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Research Paper

Characterization of Bacteriocin like inhibitory substance produced by a new Strain *Brevibacillus borstelensis* AG1 Isolated from 'Marcha'

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Abstract

In the present study, a bacterium isolated from Marcha- a herbal cake used as traditional starter culture to ferment local wine in North East India, was evaluated for bacteriocin like inhibitory substance production and was tested against six food borne/spoilage causing pathogens *viz. Listeria monocytogenes* MTCC 839, *Bacillus subtilis* MTCC 121, *Clostridium perfringens* MTCC 450, *Staphylococcus aureus, Lactobacillus plantarum* and *Leuconostoc mesenteroides* MTCC 107 by using bit/disc method followed by well diffusion method. The bacterial isolate was identified as *Brevibacillus borstelensis* on the basis of phenotypic, biochemical and molecular characteristics using 16Sr RNA gene technique. Bacteriocin like inhibitory substance produced by *Brevibacillus borstelensis* AG1 was purified by gel exclusion chromatography. The molecular mass of the *Brevibacillus borstelensis* AG1 was found to be 12 kDa. Purified bacteriocin like inhibitory substance of *Brevibacillus borstelensis* was further characterized by studying the effect of temperature, pH, proteolytic enzyme and stability. Bacteriocin like inhibitory substance was found to be thermostable upto 100 °C, active at neutral pH, sensitive to trypsin, and partially stable till third week of storage thus showing a bright prospective to be used as a potential food biopreservative.

Key words: Bacteriocin, purification, SDS -PAGE, Marcha, preservation.

Introduction

Food borne outbreaks are of major concern all over the world. Bacteria are the causative agent of 2/3rd of the food borne disease outbreaks (Kumar *et al.*, 2009). To control these food borne pathogens in food items, role of bacteriocin or bacteriocin like substances might be the best alternative to chemical preservative because bacteriocin provides safety against spoilage causing and food borne pathogens and it does not have any ill effect on health. Bacteriocin has attracted the attention of academia and industry declaring thrust area of research on its production, purification, genetics and applications (Pingitora *et al.*, 2007). Therefore, there is a growing interest to apply these antimicrobial peptide/bacteriocins of bacterial origin that target food spoilage and pathogenic organisms. Bacteriocins are ribosomally synthesized antimicrobial protein or protein complexes with bactericidal activity directed against species that are usually closely related to the producer microorganisms (Sharma and Gautam, 2008). These are heterogeneous compounds which vary in molecular weight, biochemical properties, activity spectra and mechanism of action (Klaenhammer, 1993). Bacteriocins are frequently found as secondary metabolites produced by various microorganisms, such as the gram positive bacteria of genus *Bacillus* and *Lactobacillus sp*. (Jack *et al.*, 1995).

The genus *Bacillus* and *Lactobacillus sp*. include a variety of species with a history of their safe use in different beneficial industrial products like enzymes, antibiotics and amino acids etc. (Bizani and Brandelli, 2002). Similarly for the bacteriocin production, *Bacillus* and Lactic acid bacteria have become the major focus of the study. In the present study, we report on bacteriocin producing potential of a rarely reported isolate, *Brevibacillus borstelensis* AG1 which belong to a nested genus *Bacillus*. This rarely reported bacterial isolate, isolated from least explored

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'Marcha' (a herbal cake used as starter culture to ferment local wine) was taken to produce potential bacteriocin.

Materials and Methods

Isolation of bacteriocin producing strain

A bacterial strain capable of producing bacteriocin like inhibitory substance was isolated from 'Marcha'. Marcha is a traditional herbal cake, which is used to ferment a popular local wine in North Eastern part of India. For its isolation, 1 g fermented material after crushing in sterilized pestle mortar was soaked in 10 mL of distilled water at 25 ± 2 °C for 24 h and it was serially diluted. Isolation of bacteria was carried out by standard serial dilution method on nutrient agar medium followed by incubation at 35 °C for 48 h (Chen and Hoover, 2003).

In total, 5 different bacterial colonies were initially observed on the plates followed by screening of bacteriocin producing isolates by bit/disc method and well-diffusion method (Barefoot and Klaenhammer, 1983; Kimura *et al.*, 1998) against test indicators such as *Listeria monocytogenes* MTCC 839, *Bacillus subtilis* MTCC 121, *Clostridium perfringens* MTCC 450, *Staphylococcus aureus, Lactobacillus plantarum* and *Leuconostoc mesenteroides* MTCC 107 which were mainly spoilage causing/food borne pathogens. Out of total 5 isolates, one present bacterium was selected for further study based on its strong antagonistic activity against maximum number of test strains.

Identification of bacteriocin producing isolate

Identification of selected isolate was done by observing morphological and biochemical characteristics. Further identification at genomic level was done by using 16S rRNA gene technique. Genomic DNA was extracted by using DNA prep kit (Banglore Genei, India Pvt. Ltd. Make). The PCR analysis was carried out with a volume of 50 µL mixture in thermocycler (Astec, Japan). The procedure consisted of 35 cycles of 95 °C for 2 min; 50 °C for 1 min and 72 °C for a min with final extension of 72 °C for 10 min. Amplified PCR product was harvested from agarose gel and purified with gel extraction/purification kit. The purified product was sequenced with an automated sequencer (Applied Biosystem, USA) and the sequence obtained was deposited in NCBI. The isolate was identified as *Brevibacillus borstelensis* AG1 with an accession no. JX129162.

Purification of bacteriocin like inhibitory substance produced from *Brevibacillus borstelensis* AG1

From previously prepared seed culture, 10% of *Brevibacillus borstelensis* AG1 (1.0 OD) culture was added to flask having 500 mL of nutrient broth (pH 7.0) and was kept for incubation at 35 °C at 120 rpm for 48 h. Culture supernatant was obtained by centrifugation (10,000 rpm for 10 min at 4 °C) of *Brevibacillus borstelensis* inoculated nutrient broth incubated at 35 °C for 48 h. Ammonium sulphate precipitation method was followed for partial purification of bacteriocin like inhibitory substance (Ogunbanwo, 2003). Precipitation was attained at 50 per cent salt saturation level and solution was kept at 4 °C for 12 h followed by centrifugation at 15,000 rpm for 30 min (4 °C). The pellet obtained after precipitation was dissolved separately in 1 mL of sodium phosphate buffer (0.1 M, pH 7.0) and was stored at 4 °C. The cell free extract of *Brevibacillus borstelensis* AG1 was dialysed against phosphate buffer (pH 7.0, 0.1 M). After 24 h, the dialysed cell free extract was carefully removed from dialysis bags and antimicrobial activity was checked against test indicators *viz. L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450. The resulting pellet was resuspended in 3 mL sodium phosphate buffer (0.1 M, pH 7.0) and loaded on gel filtration column and 50 fractions were eluted with same buffer.

Each fraction was tested against standard indicators for formation of inhibition zones to detect bacteriocin activity. The fractions showing positive antimicrobial activity were pooled and 12 µL of protein along with 8 µL of sample buffer were applied to 15 per cent polyacrylamid gel which was electrophoresed at 100 V for 2 h. A molecular weight marker of 13-90 kDa was also loaded along with the sample. The gel was then placed in fixing solution for overnight. Next day the gel was put in 30% (50 mL) ethanol for 30 min twice. After fixing, gel was kept in Farmer's reagent for 5 min followed by three washings of 10 min each with autoclaved distilled water. After washing, 0.1% $AgNO_3$ solution was added and the gel was put in dark for 30 min. Gel was washed for 2-3 times with distilled water for 20 s each and 100 mL of developing solution was added until brown coloured band appeared in the gel (Merril *et al.*, 1981). Arbitrary units of purified bacteriocin produced by *Brevibacillus borstelensis* AG1 were calculated by serial two fold dilution method (Barefoot and Klaenhammer, 1983). AU/mL is the reciprocal of highest dilution forming detectable zones of inhibition (Van Rennan *et al.*, 1998). Protein content of bacteriocin like inhibitory substance was estimated after every step of purification by Lowry's method (Lowry *et al.*, 1951).

Characterization of purified bacteriocin like inhibitory substance

Effect of temperature on activity of purified bacteriocin like inhibitory substance

An aliquot of 0.5 mL of purified bacteriocin like inhibitory substance added to test tubes containing 4.5 mL nutrient broth was plugged with cotton to prevent evaporation and then heated at different range of 40, 50, 60, 70, 80, 90 and 100 °C each for 10 min and autoclaved at 121 °C for 10 min. The heat treated bacteriocin like inhibitory sub-

stance samples were then assayed for antimicrobial activity by using well diffusion method against selected test pathogens.

Effect of pH on activity of purified bacteriocin like inhibitory substance

A volume of 0.5 mL bacteriocin like inhibitory substance was added to 4.5 mL nutrient broth in different test tubes and the pH values of the contents were adjusted from 2.0-12.0 individually using dilute NaOH / HCl. After allowing samples to stand at 35 °C for 30 min, the activity was assayed against indicators as already given.

Effect of Proteolytic enzyme (trypsin) on activity of purified bacteriocin like inhibitory substance

Proteolytic enzyme trypsin was selected for the study. Lawns of *L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450 were prepared. EC_1 marked as enzyme control I was 0.3 mL of phosphate buffer, EC_2 marked as enzyme control II was 0.15 mL of partially purified bacteriocin like inhibitory substance and 0.15 mL of phosphate buffer and ER marked as enzyme reaction was prepared by dissolving 0.25 mg of trypsin in 1 mL phosphate buffer (0.1 M, pH 7.0) and added to purified bacteriocin like inhibitory substance in the ratio of 1:1. The preparation EC_1, EC_2 and ER were incubated for 1 h at 35 °C. Enzyme reaction and both enzyme controls were assayed by well diffusion method against corresponding indicators.

The potency of purified bacteriocin like inhibitory substance was checked at regular interval viz. 0, 1, 2, 3, 4, 5 and 6 weeks against sensitive indicators. The activity of purified bacteriocin like inhibitory substance was seen against indicators by well diffusion method.

Results and Discussion

"Marcha" is a traditional fermented herbal cake capable of fermenting a unique local wine of India (Figure 1). Under the specified set of conditions, total 5 strains were isolated from 'Marcha'. Out of them, isolate AG1 was selected for bacteriocin production as it showed maximum antagonistic activity against most of the potent spoilage causing /food borne pathogens used as test indicators. It formed wide zones of inhibition against *L. monocytogene* MTCC 839 [10 (0.81) mm], *B. subtilis* MTCC 121 [10 (0.81) mm] and *C. perfringens* MTCC 450 [8 (0) mm].

This isolate was found to be gram positive, rod shaped having circular and creamish colonies on nutrient agar medium and found to be catalase positive. Based on morphological, biochemical examination and molecular 16S rRNA gene technique the strain was identified as *Brevibacillus borstelensis* AG1 with accession no. JX129162. Following sequences of *Brevibacillus borstelensis* AG1 was obtained after sequence analysis:

Figure 1 - 'Marcha' – A traditional fermented herbal cake.

Brevibacillus borstelensis AG1

GCAACGCTGGCGGCGTGCCTAATACATGCA
AGTCGAGCGAGTCCCTTCGGGGGCTAGCGGCGG
ACGGGTGAGTAACACGTAGGCAACCTGCCCGTA
AGCTCGGGATAACATGGGGAAACTCATGCTAAT
ACCGGATAGGGTCTTCTCTCGCATGAGAGGAGAC
GGAAAGGTGGCGCAAGCTACCACTTACGGATGG
GCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACG
GCCTACCAAGGCGACGATGCGTAGCCGACCTGA
GAGGGTGACCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TTTTCCACAATGGACGAAAGTCTGATGGAGCAAC
GCCGCGTGAACGATGAAGGTCTTCGGATTGTAAA
GTTCTGTTGTCAGAGACGAACAAGTACCGTTCGA
ACAGGGCGGTACCTTGACGGTACCTGACGAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGTGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGCGGCTATGTAAGT
CTGGTGTTAAAGCCCGGGGCTCAACCCCGGTTCG
CATCGGAAACTGTGTAGCTTGAGTGCAGAAGAG
GAAAGCGGTATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAAGG
CGGCTTTCTGGTCTGTAACTGACGCTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCGTAACGATGAGTGCTAGTGTTG
GGGGGTTTCATACCCCTCAGTGCCGCAGCTAAAC
GCAATAAAGCACCTCCCGCCCTTG

TGGCAAGGTCGAGCGAGTACCTTCGGGGGC
TAGCGGCGGACGGGTGAGTAACACGTAGGCAAC
TGCTGGTAAGCTCGGGATAACATGGGGAAACTC
ATGCTAATACCGGGATAGGGTCTTCTCTCGCATG
AGAGGAGACGGAAAGGTGGCGCAAGCTACCACT
TACGGATGGGCGTGCGGCGCATTAGCTAGTTGGT
GGGGTAACGGCCTACCAAGGCGACGATGCGTAG
CCGACCTGAGAGGGTGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATTTTCCACAATGGACGAAAGTCTGA
TGGAGCAACGCCGCGTGAACGATGAAGGTCTTC
GGATTGTAAAGTTCTGTTGTCAGAGACGAACAAG
TACCGTTCGAACAGGGCGGTACCTTGACGGTACC

TGACGAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGCGCGCGCAGGCGGC
TATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAAC
CCCGGTTCGCATCGGAAACTGTGTAGCTTGAGTG
CAGAAGAGGAAAGCGGTATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGGCTTTCTGGTCTGTAACTGACGCTG
AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAGGTGTTGGGGGTTTCAATACCCTCAGTGCCG
CAGCTAACGCAATAAGCACTCCGCCCTGGGAGA
CCTCCG

CCTAATACATGCAAGTCGAGCGAGTCCCTTC
GGGGGCTAGCGGCGGACGGGTGAGTAACACGTA
GGCAACCTGCCCGTAAGCTCGGGATAACATGGG
GAAACTCATGCTAATACCGGATAGGGTCTTCTCT
CGCATGAGAGGAGACGGAAAGGTGGCGCAAGCT
ACCACTTACGGATGGGCCTGCGGCGCATTAGCTA
GTTGGTGGGGTAACGGCCTACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGACCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATTTTCCACAATGGACGAA
AGTCTGATGGAGCAACGCCGCGTGAACGATGAA
GGTCTTCGGATTGTAAAGTTCTGTTGTCAGAGAC
GAACAAGTACCGTTCGAACAGGGCGGTACCTTG
ACGGTACCTGACGAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCG
CAGGCGGCTATGTAAGTCTGGTGTTAAAGCCCGG
GGCTCAACCCCGGTTCGCATCGGAAACTGTGTAG
CTTGAGTGCAGAAGAGGAAAGCGGTATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
CACCAGTGGCGAAGGCGGCTTTCTGGTCTGTAAC
TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAGGTGTTGGGGGTTTCAATACCCT
CAGTGCCGCAGCTAACGCAATAAGCACTCCG.

Sequence of the isolate so obtained was submitted to NCBI database and has been registered to gene bank databases. Phylogenetic tree was constructed on the basis of 16S rRNA gene sequence analysis for *Brevibacillus borstelensis* - JX129162 with the reference sequences available at NCBI through BLAST analysis as depicted in the Figure 2.

Maximum units - 1 x 10^6 AU/mL of crude bacteriocin like inhibitory substance were found at 35 °C after 48 h of incubation (Figure 3). After precipitation using ammonium sulphate, bacteriocin like inhibitory substance of *Brevibacillus borstelensis* AG1 had been concentrated producing 3 x 10^6 AU/mL. This partially purified cell free bacteriocin like inhibitory substance of *Brevibacillus borstelensis* AG1 retained its original pattern of antagonism against *L. monocytogenes* MTCC 839, *B. subtilis* MTCC121 and *C. perfringens* MTCC 450. Bacteriocin like inhibitory active precipitates when dissolved in buffer and subjected to gel exclusion chromatography showed maximum activity in fraction no. 20 to 24 expressing OD of 0.767, 0.927, 1.295, 1.452 and 1.114, respectively at 280 nm (Figure 4). The active fractions after pooling were found to exhibit strong activity against respective test indicators.

Molecular mass of the *Brevibacillus borstelensis* AG1 was confirmed through SDS PAGE and was estimated using Alfa Digi Doc software for molecular mass detection which was found to be 12 kDa (Figure 5). Molecular mass of the bacteriocin laterosporulin produced by *Brevibacillus* sp. strain GI-9 determined by MALDI-TOF experiments was found to be 5.6 kDa (Singh *et al.*, 2013). The bacteriocin Bac-GM100 was purified to homogeneity and based on MALDI-TOF/MS analysis was found to be a monomer protein with a molecular mass 4.375 kDa (Ghadbane *et al.*, 2013). In literature, very low to very high molecular weight bacteriocin ranging from 2.0 (Martrani *et al.*, 2001) to 94 kDa (Rajaram *et al.*, 2010) has been reported. In our study, molecular weight of purified bacteriocin of *Brevibacillus borstelensis* AG1 had been found slightly to be on higher side reflecting the possibility of a novel bacteriocin produced from a strain of *Brevibacillus borstelensis* AG1.

The increase in the size of inhibition zones due to the activity of bacteriocin like inhibitory substance after final purification against the respective indicator has been depicted in Figure 6. The zone sizes as noted by well diffusion assay were 18 (0.47), 14 (0) and 13 (0.81) mm for *L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450, respectively.

An increase of 80, 40 and 62.5% was recorded in zone size against *L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450 after purification. Activity unit of bacteriocin like inhibitory substance of *Brevibacillus borstelensis* AG1 were increased from 1 x 10^6 in culture supernatant to 3 x 10^6 in partially purified and 4 x 10^6 in purified bacteriocin like inhibitory substance. The specific activity was raised to 3.2 x 10^6 in the purified bacteriocin like inhibitory substance (Table 1). This proves that the bacteriocin activity enhances after purification along with the increase in the titres of bacteriocin. Similar studies related to increase in bacteriocin activity after purification has also been reported. Purified bacteriocin of *B. thurigiensis* showed 1.28 x 10^4 AU/mL (1) and AU/mL of bacteriocin of purified from *Brevibacillus brevis* strain GM100 was found to be 11,000 AU mL^{-1} (Ghadbane *et al.*, 2013).

Purified bacteriocin like inhibitory substance was found to be thermostable and retained its activity upto 100 °C for 10 min though partial loss of activity was noticed with continuous increase in temperature. The zones of diameter of 18 (0), 18 (0.47) and 16 (0.47) mm were formed against *L. monocytogenes* MTCC 839, *B. subtilis* MTCC

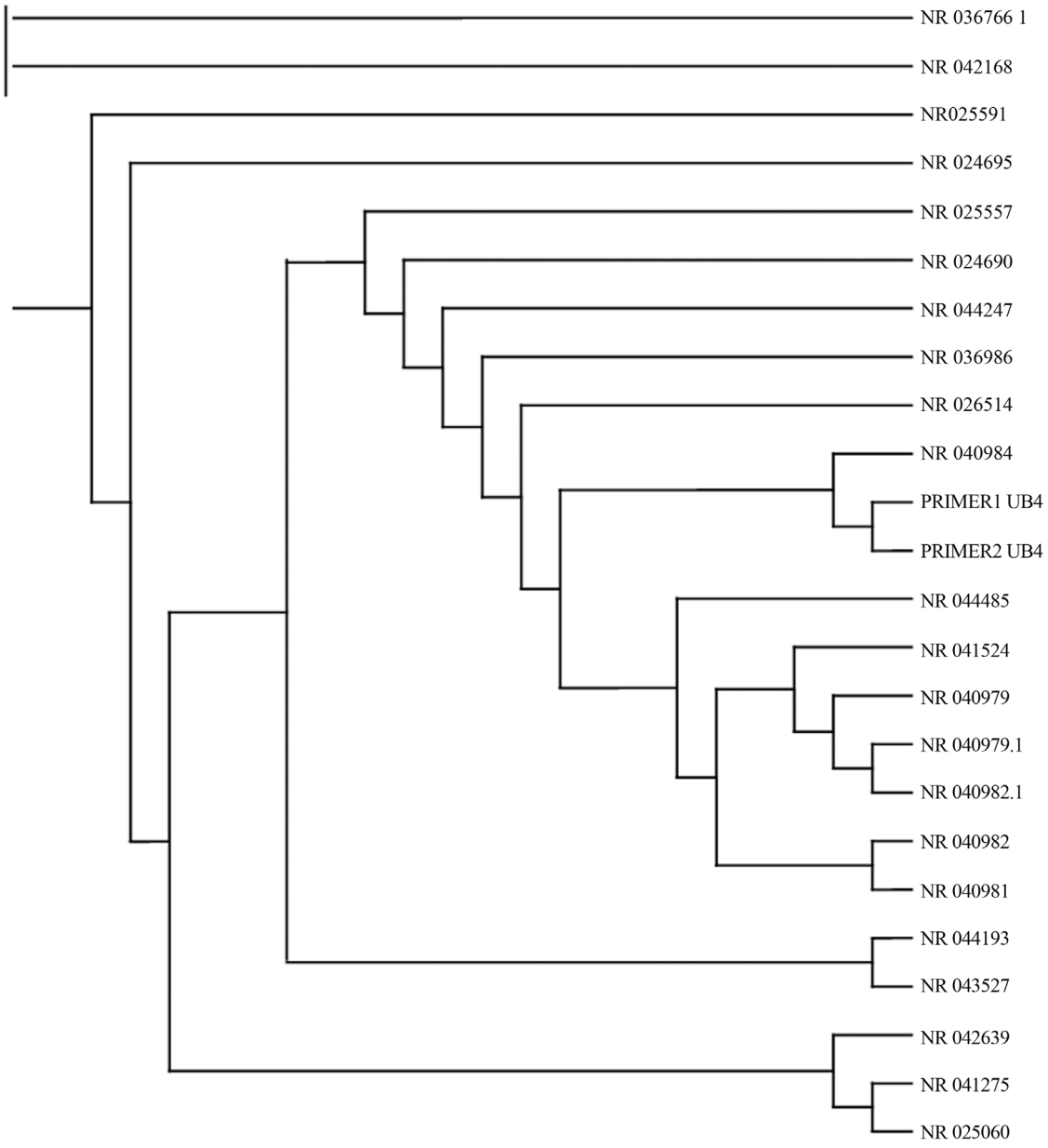

Figure 2 - Phylogenetic tree of *Brevibacillus borstelensis* – JX129162 constructed on the basis of 16S rRNA gene sequence analysis with reference to the sequences available at NCBI through BLAST analysis.

121 and *C. perfringens* MTCC 450, respectively after treatment of bacteriocin like inhibitory substance at 40 °C, 50 °C and 60 °C which decreased to 16 (0.47) mm, 15 (0.47) mm and 12 (0.47) mm for respective indicators by further increasing the temperature to 70-80 °C. The decline was more at higher temperature of 90 °C and 100 °C, zone size with residual activity of 7 (0.81) mm for *L. monocytogenes* MTCC 839, 6 (0.81) mm for *B. subtilis* MTCC 121 and 5(0) mm for *C. perfringens* MTCC 450. The bacteriocin like inhibitory substance lost its activity at very high temperature of 121 °C (Figure 7). The loss of bacteriocin activity at autoclaving temperature may be due to denaturation of three dimensional structure of protein. Earlier studies also have revealed that bacteriocin Bac-GM100 produced by *Brevibacillus brevis* strain GM100 was extremely heat-stable (20 min at 120 °C) (Ghadbane *et al.*, 2013). Bacteriocin produced by *B. megaterium* 19 also displayed heat stability upon exposure to 100 °C for 15 min but was sensitive to autoclaving temperature (Karthikeyan and Santosh, 2009). The bacteriocin produced by *Bacillus licheniformis* was stable at 100 °C for 10 min but lost its activity at 121 °C in 15 min (Khalil *et al.*, 2009) and another novel *Bacillus* sp. retained 70% of its activity after 60 min at 100 °C (Kayalvizhi and Gunasekaran, 2010). This heat stability may be useful if the bacteriocin is to be used as an antimicrobial agent in thermally processed foods.

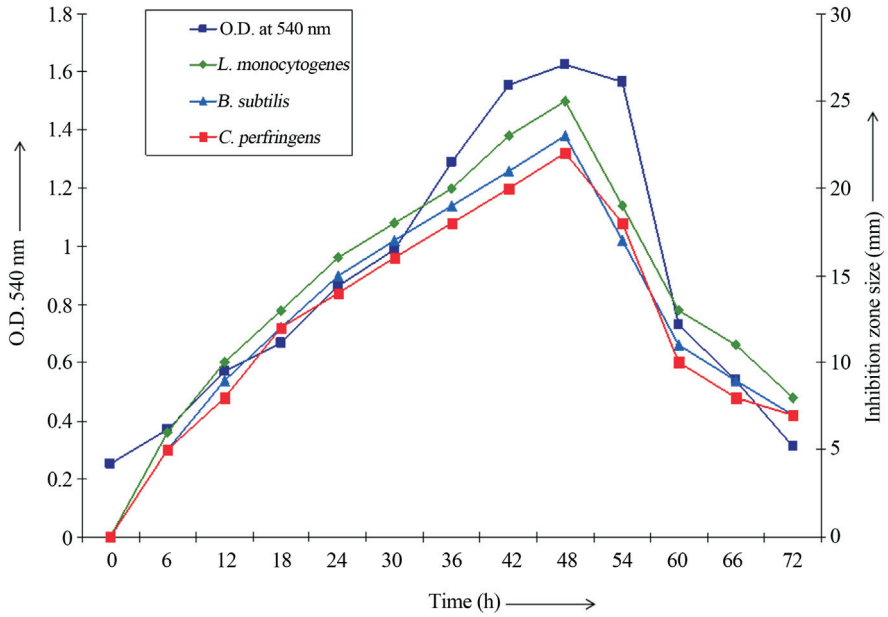

Figure 3 - Production of bacteriocin during growth cycle of *Brevibacillus borstelensis* AG1.

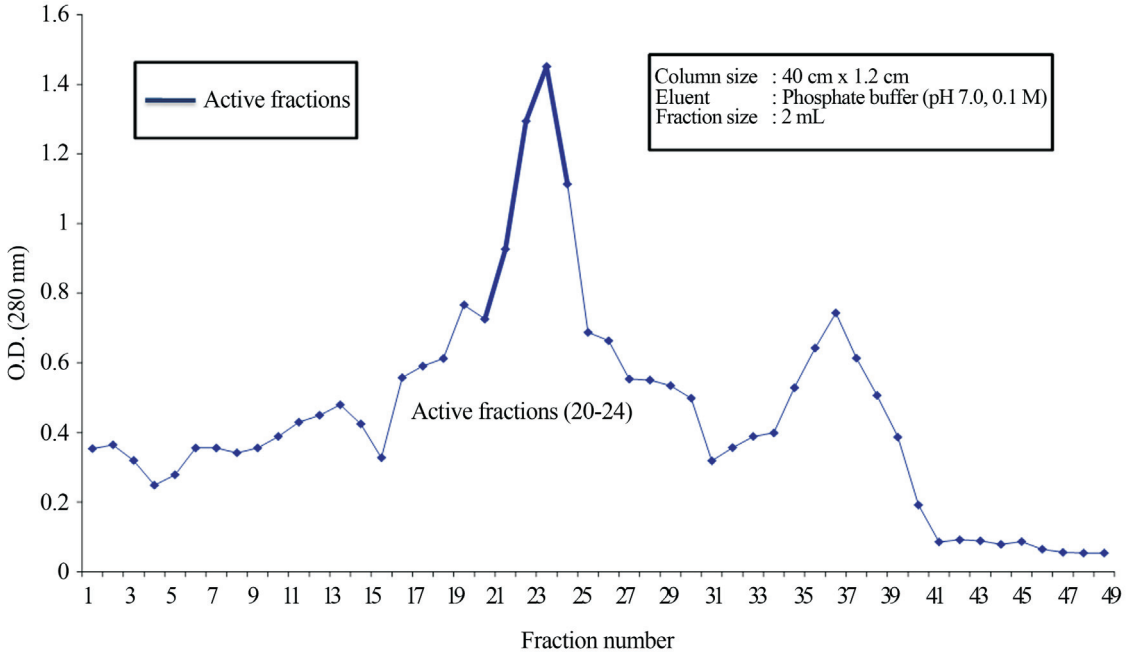

Figure 4 - Elution profile of partially purified bacteriocin of *Brevibacillus borstelensis* AG1 Sephadex G – 75 column.

Bacteriocin like inhibitory substance of *Brevibacillus borstelensis* AG1 showed the maximum activity at neutral pH 7.0 against respective indicators. Bacteriocin retained its activity when pH was changed from 4.0 to 10.0 though there was a complete loss of activity when the pH was lowered to 3.0 and 2.0 and was raised beyond 10.0 (Figure 8). Similar studies were reported where bacteriocin of *Brevibacillus brevis* strain GM100 was stable within a pH range of 3-10 (Ghadbane *et al.*, 2013). Purified bacteriocin of *B. amyloliquifaciens* showed activity in the pH ranging from 3.0-8.0 and thuricin S produced by the *Bacillus thuringiensis* was found stable at a variety of pH levels *i.e.* 3-10.5 (Lisboa *et al.*, 2006; Motta *et al.*, 2007). Thus, this bacteriocin was found to be active at wider range of pH which could be another desirable attribute to use it as food preservative in a variety of food items which may be neutral, acidic and alkaline in nature.

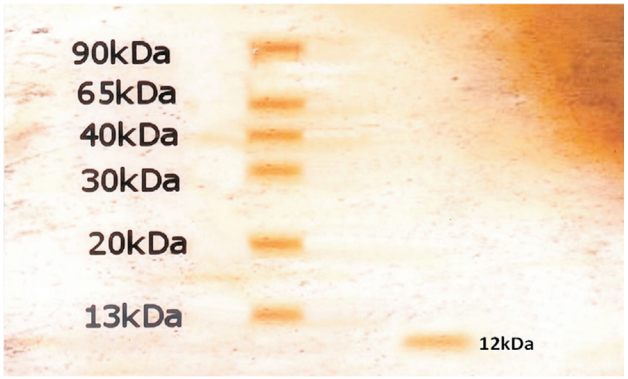

Figure 5 - SDS-Polycrylamide gel electrophoresis of purified bacteriocin produced by *Brevibacillus borstelensis* AG1.

monocytogenes MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450, respectively. This shows that enzyme trypsin had completely inactivated the bacteriocin of *Brevibacillus borstelensis* AG1. Sensitivity of bacteriocin to proteolytic enzyme proves that it is proteinaceous nature and thus can be recommended to be used as a safe food preservative. The proteolytic enzymes of digestive tract are capable of degrading it down completely in our system thus leaving no residue behind. Sensitivity of bacteriocin to proteolytic enzymes has also been observed in other studies. Bacteriocin of *B. thuringiensis* subsp. *tochigiensis* named as tochicin was found sensitive to trypsin at a concentration of 1 mg/mL (Chehimi *et al.*, 2007). Similarly, inactivation of inhibitory activity of partially purified bacte-

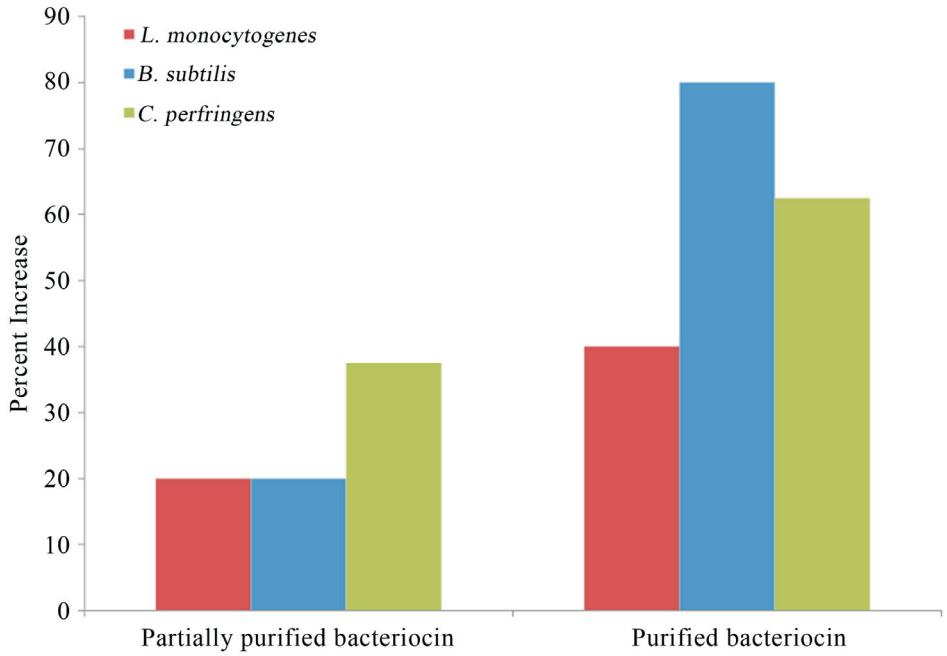

Figure 6 - Percent increase in inhibition zone size (mm) against test indicators of partially purified and purified bacteriocin of *Brevibacillus borstelensis* AG1 over culture supernatant.

Table 1 - Partial purification and purification of bacteriocin produced by *Brevibacillus borstelensis* AG1.

Purification steps	Volume (mL)	Activity units (AU/mL)	Total activity	Protein (mg/mL)	Specific activity (AU/mg)	Purification fold	Recovery (%)
Culture supernatant	800	1×10^6	8×10^8	12.8	7.8×10^4	1	100
Partially purified bacteriocin	30	3×10^6	9×10^7	10.5	2.8×10^5	3.5	82
Purified bacteriocin	10	4×10^6	4×10^7	1.22	3.2×10^6	11.4	11.6

When phosphate buffer alone and phosphate buffer containing enzyme trypsin @ 0.25 mg/mL were welled into lawns of indicator strains, no inhibition zone was formed (Figure 9). But bacteriocin like inhibitory substance treated with phosphate buffer resulted in zone formation of 11 (0.81) mm, 9 (0) mm and 7 (0.81) mm for *L.* riocin produced from *Brevibacillus brevis* strain GM100 was noticed with various prateases (Ghadbane *et al.*, 2013).

Bacteriocin like inhibitory substance produced from *Brevibacillus borstelensis* AG1 was found to be stable upto 6 weeks of storage at low temperature while there was a consistent loss of its activity afterwards at refrigerated tem-

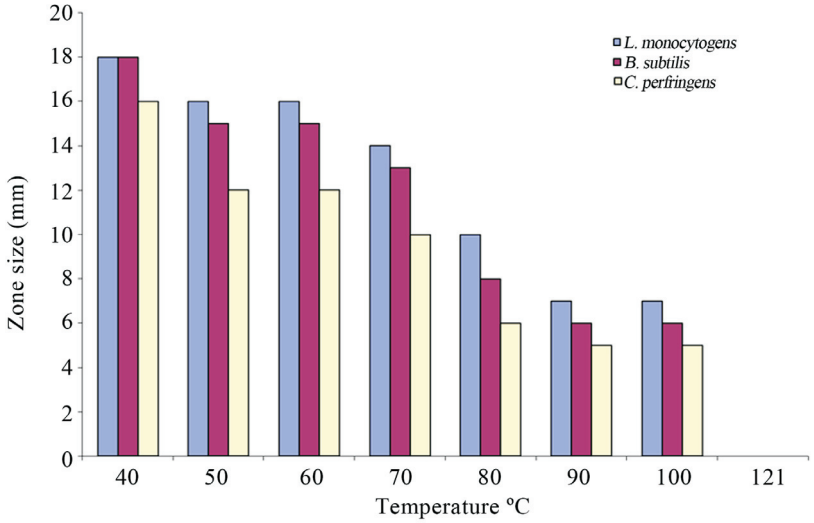

Figure 7 - Effect of temperature on activity of purified bacteriocin produced by *Brevibacillus borstelensis* AG1.

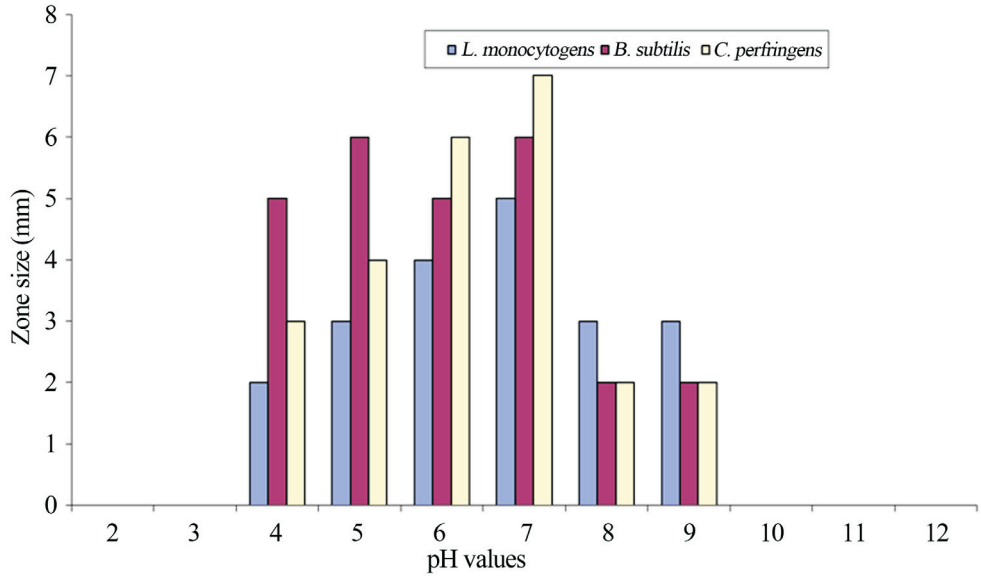

Figure 8 - Effect of pH on activity of purified bacteriocin of *Brevibacillus borstelensis* AG1.

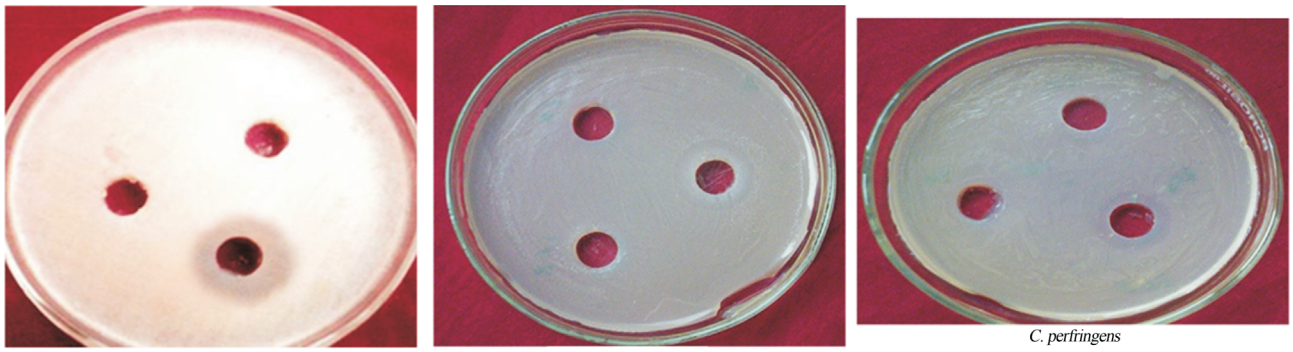

Figure 9 - Effect of proteolytic enzyme – trypsin on activity of purified bacteriocin produced by *Brevibacillus borstelensis* AG1 against *L. monocytogenes*, *B. subtilis* and *C. perfringens*.

perature. It suggested that bacteriocin of *Brevibacillus borstelensis* AG1 could be used efficiently upto six weeks time with minimal loss in its activity.

Conclusion

Bacteriocin like inhibitory substance produced from *Brevibacillus borstelensis* AG1 showed antimicrobial activity against most challenging and serious food borne pathogens like *L. monocytogenes* MTCC 839, *B. subtilis* MTCC 121 and *C. perfringens* MTCC 450. Purification of bacteriocin like inhibitory substance was done by salt saturation (ammonium sulphate) method followed by gel exclusion chromatography. The purified bacteriocin like inhibitory substance exhibited very high activity of 4 x 10^6 AU/mL and specific activity of 3.2 x 10^6. Its titre had increased at every step of purification, along with the size of zone of inhibition which was increased by 80%, 40% and 62.5% against respective indicators. The characterization of bacteriocin like inhibitory substance showed high thermostability upto 100 °C for 10 min, a wide range pH tolerance *i.e.* from 4.0-10.0 and was found to be completely sensitive to proteolytic enzyme - trypsin. The unique combination of all the above mentioned properties rendered bacteriocin like inhibitory substance of isolated strain *Brevibacillus borstelensis* AG1 as an attractive food biopreservative and thus highly desirable for preservation of food items in the food/ food processing industry.

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