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ISOLATION AND PRELIMINARY CHARACTERIZATION OF A BACTERIOCIN-PRODUCER *BACILLUS* STRAIN INHIBITING METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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In a multivalent approach to discover new antimicrobial substance, a total of 160 *Bacilli* were isolated from termitarium soil, characterized on the basis of their morphological and physiological characters and screened for their antimicrobial activity by agar well diffusion method against certain drug resistant pathogenic bacteria such as *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* and common food contaminating bacteria *Listeria monocytogenes*. After preliminary screening, sixteen isolates showed inhibitory activity against test pathogens. Among them *Bacillus* isolate TSH58 exhibited maximum inhibitory activity against MRSA, *Staphylococcus aureus* and *Listeria monocytogenes*. Based on morphological, physiological, biochemical and 16S rDNA characteristics isolate TSH58 was identified as a member of the *Bacillus cereus* species group. Various nutrient sources and culture conditions were optimized, the partially purified antimicrobial metabolite was subjected to various treatments such as heat, pH and proteolytic enzymes. Complete loss in the activity observed when the crude metabolite was treated with proteolytic enzymes suggesting its proteinaceous nature and termed as bacteriocin like inhibitory substance (BLIS). Minimal inhibitory concentration of the partially purified bacteriocin determined by microtiter plate assay was 80 µg/ml for MRSA and 40 µg/ml for *L. monocytogenes*. Tricine SDS PAGE analysis revealed that the partially purified bacteriocin produced by the *Bacillus* strain TSH58 had an apparent molecular weight of about 4.0 KDa.

Keywords: Bacillus – *Staphylococcus aureus* – MRSA – *Listeria monocytogenes* – bacteriocin

INTRODUCTION

Increasing drug resistance to antibiotics in bacteria continue to be a major health problem worldwide. The current known antibiotics and possible derivatives sooner or later lose their efficiency [8]. Treatments are urgently required for the abatement of the growing number of infections caused by antibiotic-resistant bacteria. Methicillin resistant *Staphylococcus aureus* (MRSA) is among the major one that have also acquired resistance not only to β-lactam antibiotics, but also to flouroquinolones, chloramphenicol, clindamycin, tetracycline, and aminoglycosides and more recently to vancomycin and teicoplanin around the world [30]. Therefore, urgent research for new antimicrobial agents to prevent and treat MRSA infection is needed.

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Bacillus spp. are widely distributed in nature and play an important role in inhibiting pathogenic bacteria by producing broad spectrum antimicrobial compounds, i.e. bacteriocins. These compounds exhibit strong antimicrobial activities and enable the bacterium to survive in its natural environment [29]. Bacteriocins exhibit bactericidal activity towards species that are often closely related to the producer bacteria and might warrant serious consideration as alternatives to traditional antibiotics [12]. They have a narrow or broad spectrum of activity and exhibit significant potency against bacteria, including antibiotic resistant strains [9, 24]. Bacteriocins can be used in the treatment of multidrug resistant human pathogens. One example is a bacteriocin produced by *Enterococcus faecium* has shown to inhibit 29 different strains of vancomycin resistant of enterococci [28]. In addition, synergistic effect of bacteriocins enhance the efficacy of antibiotics and reduce the animal cell toxicity [17]. On the other hand, bacteriocin such as Nisin A produced by Lactic acid bacteria (LAB) is used as food preservative in over 50 countries [2].

Termitarium or termite mound or nest is an unexplored ecological niche as far as the presence of *bacilli* is concerned. Being rich in partially digested food materials and faecal matter of termites, enriched with minerals and other organic constituents provide existence of a huge diversity of microorganisms [15]. Therefore, search for potent antimicrobial compounds producing *bacilli* was made that can combat drug resistant bacterial pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS

Isolation of aerobic endospore forming bacilli

Termitarium soil samples were collected from Haridwar district (29°58'N latitude and 78°14'E longitude), Uttarakhand in a sterile polythene bag and transported to the laboratory. For the isolation of aerobic endospore forming *bacilli* 10 g soil sample was mixed with 90 ml of sterile distilled water and placed in a water bath at 80 °C for 10 min to kill the vegetative cells [10]. A serial 10-fold dilution was then prepared in sterile distilled water up to 10^{-5}. Then, 100 µl of each dilution was spread on nutrient agar and *Bacillus* agar plates (Hi-Media Laboratories, India) and incubated at 30 °C for 24 to 48 h. The morphologically distinct colonies were picked and sub-cultured on nutrient agar (NA). All bacterial isolates were preliminary characterized according to Bergey's manual of determinative bacteriology [11].

Test organisms

Methicillin resistant *Staphylococcus aureus* isolated from clinical specimen was procured from PGIMER Dr. Ram Manohar Lohia Hospital, New Delhi, India. *Staphylococcus aureus* ATCC 25923, and *Listeria monocytogenes* MTCC 657 were obtained from local culture collrection, Haridwar, India and used as test pathogens.

Antimicrobial activity of the isolates

Bacterial isolates were grown in nutrient broth (NB) medium at 30 °C for 48 h. The broth was centrifuged at 10,000 rpm for 20 min to sediment the cells and crude supernatant was tested against *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* and *Listeria monocytogenes* MTCC 657 by agar well diffusion method. In brief, Mueller Hinton agar plates seeded with test pathogen 10^6 CFU/ml were taken. Wells of 6.0 mm diameter were made using sterilized steel borer. Then, 100 µl culture supernatant of isolated bacteria were added to the well and the plates were incubated at 37 °C for 18–24 h. Inhibition zones were recorded and bacteria showing maximum inhibition were selected for further study [31].

Molecular characterization and phylogenetic analysis

To determine the identity of the selected *Bacillus* strain TSH58, it was subjected to genomic DNA extraction and amplification of 16S rDNA [23]. The extracted DNA was dissolved in 20 µl TE buffer and used as the template for the PCR reactions. PCR amplifications were performed in a total volume of 50 µl by mixing 100 ng of the template DNA with 2.5 mM concentrations of each deoxynucleotide triphosphate and 0.3 mM of each universal primer of Bac27F (5′-AGAGTTTGATCMTGGCTCAG -3′) and Univ1492R (5′-CGGTTACCTTGTTACGACTT-3′) described by Jiang et al. [13]. The thermocycling profile was carried out with an initial denaturation at 95 °C (2 min) followed by denaturation at 95 °C (30 sec), annealing at 52 °C (30 sec), extension at 72 °C (2 min), and a final extension at 72 °C (15 min) in an Eppendorf Gradient thermocycler. The PCR amplified rDNA was purified by using the Quick PCR purification kit (Bangalore Genie, India). The amplified DNA sequences were aligned using multiple sequence alignment programs and phylogenetic analysis was performed using mega 7 software [14].

Optimization of media and culture conditions

For the production of the maximum amount of antimicrobial compound, various carbon sources, nitrogen sources and physiological parameters such as temperature, incubation period and pH of the fermentation conditions were optimized and the antimicrobial activity of the test supernatant was determined as outlined by Risoen et al. [20].

Titration of antibiotic units in culture supernatant

The titre of the antibiotic units in the culture supernatant was carried out by twofold serial dilution of supernatant in normal saline up to 12× times. A 50 µl of each dilution was placed into wells made on Mueller-Hinton agar plates seeded with 10^6 cells

of test pathogens. The plates were incubated at 37 °C for 18–24 h and the inhibition zones were measured. The antibiotic activity is defined as the highest dilution of the supernatant that inhibits the growth of test pathogens *in vitro* and is expressed as activity units AU/ml and was calculated by using the formula given by Batdorj et al. [6]. Bacteriocin activity (AU/ml) = (the highest dilution exhibiting inhibition zone ×1000)/Volume (µl).

Determination of nature of antimicrobial compound

To determine the nature of antimicrobial compound, the culture supernatant was treated with a final concentration of 1 mg/ml of proteolytic enzymes such as proteinase K and trypsin and incubated at 37 °C for 6 h.

Extraction of crude antibacterial protein

The extraction of antibacterial protein was carried out following the method of Oscariz et al. [18] with few modifications. The bacterial isolate was grown in 500 ml nutrient broth supplemented with 1% glucose at 30 °C for 36 h. The broth was centrifuged at 10,000 rpm for 20 min and the cell free supernatant was filtered through a 0.22 um bacteriological filter. Solid ammonium sulphate was added up to 60% saturation and kept overnight at 4 °C in a shaking incubator. The mixture was then centrifuged at 12,000 rpm for 20 min. The precipitate was collected and re-suspended in 10 ml of 50 mM sodium phosphate buffer (PBS pH 7.0). The solution was dialyzed against the same buffer overnight using dialysis membrane of 3.0 KDa cut off (Hi media Labs Pvt. Ltd, India) and crude dialysed metabolite was then extracted with butanol (1:2 ratio butanol: sample). Afterwards, the butanol was evaporated and the extracted crude metabolite named as bacteriocin like inhibitory substance (BLIS) was dissolved in 2.0 ml of PBS and stored at –20 °C. The antimicrobial activity of the partially purified BLIS was determined by agar well diffusion method as previously described, which exhibited inhibition zone of 23 mm for MRSA and 28 mm for *L. monocytogenes.*

Effect of heat, pH and proteolytic enzymes on crude metabolite

To determine the thermal stability, the partially purified BLIS was treated at 50, 60, 70, 80, 90, 100 and 121 °C for 10 min and antimicrobial activity was determined by agar well diffusion method as previously described. To investigate the effect of pH, antibacterial activity was measured by adjusting the pH of the BLIS ranging from 3 to 11 and stored at 4 °C for 4 h. Effect of proteolytic enzymes was determined by treating the crude BLIS with final concentration of 1 mg/ml of the enzymes trypsin and proteinase K and incubation at 37 °C for 6 h [5].

Determination of minimal inhibitory concentration (MIC) of crude protein

Minimal inhibitory concentration of crude antimicrobial metabolite was determined by microtiter plate assay as described by Wu et al. [32] with some modifications. The 2,3,5 tri phenyl tetrazolium salt at a final concentration of 0.01 percent was added as a growth indicator. A sterile 96-well plate was labelled and 200 µl of crude extract (10 mg/ml) in PBS) was pipetted into the first well of the plate. To all other wells, 100 µl of Mueller Hinton broth containing tetrazolium salt was added. Doubling dilution was performed using a micro pipette such that each well had 100 µl of the test material in descending concentrations. Then, 10 µl of bacterial suspension 5×10^6 cfu/ml was added to each well to achieve a concentration of 5×10^5 cfu/ml. Each plate was wrapped with aluminium foil to ensure that bacteria did not become dehydrated and the MIC was determined as the lowest dilution that inhibited the growth of indicator organism. Each plate had a set of controls: a row with a broad-spectrum antibiotic gentamicin as a positive control in doubling dilution, a row with all solutions except the test compound, and a row with all solutions except the bacterial solution adding 10 µl of Mueller Hinton broth instead.

Assessment of mode of action

To determine the mode of action of partially purified BLIS a pre-determined concentration of 100 µg/ml and 200 µg/ml were added to mid-logarithmic growth phase culture of MRSA in 100 ml nutrient broth. The culture was incubated at 37 °C and samples were taken at different time intervals and the optical density was measured at 600 nm and number of viable cells (CFU/ml) was measured by the standard plate counting method on NAM. Nutrient broth inoculated with MRSA was served as control [27].

Determination of molecular weight of antimicrobial compound

The molecular mass of bacteriocin produced by *B. cereus* TSH58 was estimated in a Tricine SDS-PAGE system as described by Schagger and Von Jagow [25], using 4% acrylamide in the stacking concentration gel and 16% acrylamide in the separation gel. After electrophoresis, the gel was cut in two vertical parts. Proteins were visualized after staining the first half of the gel with the Silver Stain Plus Kit (Bio-Rad, Richmond, CA, USA). The other part was assayed for antimicrobial activity, according to Oscariz et al. [19]. Briefly, the gel was rinsed with distilled water (1 h initial rinsed followed by two washes of 5 min), and overlaid with 20 ml of soft (0.8%) nutrient agar seeded with 10^6 CFU/ml of indicator strain MRSA. After incubation at 37 °C for 24 h, the gel was examined for the presence of an inhibitory zone. Molecular mass markers for peptides (3B Black Biotech India Ltd.) were used for mass standards.

RESULTS

Based on the morphological, physiological and biochemical characterization, a total of 160 *Bacillus* strains were isolated from termitarium soil (36.5% sand, 62% clay, total organic C 0.62% and pH 7.4) of the Haridwar district (29°58'N latitude and 78°14'E longitude), Uttarakhand, India. All 160 isolates were screened for their antimicrobial activity against MRSA, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* MTCC 657. Sixteen isolates showed antibacterial activity against at least one of the three test pathogens. Isolate TSH58 showed maximum activity against all the three test pathogens and selected for further study. The 16SrRNA gene sequences of the isolate TSH58 were aligned using NCBI database and phylogenetic tree was constructed using Mega 7 (Fig. 1). On the basis of 16SrRNA gene sequencing and biochemical characterization, the isolate TSH58 was identified as a member of the *Bacillus cereus* species group and the gene sequence of the isolate TSH58 was submitted in the NCBI Gene Bank with accession no. KX344724.

Several nutrient sources and physiological parameters optimized for the maximum production of antimicrobial metabolite production and peptone and glucose were found to be most suitable (data not shown). The growth analysis of the isolate TSH58 along with studies of antimicrobial metabolite production indicated that production of antimicrobial compound started after 16 h of incubation (early stationary phase)

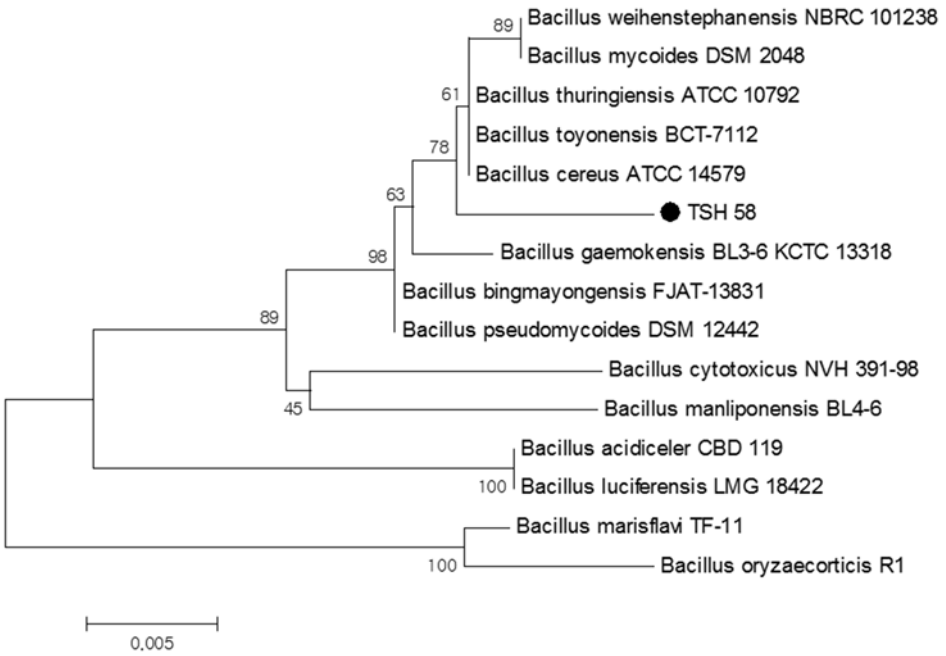

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship of the isolate TSH58 and other members of the genus *Bacillus*

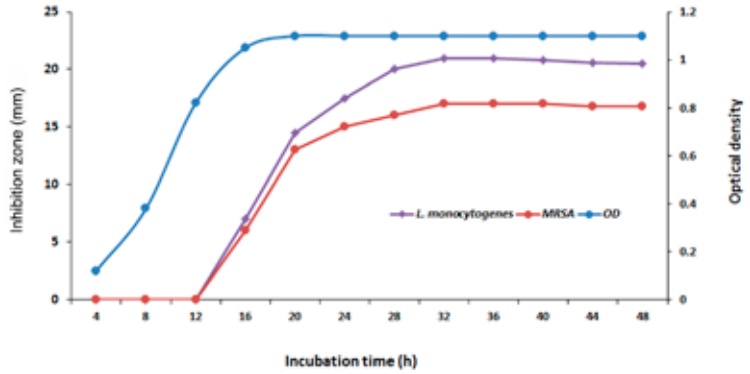

Fig. 2. Growth phase dependent production of bacteriocin by *B. cereus* TSH58. Bars representing standard error

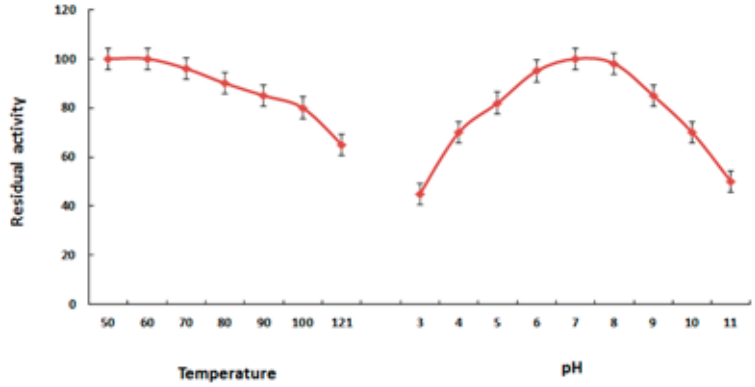

Fig. 3. Residual activity and stability of bacteriocin at various temperature and pH

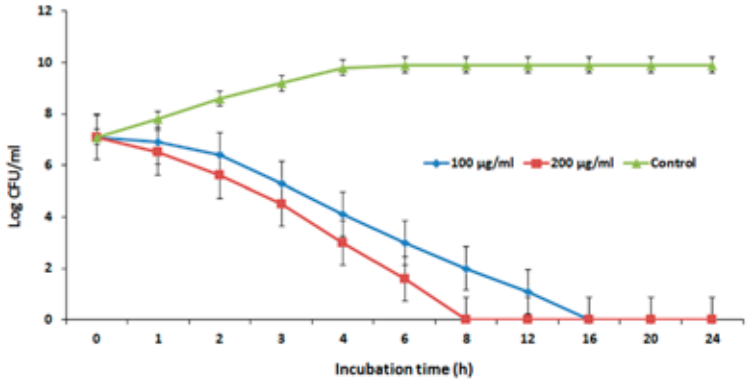

Fig. 4. Effect of partially purified bacteriocin on the growth of methicillin resistant *Staphylococcus aureus*. The results are expressed as log CFU/ml counted in the presence of 100 µg/ml and 200 µg/ml of bacteriocin. Nutrient broth without bacteriocin was used as control

and maximum production was seen between 32 to 36 h of incubation at 30±1 °C by maintaining the pH 7.0 when 0.5% peptone and 1% glucose were added as nutrient sources (Fig. 2). Complete loss of antimicrobial activity was observed when the culture supernatant of the isolate *B. cereus* TSH58 was treated with proteolytic enzymes trypsin and proteinase K, indicated the proteinaceous nature of the compound. Thus, it may be classified as bacteriocin like inhibitory substance (BLIS) with 1280 AU/ml.

The stability of the partially purified BLIS was elaborated at various pH and highest antimicrobial activity was exhibited at the pH 7.0. The BLIS showed slight change in its activity profile between pH 5.0 to 9.0. However, the activity was found sharply reduced at pH below 3.0 and above 11.0 (Fig. 3). The antimicrobial substance produced by *B. cereus* showed stability during heat treatments. The residual activity of BLIS did not exhibit a significant difference from the control. The BLIS produced by the isolate TSH58 was considered to be heat stable as the activity persists even after heating at 121 °C (Fig. 3).

The MIC analysis of the partially purified BLIS showed *L. monocytogenes* was more sensitive as compared to MRSA. The MIC of the BLIS was determined ~40 µg/ml in case of *L. monocytogenes* and ~80 µg/ml for MRSA. To determine whether the partially purified BLIS is bacteriostatic or bactericidal, BLIS at a concentration 100 µg/ml (low dose) and 200 µg/ml (high dose) were added to mid-logarithmic culture of MRSA. Addition of BLIS resulted in very fast decrease in the viability of bacterial cells over a period of 8 h and no growth was observed after 16 h of incubation. Whereas the optical density recorded was found constant during the incubation period (Fig. 4).

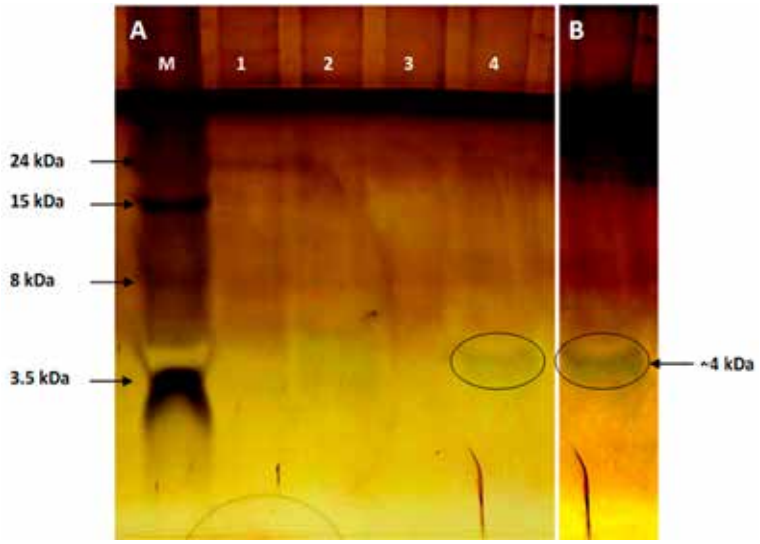

Fig. 5. Tricine SDS PAGE analysis of partially purified bacteriocin from *B. cereus* TSH58 (A) Silver stained gel (M) Peptide molecular weight marker (Lines 1, 2, and 3 are control rows inoculated with buffer only (4) Circle part showing bacteriocin from *B. cereus* TSH589 (B) Contrast image of bacteriocin from *B. cereus* TSH58

The partially purified bacteriocin was analyzed by the tricine SDS-PAGE for the determination of the molecular weight. One part of the gel was assayed for antimicrobial activity using the agar overlaying technique with nutrient soft agar containing indicator organisms. A zone of inhibition was observed at the position equivalent to a protein band, having a molecular mass of about 4 kDa, formed in the other part of the stained SDS-PAGE gel (Fig. 5). From the results obtained through SDS-PAGE, it may be concluded that the antibacterial bacteriocin produced by *B. cereus* TSH58 was a peptide with a molecular mass of about 4 kDa (Fig. 5). On the basis of its low molecular weight and proteinaceous nature, it may be classified as bacteriocin.

DISCUSSION

An increasing number of MRSA infections have become a serious global problem, hence, new therapeutic agents are urgently required to overcome this problem [21]. Bacteriocins may be used as an alternative to overcome this problem. Although a variety of bacteriocins have been discovered, very scanty research has been conducted on their use as therapeutic agents [9]. In this study, a neglected ecological habitat was explored from which *Bacillus* strain TSH58 was isolated, which exhibits antimicrobial activity against MRSA and *L. monocytogenes.* Nutrient broth supplemented with 1% glucose exhibited maximum production of bacteriocin as compared to other nitrogen sources such as tryptone and casein. From these results, it can be estimated that animal peptone and glucose are suitable for bacteriocin production as compared to soya peptone. Earlier, the partially purified antimicrobial substance extracted from the strain TSH58 completely lost its activity treated with the preparation of proteolytic enzymes, suggesting its proteinaceous nature and thus classified as bacteriocin like inhibitory substance (BLIS). The proteinaceous nature of the BLIS was clearly demonstrated by applying denaturing PAGE, which showed that the band observable after purification corresponded to the activity observed after incubation of the indicator strain with a gel slice containing BLIS. The SDS PAGE analysis revealed a clear band of ~4 KDa. *Bacillus strains* have been reported to produce several low molecular weight bacteriocins, i.e. cerein GN105 with mol. wt. 9 KDa [16], cerein 7A with mol. wt 3.95 KDa [19], cerein 7B with mol. wt 4.89 KDa [18], cerein 8A with mol. wt. 26 KDa [7] and cerein MRX1 with mol. wt. 3.14 [26]. Production of antibacterial bacteriocins is common among *Bacillus* species and most of them are active against Gram-positive bacteria. However, quite a few *Bacillus* spp. exhibit killing activity against MRSA [3, 19]. Hence, the BILS isolated in this study could be recommended as an alternative to combat drug resistant bacterial pathogens such as MRSA. It has an added advantage to the industry if it showed stability to heat and pH. The BLIS produced by *B. cereus* TSH58 was found to be thermostable and tolerant to a wide range of pH. The heat and pH stability of bacteriocins has been reported previously for the BLIS isolated from *B. cereus* [20], *Bacillus pumilus* [40], *B. coagulans* [1] and *B. subtilis* [32].

The MIC of the partially purified bacteriocin against MRSA determined by micro-titre plate assay was ~80 µg/ml. Therefore, to determine the mode of action of the partially purified bacteriocin, one lower dose (100 µg/ml) and one higher dose (200 µg/ml) concentration of the partially purified bacteriocin was added to mid-logarithmic culture of MRSA. A continuous decline in the viable cell count was observed. The optical density of the culture remained constant in the experiment indicated the bactericidal nature of the bacteriocin without cell lysis. The bactericidal nature of the bacteriocin was corresponded to concentration and was time-dependent which showed decline in the number of viable cells of MRSA, and no viable growth was observed after 12 h of incubation when 200 µg/ml of bacteriocin was used, and after 16 h when 100 µg/ml of the bacteriocin was used. The bactericidal nature of the *Bacillus* bacteriocins with or without cell lysis has also been reported previously [22, 27].

The present study proved that exploration of rare ecological habitats such as termitarium provides a rich source of microrganisms with valuable properties that may provide a tool to combat drug resistant pathogen infections via their new drug development potential which can provide novel antimicrobial compound to act as a probiotic in nature.

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