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Antagonistic *Bacillus cereus* TC-1 Isolated from Solar Salt Work in Southern India

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Abstract

Bacillus cereus TC-1 was isolated from condenser pond of manmade solar salt pan in Thamaraikulam, Tamilnadu, India effectively suppressed the shrimp bacterial pathogens of *Vibrio harveyi*, *V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus* and *V. vulnificus* by *In vitro* antagonistic assay at 9 to 15 mm of zone of inhibition. Phylogenetic analysis and evolutionary distance revealed that, *B. cereus* TC-1 was highly similar to that the *Bacillus* sp. and other *B. cereus* strains. Their optimum growth rate was ranged between of 4 to 6% of NaCl in the growth media. The alkaline protease production was significantly (P< 0.001) differed among the different NaCl enriched growth media. Based on the antagonistic activity of the *B. cereus* TC-1 against the shrimp pathogens and its antimicrobial factors, it may be used as probiotics and developing novel antimicrobial bioactive substances against aquatic pathogens.

Keywords: Antagonism; *Bacillus cereus*; Extremophiles; Halophilic Bacteria

Introduction

Microbes from extreme environments have attracted considerable attention in recent years due to the presence of stable and novel macromolecules [1]. They are often under extreme conditions of pressure, temperature, salinity, and depletion of micronutrients, with survival and proliferation often depending on the ability to produce biologically active compounds. The diversity of marine environments has exerted a driving force on bacteria selection leading to new adaptive strategies and the synthesis of new metabolites [2]. Microbial secondary metabolites have been recognized as a major source of compounds endowed with ingenious structures and potent biological activities [3].

The genus *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore. Most *Bacillus* species are not harmful to mammalians, including humans and are commercially important as producers of a high and diverse amount of secondary metabolites (antibiotics, bio insecticides, fine chemicals and enzymes) [4,5]. The genus *Bacillus* has been in use in the biotechnology industry for a very long time with a number of new cultures exhibiting a variety of benefits to humans. Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogen growth, such as kanosamine or zwittermycin A from *B. cereus* [6].

Microorganisms represent the most common candidates as sources of new enzymes because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Microbial alkaline proteases dominate the worldwide enzyme market, with a two third share of the detergent industry [7,8]. Nowadays increasing emphasis is being laid on extremophiles for the presence of such enzymes, mainly due to the mechanisms and strategies that help them to function under stressful growth conditions [9].

Antagonistic activities between micro-organisms have been widely reported [10] and in many instances the inhibition is due to the production of bacteriocins and other extracellular products (EP). Bacteria showing antagonistic activity have potential application as bio control agents. Sugita et al. [11] isolated a strain of *Bacillus* sp. that was antagonistic to 63% of the bacterial isolates from fish intestine. Some bacteria have been observed to be antagonistic to even viruses [12] and such bacteria have potential use in biocontrol of viral diseases [13]. The present study focuses isolation, identification, biochemical characterization and antagonistic studies of the halophilic *Bacillus cereus* from the crystallizer pond of the solar salt works.

Materials and Methods

Water samples were collected from the condensed ponds (C-I, II and III) from the solar salt works of Thamaraikulam, Kanyakumari, Tamilnadu, India. The physicochemical parameters of the samples were studied following the standard methods by Bhaskaran [14] and given in the Table 1.

Saline water samples were decimally diluted and spread in the specific media of *Bacillus cereus* Agar Base (Himedia, Mumbai, India) and incubated the plates at 37°C for 24 hours. A total number of 3 tentative isolates were picked based on morphological characteristics and checked by microscopy (Gram- staining and spore staining) tested for motility and for presumptive identification.

Physicochemical parameters	Sampling Area for bacterial isolation		
	Condenser-I	Condenser-II	Condenser-III
Salinity (%)	150 ± 0.0	155 ± 0.0	150 ± 0.0
pH	8.22 ± 0.33	7.91 ± 0.53	8.0 ± 0.0
DO (mg/l)	4.32 ± 0.21	4.05 ± 0.43	4.15 ± 0.04
Hardness (%)	6.3 ± 0.8	6.5 ± 0.87	6.25 ± 0.05
Chloride (mg/l)	132.55 ± 4.87	139.54 ± 11.98	142.98 ± 11.09
Calcium (mg/l)	69.0 ± 2.76	73.5 ± 3.02	72.5 ± 1.72
Magnesium (mg/l)	17.3 ± 0.67	16.75 ± 0.98	17.05 ± 2.53

Table 1: Physico chemical parameters of the condenser pond of Thamaraikulam salt pan which the sampling is made.

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The presumptive isolates subjected to biochemical characterization were based on sugar fermentation pattern in basal broth medium as per the standard method. The *Bacillus* isolates were tested using 15 carbohydrate discs (Himedia, Mumbai) for their ability to ferment different sugars. Isolates were also tested for catalase, indole, gelatin hydrolysis and lactic acid production.

Genomic DNA was isolated from *B. cereus* TC-1 strain and 100 ng was PCR amplified using 16S rRNA universal primers. The PCR product was cloned in to pTZ57R vector and transformed to DH5α following the method of Sambrook et al. [15]. The transformants were sequenced by using ABI 3700 automated DNA sequencer. Sequences were compared with other 16S rRNAs obtained from Genbank using the BLAST program. The phylogenetic tree was constructed by neighbor-joining method using Geneious Basic 5.4.6 analysis and evolutionary history was inferred using the neighbor-joining method [16]. The evolutionary distances were computed using HKY genetic distance model [17].

To optimize the *B. cereus* TC-1 growth in different NaCl concentration, Nutrient Broth was enriched with 1, 2, 3, 4 and 5% NaCl and studied the growth curve was prepared. The inoculated cultures were incubated at 37°C in a shaker at 100 rpm and bacterial growth was monitored at 0, 12, 36, 48, 60, 72, 84 and 86 hours after inoculation.

Alkaline protease assay was done in the skim milk agar base and the activity was estimated by Hagihara [18]. The enzyme (0.5 ml) was added to 3.0 ml casein (0.6 % w/v in 20 mM borax NaOH buffer, pH 10) and the reaction mixture was incubated at 37°C for 10 min before the addition of 3.2 ml of TCA mixture (0.11 M trichloroacetic acid, 0.22 M sodium acetate, 0.33 M acetic acid). The terminated reaction mixture was incubated for 30 min at room temperature. The precipitates were removed by filtration through Whatman No. 1 filter paper and the absorbance of the filtrate was measured at 280 nm. One unit of alkaline protease activity was defined as the amount of enzyme liberating 1 lg of tyrosine per minute under assay conditions. Enzyme units were measured using tyrosine (0-100 µg) as standard.

The lawn culture of the pathogenic *Vibrio harveyi*, *V. parahaemolyticus*, *V. anguillarum*, *V. alginolyticus* and *V. vulnificus* were prepared by pouring 2 ml of each young culture over Muller-Hinton Agar media plates, separately. The plates were air dried by keeping in an incubator at 30°C for 15 minutes. Wells (3 mm diameter) were punched in the plates using a sterile gel puncher. Thirty microlitres of an 18 hour culture of *B. cereus* TC-1 (16-18 hours in nutrient broth, supplemented with 5% sodium chloride) was pipette into the wells and plates were incubated for 24 hours. Zone of inhibition around the wells was recorded.

One way and two way Analysis of Variance (ANOVA) were carried out using the software PASW statistics data editor and Ky plot respectively. Means were compared at 0.05% for One Way ANOVA and 0.001% level.

Results and Discussion

Based on the morphological, physiological, biochemical and genetic identification, the rod shaped bacteria was confirmed as *Bacillus cereus* and submitted to the NCBI gene bank, accession number is GU939623.1. Neighbor-joining method with evolutionary distances revealed that *B. cereus* TC-1 was highly similar (99%) that the *Bacillus* sp. SAT1-10J1-1 (GeneBank acc. No: JQ340870.1) followed by the *Bacillus* sp. 4 (99%), *Bacillus* sp. R5 (GeneBank acc No: GU566345.1) and *B. cereus* strain ATCC 14579 (AE016877.1) (Figure 1). Many types

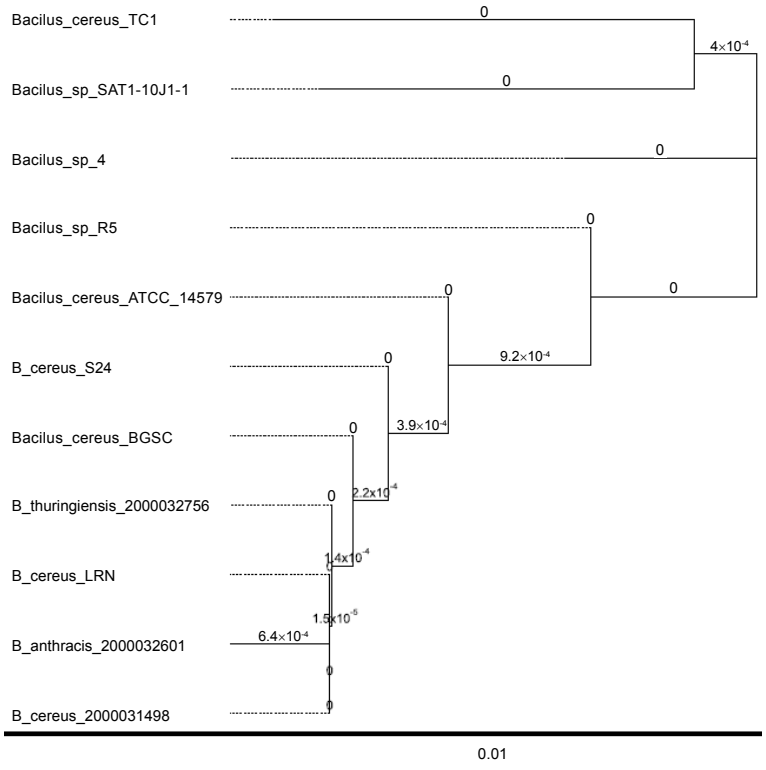

Figure 1: Graphical phylogenetic tree of halophilic *B. cereus* TC-1 based on 16S rRNA gene sequence data compare with other *Bacillus* sp. The tree was constructed using the HKY genetic distance model with neighbor-joining method.

of bacterial species have been isolated from various salt environments such as soils and salterns including the Gram-negative halophilic like species of the genera *Vibrio*, *Alteromonas*, *Acinetobacter*, *Marinomonas*, *Pseudomonas* [19] and the genera *Marinococcus*, *Sporosarcina*, *Salinococcus* and *Bacillus* [20].

NaCl played an important role for the growth of *B. cereus* TC-1. The bacterial growth in 2% NaCl-supplemented media reached the stationary phase at 24 hours with less number of bacterial counts. The NaCl concentration of 4, 5 and 6% in the growth media help to faster growth of the *B. cereus* TC1 significantly ($P< 0.05$) in the growth media and the cells reached the stationary phase around 48 hours. Also two way ANOVA revealed that the growth rate varied significantly among the different groups ($F=4.4333$; 32. 0493; $P \leq 0.001$). The 12 and 15% of NaCl enriched growth media were fails to induce the *B. cereus* TC1growth due to the higher concentrations and it may cause the stress to the bacterial cells and unable to divide. Based on the faster growth time and higher cell density, the present study revealed that, the optimum NaCl concentrations in the growth media were 4, 5 and 6% (Figure 2). Patel et al. [21], isolated and identified the haloalkaliphilic, Gram-positive, aerobic, coccoid *Bacillus pseudofirmus*-Po2 by 16S rRNA sequencing analysis from the seawater sample in Gujarat, India. They also screened and optimized the production of alkaline protease using NaCl, nitrogen sources, metal ions, etc.

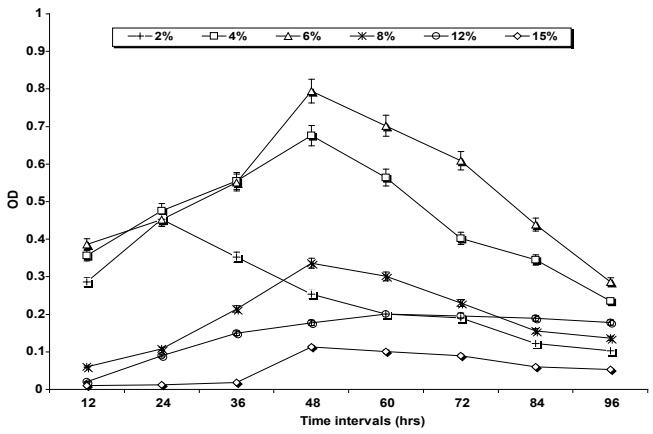

Figure 2: Growth optimization of halophilic *Bacillus cereus* TC-1 with reference to various NaCl concentrations.

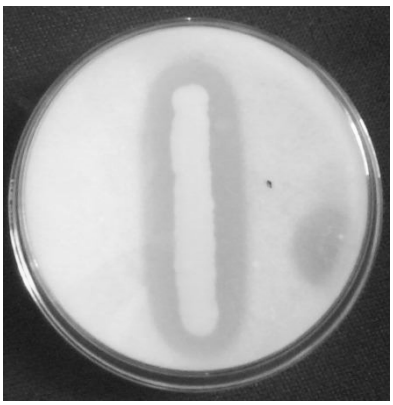

Figure 3a: Alkaline protease activity of halophilic *Bacillus cereus* TC-1 streaked on alkaline agar plate containing skimmed milk. The clear zone indicated the hydrolysis of casein as a result of alkaline protease production.

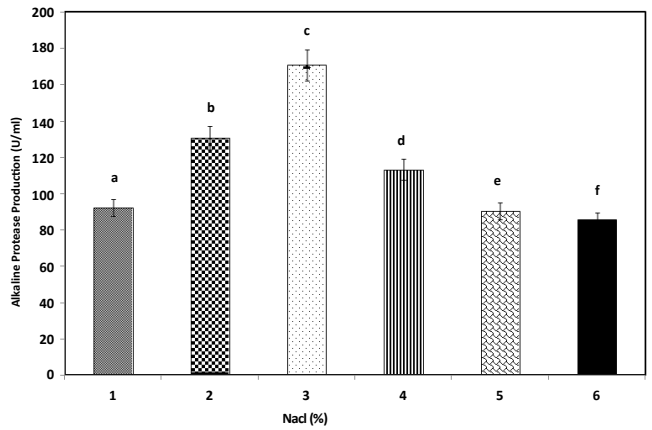

Figure 3b: Alkaline protease production (U/ml) of the halophilic *Bacillus cereus* TC-1 with reference to different NaCl concentration. Means with the same superscripts (a-f) do not differ from each other ($P< 0.001$). One way ANOVA.

Sl. No	Pathogenic *Vibrio*	Zone of inhibition (mm)
1.	*Vibrio harveyi*	15.33 ± 1.57
2.	*Vibrio* parahaemolyticus	12.87 ± 1.64
3	*Vibrio* anguillarum	11.05 ± 0.77
4	*Vibrio* alginolyticus	13.33 ± 1.24
5	*Vibrio* vulnificus	9.25 ±0.85

Table 2: *In vitro* antagonistic activity of halophilic antagonistic *Bacillus cereus* TC-1 against the pathogenic *Vibrio* sp.

Figure 3a shows the positive alkaline protease activity produced by *B. cereus* TC-1 in the skimmed milk agar base. The clear zone indicated the hydrolysis of casein as a result of alkaline protease production. The lowest alkaline protease production was observed in 1% NaCl of 92.24 U/ml. This was significantly ($P<0.05$) increased to 130.55 and 170.85 U/ml respectively in the 2 and 3% NaCl enriched growth media. Further the production was decreased in the 4 to 6% NaCl enriched growth media (Figure 3b). Similar trends were also evident in *Salinicoccus alkaliphilus* sp. nov., a moderately halophilic and alkaliphilic coccus isolated form Baer Soda Lake in Mongolia, which could grow over a wide range of NaCl, 0-25% (w/v) with optimum at 10% (w/v) [22]. In the present study, alkaline protease production was also higher in 2 and 3% NaCl enriched growth media and it reflected that due to the moderate halophilic nature of the bacterial strain. The more enzyme production got failed at lower and higher concentrations. Po2 produced protease in the range of 5-20% (w/v) NaCl, optimally (162-170 U/ml) at 10% (w/v) NaCl [21]. Similar results have also been reflected by the haloalkalophilic archaeon, *Natronococcus occultus* in which protease secretion was optimum at 1-2 M NaCl [23]. However, in the case of the archaebacterium *Halobacterium mediterranei*, a much higher salt requirement (25%, w/v) for serine protease secretion was reported [24].

In vitro antagonistic activity of *Bacillus cereus* TC-1 against the five pathogenic *Vibrio* sp was tabulated in Table 2 and the Figure 4. The highest inhibitory activity was shown against *V. harveyi* (15.33 ± 1.57 mm). The lowest activity was against *V. vulnificus* (9.25 ± 0.85). The activity against other species likes *V. parahaemolyticus*, *V. anguillarum* and *V. alginolyticus* were 12.87 ± 1.64, 11.05 ± 0.77 and 13.33 ± 1.24 mm respectively. *Bacillus* species produce is an interesting genus to investigate for antimicrobial activity since *Bacillus* species production a diverse array of antimicrobial peptides representing several different basic chemical structures [25], with a distinct diversity in their inhibitory activities against a variety of microorganisms [26]. Bacteriocins have

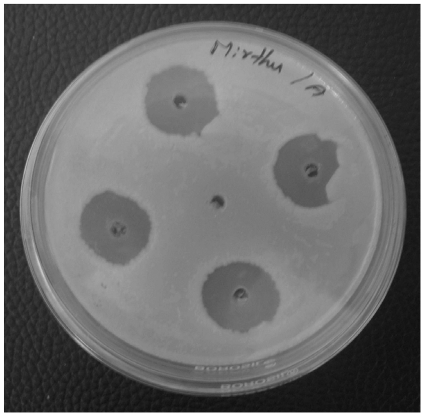

Figure 4: *In vitro* antagonistic activity of halophilic antagonistic *Bacillus cereus* TC-1 against the pathogenic *Vibrio harveyi*.

been studied in different species including: *B. subtilis*, *B. cereus*, *B. stearothermophilus*, *B. licheniformis*, *B. thuringensis* and other *Bacillus* sp. [27]. *Pseudoalteromonas* sp. A1-J11 isolated from coastal seawater of Kagoshima Bay, Kagoshima Prefecture, Japan was found to produce anti-*Vibrio* substances extracellularly [28]. In our previous study, the *Bacillus cereus* TC-1 isolated from coconut retting water effectively suppressed the pathogenic *Vibrio harveyi* and *Aeromonas hyrophila* by *in vitro* and *in vivo* level. They significantly decreased the bacterial loads in the culture tanks [29,30]. *Bacillus* strains are one of the most recognized beneficial bacteria used against bacterial or viral disease in shrimp aquaculture; they release antibacterial substances [31]. There is less evidence that *Bacillus* strains exert harmful effects on shrimp or the environment [32].

Conclusions

The *B. cereus* TC-1 isolated from solar salt works having higher antagonistic activities against the pathogenic *Vibrio* sp. Further works are need to *in vivo* treatments against the aquatic pathogens in laboratory as well as field trails and characterize the virulence factors at molecular level.

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