test, Urease test, Starch Hydrolysis test, Triple sugar iron agar test, Bile esculin test, Nitrate reduction test, Oxidase test and Amino Acid Deamination test were carried out, following the standard methods described by Bailey & Scott and Holt et al. [6,7].

2.2 Experiments for the Detection of Thermophiles

2.2.1 Protocol 1

Protocol 1 was applied to all 444 bacterial isolates to determine whether or not these cultures survive autoclaving at 15 psi (121°C) for 30 min and consistently exhibit extreme thermotolerance. Nutrient broth (25 ml) was seeded with a loop full of well grown bacterial culture from a nutrient agar plate, incubated in an orbital shaker incubator at 37°C overnight, and the well grown (turbid; optical density 1.8-2) bacterial broth was autoclaved at 15 psi (121°C) for 30 min. One hundred µl of the autoclaved broth was placed onto freshly prepared nutrient agar plates and incubated overnight at 37°C.

2.2.2 Protocol 2

In protocol 2, nutrient broth (5 ml) was seeded with a loop full of well grown thermotolerant bacterial culture from a nutrient agar plate, incubated in an orbital shaker incubator at 37°C overnight and the well grown (turbid; optical density 1.8 -2) bacterial broth was centrifuged at 10,000 rpm for 5 min in Eppendorf tubes. The pellet was resuspended in 100 µl of nutrient broth and autoclaved (15 psi; 121°C) for 30 min. The pellet inocula were then plated on nutrient agar plates and incubated overnight at 37°C.

2.2.3 Protocol 3

This protocol was designed to test whether or not the cultures that were autoclaved in test tubes would produce different results from those autoclaved in Eppendorf tubes (protocol 2). The thermotolerant bacterial pellet cultures were autoclaved for 30 min at 15 psi (121°C) in glass test tubes and plated on nutrient agar plates. Incubation was carried overnight at 37°C and the growths of bacterial colonies (positive+ or negative-) were recorded. The experiment was repeated 11 times (Table 2) at various intervals.

2.2.4 Protocol 4

To determine the growth rate of the thermotolerant *Bacillus* sp. isolated from the pelletized fish feed, cells from an overnight broth culture (16 hrs) at 37ºC were inoculated into nutrient broth in triplicates. Initial Optical Density (OD) readings and OD readings after incubation at various time intervals up to 68 hrs and temperatures (37ºC, 40ºC, 45ºC and 50ºC) were taken using a UV spectrophotometer (UV-1601 Shimadzu) at 660 nm. Sterile nutrient broth served as control. An increase in OD from the initial OD was taken as positive for growth of the bacteria inoculated. Bacterial isolates which exhibited growth at 50ºC were termed "thermophilic bacteria".

2.2.5 Statistics and data analysis

Mean ± Standard error and one way ANOVA were determined by using MS Office Excel 2007.

2.3 Antimicrobial Sensitivity of the Bacterial Isolates

The antibiotic sensitivity of the thermotolerant bacteria isolated from the pelletized fish feed was tested against various antibiotics after plating the bacteria on *Staphylococcus* Baird Parker agar, *Salmonella* agar ÖNÖZ or *E. coli* FAGI agar (Tables 4-6). Susceptibility to several commonly used antibiotics was tested using the Agar-disc diffusion method of Bauer et al. [8]. Commercially available antibiotic discs obtained from Hi-Media-Bombay were used. The antibiotic discs were: Ampicillin (10 µg), Azithromycin (15 µg), Amikacin (30 µg), Chloramphenicol (30 µg), Cefaclor (30 µg), Ciprofloxacin (10 µg), Cephotaxime (30 µg), Cephadroxil (30 µg), Cephalexcin (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Neomycin (30 µg), Nalidixic acid (30 µg), Penicillin (10 units), Streptomycin (10 µg) and Tetracycline (30 µg). The bacteria were initially grown in nutrient broth incubated at 37ºC. The well grown cultures were diluted with sterile distilled water (1:2 ratio) and plated on nutrient agar plates, after which the antibiotic discs were placed on the agar surface. Inhibition zones were measured after a 24 hour incubation period [8]. The identification and characterization of thermotolerant bacteria was performed as described by Bergey [7].

2.4 Membrane Protein Profiles of Thermotolerant *Bacillus* sp.

A single colony of *Bacillus* sp. from fish feed was isolated from the master stock, inoculated in 300ml LB medium and incubated at 40ºC with constant shaking. At log phase of growth the culture was harvested by centrifugation at 10,000 rpm for 10 minutes. At this phase, maximum growth is occurring [9,10,11]. The pellet was resuspended in phosphate buffer saline (pH 7.2-7.4) and spun down at 5,000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml of TE buffer (pH8.0) with 0.1% SDS, mixed well using a vortex for 60 seconds and then centrifuged at 5,000 rpm for 10 minutes. The total membrane protein fractions were recovered, aliquoted and stored at -20ºC for further use. For each use, the membrane protein stock solution was diluted 2:3 with 12.5 mM Tris-buffer, (pH-6.8) containing 20% (w/v) glycerol, 4% SDS, 10% 2- mercaptoethanol and bromophenol blue. The proteins were solubilised by boiling for 5 minutes then applied to each well of the SDS-gel. The preparation of buffers and gels was performed as described by Laemmli [12]. The stacking gel concentration was 3.5% and the separating gel was 10%. Electrophoresis was carried out at a constant current of 15 MA through the stacking gel and 20 MA through the separating gel. The gels were stained with Coomassie blue (0.05% w/v) dissolved in methanol, acetic acid and water, 5:5:1. De-staining was performed by three successive washes with methanol, acetic acid and water. The molecular weight of the protein bands was determined with the help of a Gel- doc system.

2.5 Pathogenicity of *Bacillus* sp. against Gold Fish (*Cyprin macrophthalmus*)

Thermotolerant *Bacillus* sp. protein was extracted from overnight-incubated culture broth and was subjected to gold fish (*Cyprin macrophthalmus*) at various quantities (10 µg, 20 µg, 40 µg, 80 µg and 100 µg, respectively). The mortality of the challenged fishes was tabulated. The proteins from the *Bacillus* sp. were estimated by using the method of Bradford [13]. They were separated by discontinuous NATIVE-Polyacrylamide Gel Electrophoresis (NATIVE-PAGE) and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) [12] and were visualized by staining with silver nitrate staining procedures and Coomassie Brilliant Blue (for general proteins).

3. RESULTS

3.1 Growth of Bacterial Isolates on *Staphylococcus* Baird Parker Agar, *Salmonella* Agar ÖNÖZ, *E. coli* FAGI Agar and TCBS Agar

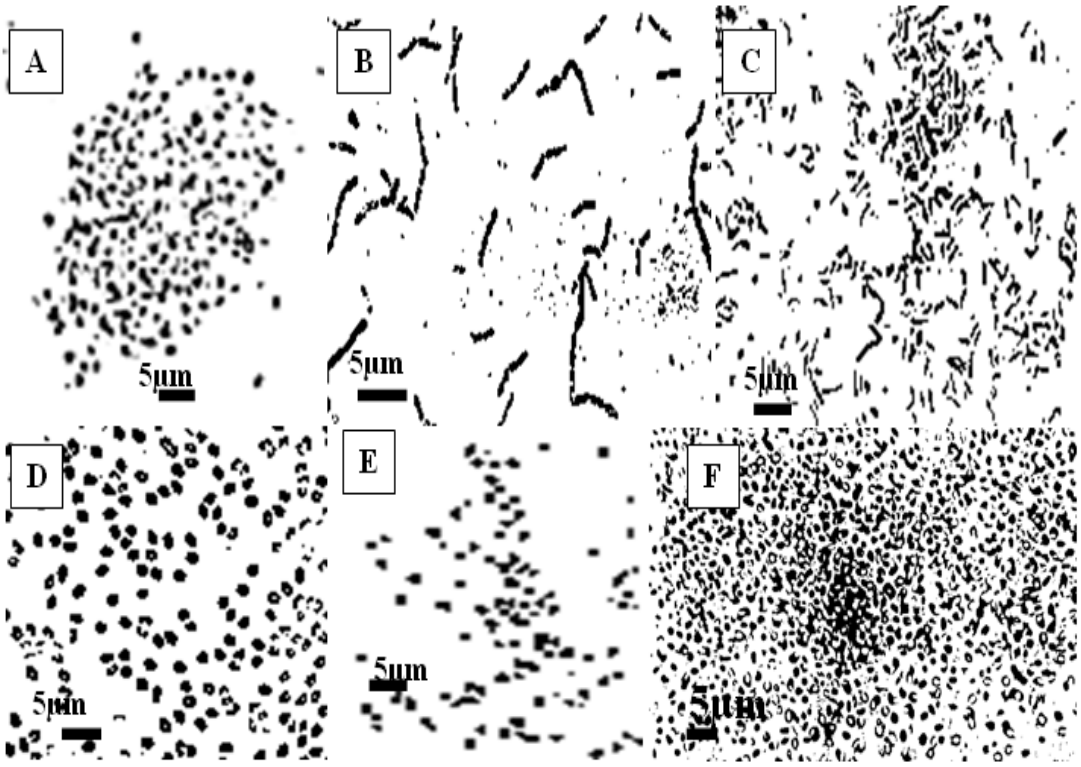
In our study, a total of 444 different bacteria isolates were obtained from the water samples collected from the Adyar River, Marina coast, Muttukadu Backwaters, Kovalam coast and a prawn hatchery. Amongst the isolates, 134 grew on *E. coli* FAGI agar, 72 isolates grew on *Staphylococcus* Baird Parker agar, 128 grew on *Salmonella* agar ÖNÖZ and 110 grew on TCBS agar (*Vibrio* sp.). Only twenty three of the isolates that grew on *E. coli* FAGI agar, 10 that grew on *Staphylococcu*s Baird Parker agar and 10 that grew on *Salmonella* agar ÖNÖZ exhibited thermotolerance at 15 psi for 30 min. (Fig. 1A-Zp; Tables 1, 2 and 3). None of the 110 isolates that grew on TCBS agar (*Vibrio* sp.) exhibited thermotolerance, although they were isolated from the same regions as the others. The results of phenotypic characterization of the 44 thermophilic bacterial isolates revealed that they were either Gram-positive, aerobic, motile rods occurring singly or in pairs (Fig. 1A, C-F) or streptobacilli rods in chains (Fig. 1B). The figures (1A-F) illustrate the light microscopy morphology of thermophilic bacteria that grew on *Salmonella* agar ÖNÖZ, (Fig. 1E), and (Figs. 1C, D, F) were thermophilic bacteria that grew on *E. coli* FAGI agar. All the thermophilic bacterial isolates were capable of tolerating 2-7% NaCl salt concentration while one isolate (Fig. 1E) was able to grow at 8% (*w/v*) NaCl concentration. Endospores were formed when thermophilic bacterial isolates were grown on nutrient agar.

3.1.1 Occurrence of thermotolerant bacterial isolates

Only 43 of the bacterial isolates showed extreme thermotolerance (Table 2). Out of 43 isolates, only 12 were found to grow profusely after autoclaving the cultures at 15 psi (121°C) for 30 min, while in the others, the colonies were comparatively fewer. The study revealed that the thermotolerant bacteria are inconsistently extremely thermotolerant except the cultures 4, 9, 12, 15, 16, 17, 21, 30 which showed consistency in being extremely thermotolerant. The presence of a few colonies was noticed each time when they were plated after autoclaving at 15 psi (121°C) for 30 min.

Table 1. Total number of bacterial isolates and thermotolerant bacteria that grew on *Salmonella* agar ÖNÖZ; *Staphylococcus* Baird Parker agar; *E. coli* FAGI agar; *Vibrio* TCBS agar from the Adyar River, Marina Coast, Muttukadu Backwater, Kovalam Coast and a Prawn hatchery in and around Chennai-India

S. no	Site	Bacteria grown on *E. coli* FAGI agar			Bacteria grown on *Staphylococcus* Baird Parker agar			Bacteria grown on *Salmonella* agar ÖNÖZ			Bacteria grown on *Vibrio* TCBS agar			Total no of bacterial isolates	Total no of thermo-tolerant isolates	Total % of thermo-tolerant isolates
		Total number of isolates	No. thermo-tolerant isolates	% thermo-tolerant isolates	Total number of isolates	No. thermo-tolerant isolates	% thermo-tolerant isolates	Total number of isolates	No. thermo-tolerant isolates	% thermo-tolerant isolates	Total number of isolates	No. thermo-tolerant isolates	% thermo-tolerant isolates			
1	Adyar River	34	5	15	12	5	42	50	4	8	50	0	0	444	43	9.6
2	Marina Coast	29	5	17	7	1	14	23	1	4	16	0	0			
3	Muttukadu Backwater	15	5	33	16	1	6	28	1	4	13	0	0			
4	Kovalam Coast	21	4	19	16	2	13	13	1	8	15	0	0			
5	Hatchery	35	4	11	21	1	5	14	3	21	16	0	0			
Total		134	23	17	72	10	14	128	10	8	110	0	0			



Figs. 1(A-F). Light microscopy morphology of some of the selected thermotolerant bacteria
Fig. 1A. Illustrates the light microscopy morphology of thermophilic bacteria that grew on Salmonella agar ÖNÖZ; Fig. 1E was thermophilic bacteria that grew on Staphylococcus Baird Parker agar; Figs. 1C, D, F was thermophilic bacteria that grew on E. coli FAGI agar. All thermophilic bacterial isolates were motile rods occurring singly or in pairs (Figs. 1A, C-F) or streptobacilli rods in chains (Fig. 1B)

The results of this study are presented in Tables 2 and 3. The study revealed the occurrence of consistently profusely growing extremely thermotolerant bacteria covering the entire Petri plate each time they were plated after autoclaving the cultures at 15 psi (121°C) for 30 min. The experiments were performed using four different autoclaves at different intervals.

3.2 Antibiotic Sensitivity of Thermo-tolerant Bacteria

The antibiotic sensitivity of the thermotolerant bacterial isolates was studied. The isolates that grew on *Staphylococcus* Baird Parker agar showed more resistance to antibiotics than those grown on *Salmonella* agar ÖNÖZ and *E. coli* FAGI agar (see Tables 4-6).

While the thermotolerant bacterial isolates # 12, 14, 15, 17 that grew on *Staphylococcus* Baird Parker agar exhibited resistance to 16 different antibiotics tested, others (#16 and 20) exhibited resistance to 15 antibiotics. The isolate # 1 that grew on *Salmonella* agar ÖNÖZ was resistant to all 16 antibiotics tested. In contrast, the isolate # 4 was sensitive to 14 antibiotics. The isolates # 21, 23, 24 and 40, respectively, that grew on *E. coli* FAGI agar were resistant to most antibiotics, whereas the isolate # 29 was sensitive to 15 antibiotics and was resistant to only Nalidixic acid.

3.2.1 Growth of the thermotolerant bacterial isolates at different temperatures

The optimal growth of the thermotolerant bacterial isolates at different temperatures (37°C, 40°C, 50°C, 55°C and 60°C) was determined by inoculating the bacteria in nutrient broth. The optimum temperature for growth of all the thermotolerant bacteria and the *Bacillus* sp. was found to be 45°C except for seven cultures that grew at 50°C, one at 52ºC and another one that grew profusely at 55°C (Table 7).

Table 2. Presence of extremely thermotolerant bacteria

Culture no.	Number of times the extremely thermotolerant bacterial broth was autoclaved, plated and grown										
	1	2	3	4	5	6	7	8	9	10	11
1	+	+	+	+	+	+	-	+	-	+	+
2	+	+	+	+	+	-	-	+	-	-	-
3	+	+	+	+	+	+	-	-	+	-	-
4 ✳	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	-	+	-	-
6	+	+	+	+	+	-	-	+	+	-	-
7	+	+	+	+	+	+	-	+	+	+	-
8	+	+	+	+	+	-	+	-	+	-	-
9 ✳	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	-	+	-	+	-	-
11	+	+	+	+	+	+	-	+	-	-	-
12 ✳	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	-	+	+	+
14	+	+	+	+	+	-	+	+	-	-	-
15 ✳	+	+	+	+	+	+	+	+	+	+	+
16 ✳	+	+	+	+	+	+	+	+	+	+	+
17 ✳	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	-	+	-	+	-
19	+	+	+	+	+	-	+	+	-	+	+
20	+	+	+	+	+	+	-	+	-	+	-
21 ✳	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	-	+	+	-	+	+
23	+	+	+	+	+	+	-	+	-	+	-
24	+	+	+	+	+	-	+	+	+	+	-
25	+	+	+	+	+	+	-	+	-	+	-
26	+	+	+	+	+	-	+	-	+	-	+
27	+	+	+	+	+	-	-	-	+	+	-
28	+	+	+	+	+	-	-	+	-	+	+
29	+	+	+	+	+	+	-	-	+	-	-
30 ✳	+	+	+	+	+	+	+	+	+	+	+
31	+	+	+	+	+	-	+	+	+	-	-
32	+	+	+	+	+	+	-	+	+	-	-
33	+	+	+	+	+	-	+	+	-	-	+
34	+	+	+	+	+	-	-	+	+	+	-
35	+	+	+	+	+	-	-	+	+	-	-
36	+	+	+	+	+	+	-	+	-	+	-
37	+	+	+	+	+	+	+	-	-	+	-
38	+	+	+	+	+	-	+	+	-	+	-
39	+	+	+	+	+	+	-	-	+	+	-
40	+	+	+	+	+	+	-	-	+	-	-
41	+	+	+	+	+	+	-	-	-	+	-
42	+	+	+	+	+	-	+	-	+	-	+
43	+	+	+	+	+	+	-	+	+	+	+

✳*Consistently thermotolerant;*

+ indicates growth of thermotolerant bacteria; - indicates no growth of thermotolerant bacteria

Table 3. Presence of consistently profusely growing extreme thermotolerant bacteria after autoclaving at 15 psi (121°C) for 30 min in Eppendorf tubes by using different autoclaves

Culture no.	Amount of inoculum (µl)	Autoclaves used							
		1					2	3	4
		Number of times the experiments were carried out							
		1	2	3	4	5	1	1	1
21	100	+	+	+	+	+	+	+	+
30	100	+	+	+	+	+	+	+	+
12	100	+	+	+	+	+	+	+	+
13	100	+	+	+	+	+	+	+	+
15	100	+	+	+	+	+	+	+	+
16	100	+	+	+	+	+	+	+	+
17	100	+	+	+	+	+	+	+	+
1	100	+	+	+	+	+	+	+	+
4	100	+	+	+	+	+	+	+	+
9	100	+	+	+	+	+	+	+	+
42	100	+	+	+	+	+	+	+	+
43	100	+	+	+	+	+	+	+	+

'+' indicates the growth of thermotolerant bacteria;
''- indicates the absence of growth

Table 4. Antibiotic sensitivity of thermotolerant isolates grown on *E. coli* FAGI agar

Sl. no.	Antibiotic	Thermotolerant isolates number										
		29	25	38	23	36	40	24	30	21	42	43
1.	Amikacin 30 µg/disc	S	S	S	S	S	S	R	S	S	S	S
2.	Azithromycin 15 µg/disc	S	S	S	R	S	R	R	S	R	S	R
3.	Cephalaxine 30 µg/disc	S	S	S	R	S	R	R	S	R	R	R
4.	Streptomycin 10 µg/disc	S	S	R	S	R	S	R	S	S	S	S
5.	Ampicillin 10 µg/disc	S	R	S	R	S	R	S	R	R	R	R
6.	Nalidixic acid Na 30 µg/disc	R	R	R	R	R	R	R	R	R	R	R
7.	Ciprofloxacin 10 µg/disc	S	R	R	S	S	S	R	S	R	R	R
8.	Cefaclor 30 µg/disc	S	S	S	R	S	R	S	R	R	R	R
9.	Rifampicin 5 µg/disc	S	R	R	R	S	R	R	R	R	R	R
10.	Cloxacillin 1 µg/disc	S	S	S	R	S	R	S	R	R	R	R
11.	Chloroamphenicol 30 µg/disc	S	S	S	R	S	R	R	R	S	R	R
12.	Erythromycin 30 µg/disc	S	R	R	R	S	R	R	R	R	S	R
13.	Oxacillin 5 µg/disc	S	R	R	R	S	R	R	R	R	R	R
14.	Neomycin 30 µg/disc	S	R	R	R	S	R	R	R	S	S	S
15.	Gentamicin 10 µg/disc	S	S	R	R	S	R	R	S	R	S	R
16.	Vancomycin 30 µg/disc	S	S	S	R	S	R	R	S	R	S	R

3.2.2 Thermotolerant *Bacillus* sp.

A novel thermotolerant *Bacillus* species was isolated from the pelletized feed of fish (Figs. 2a, b, c). Phenotypic characterization revealed the bacteria to be Gram positive, motile and rod shaped (Fig. 2a). Biochemical characterization revealed that the bacteria were thermotolerant *Bacillus* sp. The isolated bacteria survived autoclaving at 121ºC and 15 psi for 30 min. They were found to exhibit growth on nutrient agar plates and in LB broth at 50ºC (Figs. 2b and 2c). The thermotolerant *Bacillus* sp. exhibited an optimal exponential growth rate at 50ºC with 2 hrs of lag phase, a log phase up to 16 hrs and thereafter a slow declining stationary phase up to 24 hrs (Fig. 2b). The lag phase was 4 hrs at 40ºC and 35ºC and 6 hrs at 45ºC. Thereafter the exponential growth (log phase) was up to 16hrs at 40ºC and 35ºC, and up to 22 hrs at 45ºC. The pattern of growth of the *Bacillus* for 68 hrs is illustrated in Fig. 2c. Analysis of variance of the data, presented in Fig. 2b, showed that variations of the growth rate of the *Bacillus* (at 35ºC, 40ºC, 45ºC and 50ºC) were significant at the 5% level (P=0.0001) for different time intervals and temperatures.

Table 5. Antibiotic sensitivity of thermotolerant isolates grown on *Staphylococcus Baird* Parker agar

Sl. no.	Antibiotic	Thermotolerant isolates number								
		18	16	13	20	11	14	15	17	12
1.	Azithromycin 15 µg/disc	R	R	R	R	S	R	R	R	R
2.	Cephalaxine 30 µg/disc	R	R	R	R	R	R	R	R	R
3.	Cephadroxil 30 µg/disc	R	R	R	R	R	R	R	R	R
4.	Streptomycin 10 µg/disc	R	R	R	R	R	R	R	R	R
5.	Amikacin 30 µg/disc	R	R	S	R	R	R	R	R	R
6.	Ciprofloxacin 10 µg/disc	R	R	R	R	R	R	R	R	R
7.	Nalidixic acid 30 µg/disc	R	R	R	R	R	R	R	R	R
8.	Ampicillin 10 µg/disc	R	R	R	R	R	R	R	R	R
9.	Cephotaxime 30 µg/disc	S	R	R	R	R	R	R	R	R
10.	Cefaclor 30 µg/disc	S	R	R	R	R	R	R	R	R
11.	Cloxacillin 1 µg/disc	R	R	R	S	R	R	R	R	R
12.	Erythromycin 30 µg/disc	R	R	R	R	R	R	R	R	R
13.	Chloroamphenicol 30 µg/disc	R	S	R	R	R	R	R	R	R
14.	Norfloxacin 10 µg/disc	R	R	R	R	S	R	R	R	R
15.	Tetracycline 30 µg/disc	S	R	R	R	R	R	R	R	R
16.	Gentamicin 10 µg/disc	S	R	S	R	S	R	R	R	R

Table 6. Antibiotic sensitivity of thermotolerant isolates grown on *Salmonella* agar ÖNÖZ

Sl. no.	Antibiotic	Thermotolerant isolate number							
		4	2	3	1	5	6	9	8
1.	Amikacin 30 µg/disc	S	R	S	R	S	S	S	S
2.	Azithromycin 15 µg/disc	S	R	S	R	S	S	S	R
3.	Cephalaxine 30 µg/disc	S	R	S	R	R	R	R	R
4.	Streptomycin 10 µg/disc	S	R	S	R	R	S	S	S
5.	Ampicillin 10 µg/disc	S	R	R	R	S	R	S	R
6.	Nalidixic acid 30 µg/disc	S	R	R	R	R	R	S	S
7.	Ciprofloxacin 10 µg/disc	S	R	S	R	S	S	S	S
8.	Cefaclor 30 µg/disc	S	R	S	R	S	R	R	R
9.	Rifampicin 5 µg/disc	S	S	R	R	R	R	S	R
10.	Cloxacillin 1 µg/disc	S	R	S	R	R	R	R	R
11.	Chloroamphenicol 30 µg/disc	S	R	R	R	S	R	R	S
12.	Erythromycin 30 µg/disc	R	R	R	R	R	R	R	R
13.	Oxacillin 5 µg/disc	R	R	R	R	R	R	R	R
14.	Neomycin 30 µg/disc	S	S	R	R	S	S	S	S
15.	Gentamicin 10 µg/disc	S	S	S	R	S	S	S	S
16.	Vancomycin 30 µg/disc	S	R	S	R	S	R	R	R

3.3 Isolation and Identification of Proteins from Thermotolerant Bacteria and their Pathogenicity

A major 43 kDa protein was identified in the membrane fraction of the thermotolerant *Bacillus* sp. In addition, 15 other minor protein subunits were recognised (Fig. 3). The major 43 kDa protein extracted from overnight broth cultures of the *Bacillus* sp. was administered to gold fish (*Cyrprin macrophthalmus*) at various quantities: 10 µg, 20 µg, 40 µg, 80 µg and 100 µg, respectively (Fig. 3).This bacterial protein was found to be toxic to gold fish and caused mortality. With increased amount of protein there was an increase in the mortality of fish within 24 hrs of administration. However, the fish that were not exposed to protein (controls) remained healthy and survived for several days. The results are shown in Table 8 and Figs. 4A and B and 5.

Table 7. Growth of the thermotolerant bacteria at different temperatures

Culture no.	Growth of the thermotolerant bacteria at different temperatures						
	37°C	40°C	45°C	50°C	52°C	55°C	60°C
1.	+	+	+	-	-	-	-
2.	+	+	+	-	-	-	-
3.	+	+	+	-	-	-	-
4.	+	+	+	-	-	-	-
5.	+	+	+	-	-	-	-
6.	+	+	+	-	-	-	-
7.	+	+	+	-	-	-	-
8.	+	+	+	-	-	-	-
9.	+	+	+	-	-	-	-
10.	+	+	+	+	-	-	-
11.	+	+	+	-	-	-	-
12.	+	+	+	-	-	-	-
13.	+	+	+	-	-	-	-
14.	+	+	+	+	-	-	-
15.	+	+	+	-	-	-	-
16.	+	+	+	-	-	-	-
17.	+	+	+	-	-	-	-
18.	+	+	+	-	-	-	-
19.	+	+	+	+	-	-	-
20.	+	+	+	-	-	-	-
21.	+	+	+	-	-	-	-
22.	+	+	+	-	-	-	-
23.	+	+	+	-	-	-	-
24.	+	+	+	-	-	-	-
25.	+	+	+	-	-	-	-
26.	+	+	+	-	-	-	-
27.	+	+	+	-	-	-	-
28.	+	+	+	+	-	-	-
29.	+	+	+	+	-	-	-
30.	+	+	+	-	-	-	-
31.	+	+	+	-	-	-	-
32.	+	+	+	-	-	-	-
33.	+	+	+	-	-	-	-
34.	+	+	+	-	-	-	-
35.	+	+	+	-	-	-	-
36.	+	+	+	+	-	-	-
37.	+	+	+	+	+	-	-
38.	+	+	+	-	-	-	-
39.	+	+	+	-	-	-	-
40.	+	+	+	-	-	-	-
41.	+	+	+	-	-	-	-
42.	+	+	+	+	+	+	-
43.	+	+	+	+	-	-	-

Note: (+) - indicates the growth of the thermotolerant bacteria, while (-) – indicates the absence of the growth of the thermotolerant bacteria

At higher amounts (80-100 µg) of 43 kDa protein, abnormalities such as color change (from orange to yellow or pale yellow), behavioural changes (such as non-consumption of feed) and swelling of the abdomen followed by mortality occurred between 2 and 12 hrs. At lower concentrations of the protein, the above changes were also seen, but they developed more slowly, and mortality of some fish occurred between 8 and 24 hrs.

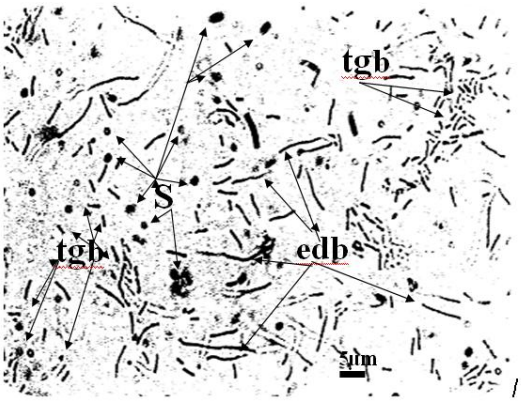


Fig. 2a. Light microscope micrograph showing thermo tolerant *Bacillus* sp. Note the spores (S), and variations in the morphology of the *Bacillus* tiny growing bacilli (tgb) and elongated dividing bacilli (edb)

The thermotolerant *Bacillus* sp. was resistant to ampicillin (10 µg), Penicillin (10 units), cloxacillin (10 µg), and amoxycillin (10 µg), but sensitive to cephadroxil (30 µg), cephalexcin (30 µg), cefaclor (30 µg), cephotaxime (30 µg), tetracycline (30 µg), and sensitive to streptomycin (10 µg), erythromycin (15 µg), amikacin (10 µg), neomycin (30 µg), ciprofloxacin (30 µg), and chloramphenical (30 µg) (Table 9).

4. DISCUSSION

In the current study, a total of 43 (9.6%) bacterial isolates that were isolated from water samples from the Adyar River, Marina coast, Muttukadu backwaters, Kovalam coast and a prawn hatchery (Chennai, India) were found to be thermotolerant (Tables 1-3). McLellan et al. [14] reported that 24.37% thermotolerant *Enterobacteriaceae* were found in recreational waters, Wisconsin, USA. Another group [15] reported only 2.47% thermotolerant coliform bacteria (*Enterobacter* sp. and *Klebsiella* sp.) from fresh produce (vegetables) from Norway. Thermotolerant coliform bacteria were also found in a Mediterranean lagoon (Thau Lagoon) and in the Vene and Pallas rivers [16]. Surface water sources, reservoirs and canals showed higher levels of thermotolerant coliform bacteria than ground water [17]. Thermotolerant coliform bacteria have been used as indicators of fecal

contamination as they are easily detectable [18,19,20]. Non-*E. coli* thermotolerant coliform bacteria were recovered from water samples collected from different environmental sources in the area of Valencia, Spain [1]. *Staphylococci* are amongst the hardiest of all non-spore forming bacteria and some strains are relatively resistant to heat (withstanding 60°C for 30 min) [21].

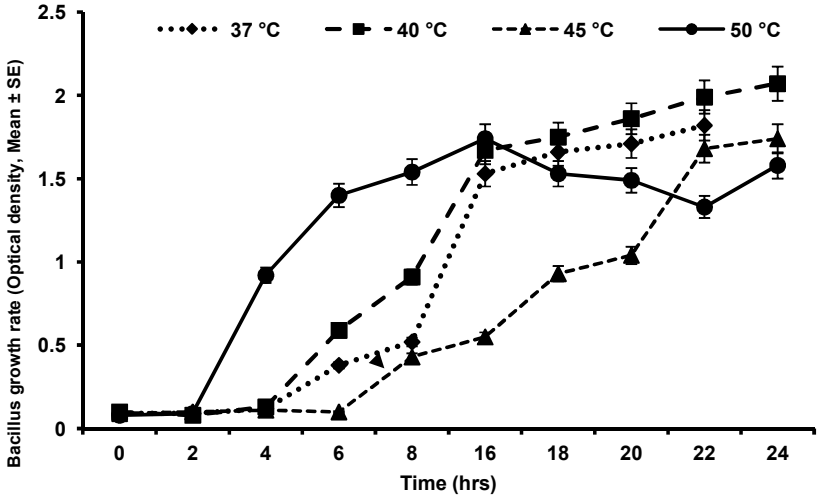


Fig. 2b. Growth curve (24 hrs) of the thermo tolerant *Bacillus* sp at 37°C, 40°C, 45°C and 50°C. Note the optimal exponential growth rate at 50ºC from lag phase up to 16 hrs and thereafter the growth rate fluctuates up to 24 hrs. Note that the initial exponential growth rate at 40ºC and 37ºC was relatively lower than at 50°C till 16 hrs and thereafter it was relatively better and optimal at 40ºC and 37ºC. In contrast the growth rate of the *Bacillus* at 45ºC was relatively slow

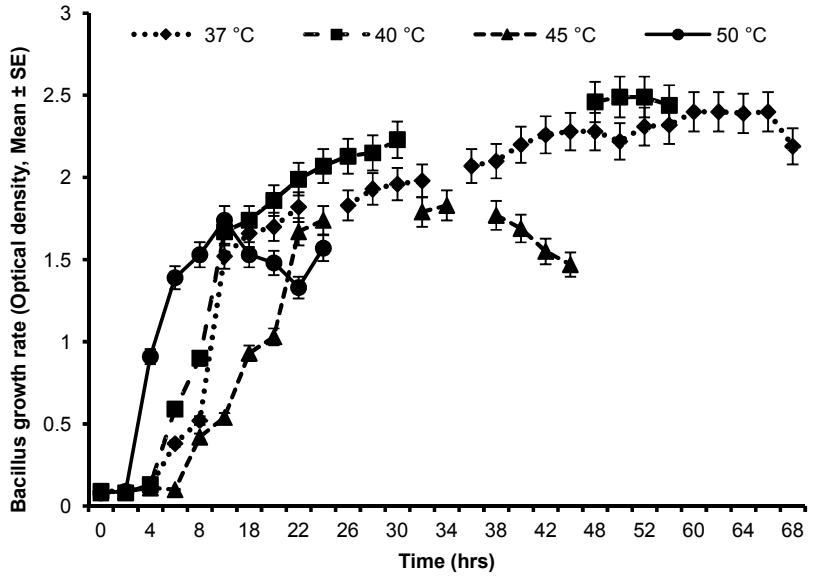


Fig. 2c. Growth curve (68 hrs) of the thermotolerant *Bacillus* sp. at 37°C, 40°C, 45°C and 50°C. Note the optimal exponential growth rate at 50ºC from 2 hrs of inoculums up to 16 hrs and thereafter the growth rate declines up to 24 hrs. Initially the exponential growth rate at 40ºC and 37ºC was lower than at 50°C till 16 hrs and thereafter the growth at 40ºC and 37ºC was better and optimal, showing a continuous growth and peaked at 48-50 hrs. In contrast the growth rate of the *Bacillus* at 45ºC was relatively very slow

Table 8. Protein extract (toxins) of thermotolerant *Bacillus* sp. cause fish *(Cyprin macrophthalmus)* mortality

Quantity of toxins-proteins in µg		Number of fish challenged	Number of fish that died (mortality)	Percentage mortality
	Control	5	0	0
10	Experimental	5	1	20
	Control	5	0	0
20	Experimental	5	2	40
	Control	5	0	0
40	Experimental	5	3	60
	Control	5	0	0
60	Experimental	5	4	80
	Control	5	0	0
80	Experimental	5	5	100
	Control	5	0	0
100	Experimental	5	5	100

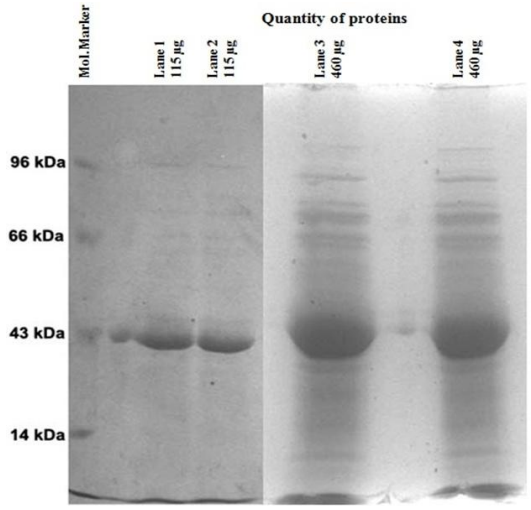
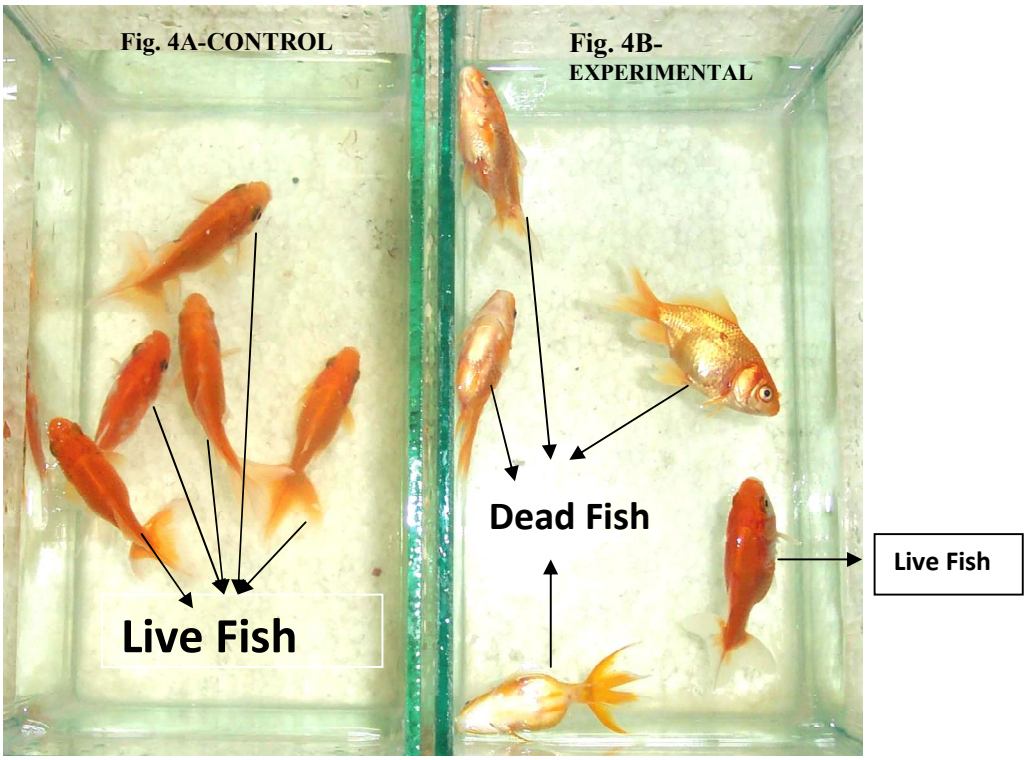


Fig. 3. Membrane Protein profile of thermotolerant *Bacillus* sp. in 10% SDS-page

Table 9. Antimicrobial sensitivity of thermotolerant *Bacillus* sp.

S. no	Antibiotics	Concentration In µg /units	Zone of inhibition in mm	Resistant (R)/ Sensitive (S)
1.	Streptomycin	10 µg	21	S
2.	Erythromycin	15 µg	25	S
3.	Amaikacin	30 µg	20	S
4.	Azithromycin	15 µg	18	R
5.	Cephadroxil	30 µg	13	R
6.	Cephalexcin	30 µg	12	R
7.	Ampicillin	10 µg	0	R
8.	Nalidixicacid	30 µg	21	S
9.	Cefaclor	30 µg	12	R
10.	Ciprofloxacin	10 µg	24	S
11.	Penicillin G	10 units	0	R
12.	Cephotaxime	30 µg	15	R
13.	Cloxacillin	10 µg	0	R
14.	Amoxycillin	20 µg	0	R
15.	Neomycin	30 µg	23	S
16.	Tetracycline	30 µg	12	R
17.	Gentamycin	10 µg	18.5	S
18.	Chloramphenicol	30 µg	17	R



Figs. 4A and B. Protein extract (toxins) of thermotolerant *Bacillus* sp. caused fish (*Cyprin macrophthalmus)* mortality. Note the normal bright red color of alive fish in the control (A), whereas in the experimental fish (B) four of the dead fish show a pale red color with enlarged abdomen while a single live fish showing normal bright red colour

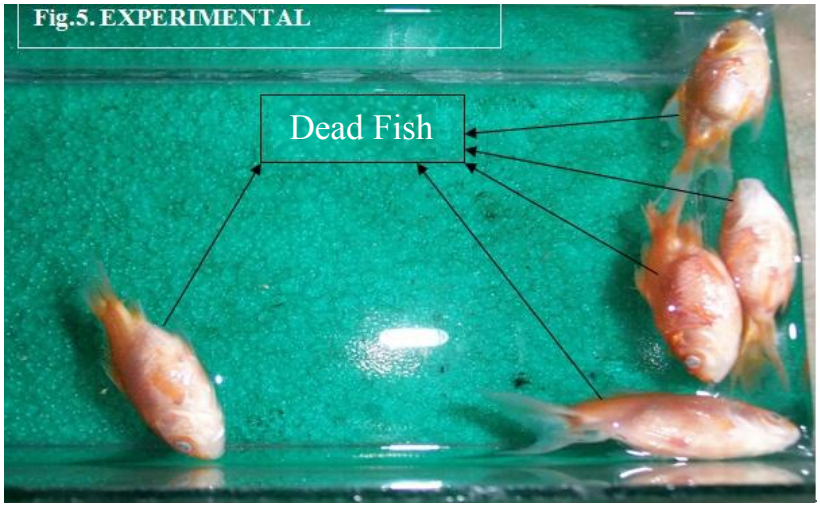


Fig. 5. Protein extract (toxins) of thermotolerant *Bacillus* sp. showing mortality of fish (*Cyprin macrophthalmus)* in experimental conditions

Note the normal bright red color of alive fish in the control (Fig. 4A), whereas in the experimental fish (Fig. 5), all five dead fish showing enlarged abdomen

In this study we report for the first time in detail on the presence of several thermotolerant bacterial isolates in the Chennai regions as described above (Tables 1, 4-6). Twenty three (17%) of the thermotolerant bacterial isolates obtained in the study grew on *E. coli* FAGI agar,

10 (13%) of the thermotolerant isolates grew on *Staphylococcus* Baird Parker agar and 10 (8%) of the thermotolerant isolates grew on *Salmonella* agar ÖNÖZ. None of the 110 isolates that grew on TCBS agar (*Vibrio)* were thermotolerant. The abundant natural occurrence of such a high percentage of thermophiles and extremely thermotolerant bacteria in the aquatic environment at Chennai indicates a risk to both human and aquatic animal health.

In this study, the optimal temperature for the growth of all the thermophiles was found to be 45ºC except for seven of the isolates which were found to grow at a maximum temperature (optimal growth temperature) of 50ºC, one at 52ºC and another at 55ºC respectively (Table 7). In these respects these thermotolerant bacteria are unique and the present report is the first relating to thermotolerant bacteria from the Chennai region. Similarly a thermotolerant bacterium *Bacillus kokeshiiformis* isolated from a marine animal resources compost was reported to grow at 35–61.6°C (optimum 50.6C) [5]. Earlier studies have revealed that the optimal temperature for growth of most thermotolerant bacteria is between 40°C and 100°C while extremely thermotolerant and hyper-thermotolerant bacteria grow between 45°C and 110°C. Ultra-thermotolerant bacteria grow between 100°C and 103°C [2,3,4,5,6]. The optimum temperature for growth of psychrophilic bacteria is between 0°C and 20°C while the mesophilic bacteria grow best between 20°C and 40°C [2,3,4,6,7]. Temperatures in the range 50°C to 100°C are usually deadly for bacterial cells and spores and in general, most bacteria are badly affected by temperatures above 50°C [2,3,10].

The present study has revealed the occurrence of a few extremely thermotolerant bacterial isolates, exhibiting consistency in profuse growth after autoclaving at 15 psi (121°C) for 30 min. Other isolates showed inconsistency in their growth after autoclaving, indicating that there could be considerable variation in the length of time required to kill extremely thermotolerant bacteria (Tables 1-3, and 7). Some of the bacteria are extremely resistant to destruction when they are exposed to a range of temperature that would be lethal for most isolates. The occurrence of thermotolerant bacteria resistant to destruction by long exposure to lethal temperature has been reported previously, and such exceptions are not uncommon in the environment [2,3,22,23].

We noted the occurrence of endospores in the extremely thermotolerant bacteria, which may be consistent with their resistance to high temperature and may protect them from radiation, desiccation and chemical agents such as disinfectants. Endospores may display as many as sixty different proteins involved in coat formation [24] and may exhibit inconsistencies in growth after autoclaving. Beishir [22] claimed that spores are not formed in response to adverse conditions and stated that the factors that stimulate bacteria to form spores remain unknown.

Our studies have demonstrated the occurrence of widespread antibiotic resistance in thermotolerant bacterial isolates, especially those isolates that grew on *Salmonella* agar ÖNÖZ, *E. coli* FAGI agar and *Staphylococcus* Baird Parker agar. This finding indicates indiscriminate usage of antibiotics in the Chennai region with consequential development of antibiotic resistance (Tables 4-6, and 9). The development and spread of antibiotic resistance in bacterial pathogens is universal and is normally not preventable [25,26,27]. Some of these antibiotic resistant bacteria pathogens might represent potential health hazards for animals and man, and need to be addressed cautiously and meticulously. Other isolates that are antibiotic sensitive, non-pathogenic and thermotolerant offer the potential for exploitation in biotechnological applications.

The current study reports the occurrence of a major over-expressed membrane protein (43kDa protein) in a thermotolerant *Bacillus* sp. isolate. The predominance of the major membrane protein of the *Bacillus* sp. isolate might be an important small heat shock protein (sHSP) representing a specialized adaptation for the survival of the bacteria at elevated temperatures. Expression of such heat shock proteins (HSPs) is universal in nature and is involved in diverse cellular processes in all living organisms [28,29,30,31]. The present investigation has also shown for the first time that the aforementioned major 43 kDa protein from thermotolerant *Bacillus* sp. is toxic for gold fish (*Cyrprin macrophthalmus*) within 24 hrs of administration (Table 8). Similarly, Cry toxins from *Bacillus thuriensis* were documented to have specific activities against several species of insect and even nematodes, while the insecticidal Bt cry2Aa2/ OD Cry2Aa2 were shown to cause mortality in insects within 3-5 days after administration [32].

5. CONCLUSIONS

This study documents 43 thermotolerant bacterial isolates from water samples from the Adyar River, Marina coast, Muttukadu Backwaters, Kovalam coast and a prawn hatchery in the Chennai district, India. Further, it describes a novel thermotolerant Gram positive *Bacillus*-type bacterium from pelletized fish feed. The optimal temperature for growth for all the thermophiles was found to be 45ºC, except for 7 of the isolates, which grew at a maximum temperature of 50ºC, one at 52ºC and another at 55ºC, respectively. In addition, we found a few extremely thermotolerant bacterial isolates exhibiting consistency in profuse growth, while others showed inconsistency in growth after autoclaving the cultures at 15 psi (121°C) for 30 min. Isolates of thermotolerant bacteria that grew on *Staphylococcus* Baird Parker agar and *Salmonella* agar ÖNÖZ were predominantly antibiotic resistant when compared to those grown on *E. coli* FAGI agar. A major 43 kDa protein was detected from the thermotolerant *Bacillus* sp. as well as 15 other minor protein subunits. These proteins were found to be toxic to gold fish (*Cyprin macrophthalmus*).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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