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# Detection of heat stable bacteriocin from *Lactobacillus acidophilus* NCIM5426 by liquid chromatography/mass spectrometry

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## Abstract

Bacteriocins from lactic acid bacteria (LAB) are natural antimicrobial peptides or proteins with interesting potential applications in food preservation and health care. The present study was aimed to isolate bacteriocinogenic LAB from dairy products, fermented food and environmental waste samples. One thirty five colonies of LAB were isolated and screened for bacteriocin production by agar overlay method. Among them, Lactobacillus acidophilus NCIM5426 isolated from home made paneer showed maximum zone of inhibition i.e 22-24mm in diameter against food borne (Listeria monocytogenes, Staphylococcus aureus) and human pathogens (Escherichia coli, Salmonella typhi). The antibacterial compound from the culture supernatant was found to be proteinaceous in nature and therefore, identified as bacteriocin. The bacteriocin of L. acidophilus NCIM5426 was found to be heat-stable (1210 for 15 min) and active over a wide pH range of 4.0-10.0. It showed stability (60%) for 30 days at room temperature (30-32°C). Addition of surfactants (EDTA, SDS, Hexadecyl trimethylamonium bromide) up to 1% to crude bacteriocin showed increase in antibacterial activity where as metal ions (Calcium Chloride, Zinc Sulphate and Mercuric Chloride) in low concentration (0.5-1mgL<sup>-1</sup>) decreased the activity. The bacteriocin was purified to its homogeneity by ammonium sulfate precipitation followed by gel filtration chromatography and HPLC. Molecular weight of bacteriocin was found to be 5.6 and 5.8 KDa by SDS page and LC/MS respectively. Production and activity of bacteriocin was significant (50%) even at higher salt (NaCl) concentration i.e 3%. Our present study demonstrates the possibility of using L. acidophilus NCIM5426 or its bacteriocin as a biopreservative in dairy industry.

Keywords: Antibacterial activity, Bacteriocin, Lactic acid bacteria, Lactobacillus acidophilus

## Introduction

Lactic acid bacteria (LAB) are traditionally used as starter cultures for the fermentation of foods and beverages because of their contribution to flavour and aroma development and to spoilage retardation. The preservative effect is mainly due to the acidic condition developed by the bacteria in food, but they are capable of producing and excreting inhibitory substances other than lactic and acetic acid. These include bacteriocins, hydrogen peroxide, ethanol, diacetyl and carbon dioxide (Rajaram et al., 2010). Bacteriocins are antimicrobial peptides synthesized by ribosomes of bacteria which have the property of inhibiting other bacteria, either of the same species (narrow spectrum) or across genera. Some of them are inhibitory towards food spoilage and foodborne pathogenic bacteria including Bacillus, Clostridium, Staphylococcus and Listeria. Therefore, bacteriocins of LAB are of particular interest because of their existing and potential applications as natural preservatives in food. The first bacteriocin to appear on both the European food additive list and the United States FDA list was intended for the use in the production of pasteurized processed cheese (Forouhandeh et al., Bacteriocins have been grouped into four main classes based on their chemical and genetic properties (Ennahar et al., 2000; Garneau et al., 2002). Class I, the lantibiotics; class II, the non-lantibiotic peptides, which are divided into the subgroups IIa or pediocin-like bacteriocins strong activity, with antilisterial bacteriocins whose activity depends the on

complementary action of two peptides, and IIc secdependent secreted bacteriocins; class III, large, heatlabile protein bacteriocins. The class IV bacteriocins are a group of complex protein, associated with other lipids or carbohydrate moieties. They are relatively hydrophobic and heat stable (Alpay et al., 2003). Bacteriocinproducing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality. Most of the bacteriocins from LAB have been isolated from species of the genus Lactobacillus, probably because of the diversity of its species and habitats. The L. acidophilus group of LAB is widely used in (fermented) milk products such as acidophilus yoghurt and sweet acidophilus milk. It has been found that, majority of L. acidophilus strains produce bacteriocins. Lactacin B was the antimicrobial peptide from L. acidophilus that was undoubtedly identified as a bacteriocin (Oh et al., 2000). Besides their application in food industry and their relevance in the improvement of food quality and safety. the increased interest in bacteriocinogenic LAB strains have been ascribed to their potential to modulate the immune system. It has been suggested that some as yet unidentified molecules from probiotic LAB can cross the epithelial layer and inhibit the lipopolysaccharide induced secretion of tumor necrosis factor -  $\alpha$  by monocytic action (Kaiserlian et al., 2005). In vivo studies have documented the ability of probiotics to modulate T cell-mediated allergic responses (Kaiserlian et al., 2005, Dimitrijevic et al., 2009). Although probiotic LAB have

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important benefits to health, caution should be exercised when considering their administration to immuno-compromised subjects (Avlami *et al.*, 2001; Land *et al.*, 2005; Z'Graggen *et al.*, 2005; Brahimi *et al.*, 2008; Tommasi *et al.*, 2008). The main objective of the present study is to evaluate the nature of the antibacterial compound (bacteriocin) from *L. acidophilus* NCIM5426 isolated from dairy sample.

# Materials and methods

Collection of sample

One hundred and thirty five (135) different samples of Milk (Heritage, Nandini, Amul etc.), milk products (cheese, paneer, yoghurt; 50 samples), food products (30 samples such as sausages, pickles etc.), fermented food products (10 samples such as olives, gherkins, beer etc.), environmental waste (10 samples) chicken and mutton sausages (20 samples) were collected from different areas of Bangalore, India and used for isolation of LAB. Sour dough (5 sample), breast milk (10 samples) and faeces of infants (5 samples) were also analyzed.

Bacterial indicator strain and Media

Indicator strains such as *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi* were maintained in Brain heart infusion agar for 24 h at 37°C before experimental use. De Mann Rogosa Sharpe (MRS) and Brain Heart Infusion (BHI) agar plates were prepared by the addition of 1.5% agar. BHI soft agar prepared by adding 0.75% agar to broth media. *Isolation of LAB* 

Lactic Acid Bacteria were isolated from different samples by standard spread plate technique. Samples were serially diluted (up to 10<sup>5</sup>), 0.1 ml was spread on to MRS agar. Plates were incubated at 35°C for 24h. Colony characteristics of the isolated organisms were noted and sub-cultured on MRS agar for further identification. Isolates were maintained at 4°C in MRS soft agar by stab inoculation and overlaid with glycerol (Forouhandeh *et al.*, 2010).

## Identification of LAB

Identification was done by studying the morphological and biochemical characterization (based on carbohydrate fermentation profile, catalase, gelatinase, arginine hydrolysis, motility test). MRS agar without yeast extract was used for the study of carbohydrate fermentation with phenol red as indicator (Bettache & Mebrouk, 2004). Identification of the isolate was further confirmed by VITEK 2 system (Biomeriux, USA).

# Screening for antibacterial activity

All the isolates of LAB were screened for the production of antibacterial compound by testing them against food borne (*L. monocytogenes, S. aureus*) and human pathogens (*E. coli, S. typhi*) by agar overlay method. Strain was spot inoculated on MRS agar, incubated at 35°C for 22 - 24h. Pathogenic organisms (Optical density at 660nm - 0.5) were inoculated into Brain heart infusion soft agar and overlaid on these LAB

culture. Plates were incubated at 37°C for 22h and zone of inhibition was measured (Alvarado *et al.*, 2006).

Production of antibacterial compound and determination of bacterioicn production

The selected strain was subcultured twice at 35°C in MRS broth (pH-6.0) without Tween 80. Inoculum, (2% v/v) from overnight culture was added to MRS broth. incubated at 35°C for 18-20h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4°C). The cell-free supernatant was adjusted to pH 6.0 with NaOH and used as crude antibacterial compound for further study (Ogunbanwo et al., 2003). The antibacterial titer of the bacteriocins in study was quantified by serial dilution. Two fold serial dilutions of crude samples were made in saline solution and aliquots of 50 µl from each dilution were placed in wells in agar plates seeded with the indicator strain. A prediffusion at 4°C for 2-4h was allowed prior to incubation at 37°C for 24h. The diameter of the inhibition zone was taken and recorded in millimeters (mm). The antibacterial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units per ml (AU ml-1) (Rammelsberg & Radler, 1990).

## Characterization of Bacteriocin

The crude bacteriocin sample from *L. acidophilus* NCIM5426 was characterized for stability to proteolytic enzyme (trypsin), heat, pH, surfactants, storage and metal ion. After the treatment, the samples were tested for bacteriocin activity by agar well diffusion method. Indicator organism was spread on BHI or Nutrient soft agar before boring the well. Aliquots of these treated samples were loaded into the well; the plates were allowed for diffusion process at 4°C for 8h then incubated at 35°C for 24h. Zone of inhibition against *L. monocytogenes* and *S. aureus* were measured.

Effect of Protease

Effect of proteolytic enzyme trypsin on activity of crude bacteriocin was studied. Three types of preparation were taken *i.e.*, a) 0.15ml of phosphate buffer + 0.15 ml of bacteriocin + 0.15 ml of trypsin (0.25mg ml<sup>-1</sup>) (b- control 1) 0.15ml of phosphate buffer + 0.15 ml of bacteriocin (c-control 2) 0.15ml of phosphate buffer + 0.15 ml of trypsin (0.25mg ml<sup>-1</sup>).The effect was studied by agar well diffusion method (Neha & Nivedita, 2009).

## Effect of heat

Crude bacteriocin was exposed to various heat treatment (15, 30, 45, 60, 75, 90, 100, 121°C) in boiling water bath for 60 min and every 15 min of time interval, an aliquot of crude bacteriocin was removed to determine the activity (Ogunbanwo *et al.*, 2003). *Effect of pH* 

Active Crude bacteriocin from late log phase of selected LAB was adjusted to different pH (2.0 - 10.0), incubated for 4hrs and aliquots were removed to determine the bacteriocin activity (Ogunbanwo *et al.*, 2003).

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#### Effect of surfactants

Crude bacteriocin was treated with different detergents (Tween-20 and 80, SDS, Ethylene Diamine Tetra Acetic acid (EDTA), Hexadecyl trimethylamonium bromide), concentration ranging from 0.05 - 1.0% and incubated at  $35^{\circ}$ C for 3h, and activity was determined (Mojgani *et al.*, 2009).

## Effect of storage

Crude bacteriocin was stored at different temperatures (-20, 4, 32,  $^{0}$ C). Each day the activity was checked for a period of 30 days (Tenbrink *et al.*, 1994). *Effect of metal ions* 

Crude bacteriocin was treated with metal ions such as Calcium Chloride, Copper Sulphate, Zinc Sulphate, Ferrous Sulphate and Mercuric Chloride at concentration ranging from 0.05 - 100 mg L<sup>-1</sup> (Nusrat *et al.*, 2009).

# Purification of bacteriocin

Ammonium sulphate precipitation

For the extraction of bacteriocin, the bacterial strain was cultivated in MRS Broth for 24 h at 30°C. The crude bacteriocin sample was adjusted to pH 6.0 using 1N NaOH and further precipitation carried out by treating with ammonium sulphate at different percentage of saturation (0-40, 40-60, 60-80) for 24h at 4°C with gentle stirring. Protein precipitated at each saturation was extracted by centrifugation at 10,000g for 30 min. The obtained protein (pellet) was dissolved in phosphate buffer (0.1M, pH-7.0) and checked for antibacterial activity (Liliana *et al.*, 2008). *Gel filtration* 

The protein sample showing bacteriocin activity in ammonium sulphate precipitation was purified by sephadex G 75 (Sigma) gel filtration chromatography. Column was equilibrated by phosphate buffer of 0.1M (pH 7.2) for 30 min and proteins were eluted with the same buffer at a flow rate of 2ml min<sup>-1</sup>. Protein concentration in each eluted fraction was determined at A280nm. Column fractions were again tested for bactericidal activity by well diffusion assay (Liliana *et al.*, 2008). Concentration of protein at each step was determined by Lowry *et al.* (1951) method using BSA (300 μg ml<sup>-1</sup>) as standard. *HPLC* 

Active fractions from gel filtration were further checked for homogeneity by HPLC (Thermo Finnigan Surveyor) using C18 column. The bacteriocin was eluted with acetonitrile and formic acid (0.2 %) in 50: 50 ratio. The flow rate was maintained at 0.2 mLmin<sup>-1</sup> throughout the run, and 0.2-mL fractions were collected and tested for their antibacterial activity against pathogens (*L. monocytogenes and S. aureus*) by agar-well diffusion method (Esther *et al.*, 2008).

Molecular weight determination by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE)

Purified bacteriocin was analyzed by SDS PAGE with 20 % and 4 % concentration of acrylamide in separating and stacking gel respectively. Active fractions were pooled and loaded into the well along with a broad range

marker protein (3 - 200 kDa). The gel was run at constant current 25mA until the tracking dye (bromophenol blue) had migrated to the end. After running, gel was stained overnight with coomassie blue stain and visualized the protein band (Yang *et al.*, 1992).

Bacteriocin activity on SDS PAGE

Bacteriocin activity was checked on SDS page with 20% and 4% concentration of acrylamide in separating and stacking gel respectively. After running, the gel was washed with deionized water for 4h and then placed on nutrient agar prepoured plate. The gel was overlaid with BHI soft agar seeded with pathogen such as *L. monocytogenes* and *S. aureus*. Plates were kept at 4°C for 4h for diffusion and incubated at 35°C for 16h, observed for the zone of inhibition (Yang *et al.*, 1992).

Molecular weight determination by Liquid Chromatography/Mass Spectrometry

Bacteriocin detection was determined by LC/MS using C18 column (Thermo LCQ Deca XP MAX). The bacteriocin was eluted with acetonitrile and formic acid (0.2 %) in 50:50 ratio and detection was performed from 10min to 35min of the elution programme. The flow rate was maintained at 0.2 mL min<sup>-1</sup> throughout the run. The conditions of ESI-MS were as follows: ionization mode, positive; desolvation temperature, 260°C; needle voltage, 2000 V; orifice voltage, 75 V; ring lens voltage, 10 V. The total ion chromatograms were taken in a mass range from m/z 50 to 2000 (Zendo *et al.*, 2008).

# Effect of salt (Sodium Chloride)

The isolate was grown in Mann Rogosa Sharpe (MRS) broth with different concentration of sodium chloride ranging from 0.1 to 5%. Culture was incubated for 24 h at 35° C. After incubation, the growth and bacteriocin activity of the organism was determined by measuring the optical density at 660nm.

# Statistical analysis

All experiments were carried out in triplicates. Results were expressed with average and standard deviation (MS Excel 3). One way and Multivariate ANOVA test (SPSS 16) has been applied for the analysis of results. Mean which is significantly different are expressed in different alphabetical superscripts (P<0.05).

## Results

## Isolation and Identification

Out of 130 test samples, 135 Lactic acid bacteria were isolated. Based on their morphological and microscopical characteristics, they were found to be cocci, coccobacilli and rods. Biochemical characterization of these isolates showed that they belong to the genera *Lactobacillus, Lactococcus, Pediococcus* and *Bifidobacterium.* The selected species of Lactobacillus was given a code as NCIM5426 and identified as *Lactobacillus acidophilus*. Culture has been deposited in NCIM, Pune, India and the accession number is NCIM5426.

Screening of LAB for antibacterial activity

The *L. acidophilus* NCIM5426 showed maximum zone of inhibition *i.e.*, 22-24 mm in diameter against *Listeria* 

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monocytogenes, Staphylococcus aureus, Escherichia coli, Salmonella typhi (Fig.1). Gram positive organisms showed more sensitivity towards crude Bacteriocin than gram negative organisms

Fig.1. L.acidophilus NCIM5426 showing bacteriocin activity against L. monocytogenes A: control, B and C: Culture supernatant (300µl) showing bacteriocin activity

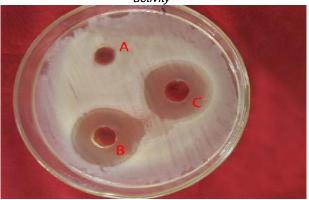


Fig. 2.Effect of Trypsin on stability of bacteriocin activity; A: Control; B: Bacteriocin treated with trypsin

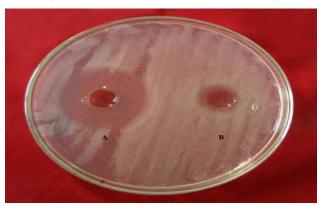
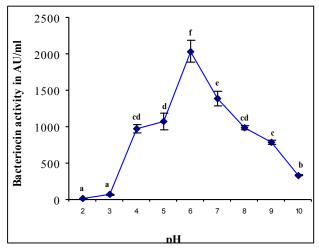


Fig. 3. Effect of pH on bacteriocin activity. Scheffe post hoc test: Means sharing different superscripts is significantly different (P<0.05).



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Table 1. Effect of heat on stability of bacteriocin								
Time interval (min)	15ºC	30ºC	45ºC	60ºC	75ºC	90º C	100ºC	
(Zone of inhibition in mm)								
15	10.33 <sup>a</sup>	9.66 <sup>a</sup>	8.23 <sup>a</sup>	6.90°	5.60 a	5.33 a	5.73°	
30	9.83 <sup>a</sup>	9.33 <sup>a</sup>	7.33 <sup>ab</sup>	7.00 <sup>a</sup>	4.93 <sup>a</sup>	6.0 <sup>a</sup>	5.70 <b>a</b>	
45	9 66 <sup>a</sup>	8 33 a	7 0 <sup>ab</sup>	5 90 a	5 26ª	5 90 a	5 63 a	

Scheffe post hoc test: Means sharing different superscripts in a column significantly different (P<0.05).

5.96 a

5.83 <sup>a</sup>

6.5 b

#### Characterization of Bacteriocin

8.66 a

Culture free supernatant of L. acidophilus NCIM5426 was found to be sensitive to trypsin (Fig. 2) indicating that the inhibitory substance is proteinaceous in nature. The inhibitory activity was unaffected by heating upto 100°C for 60min and 70% of activity retained after heat treatment at 121°C for 15min (Table 1). Bacteriocin produced by the selected strain was stable at broader pH range of 4.0- 10.0 (Fig. 3). Surfactants tested did not have profound effect on bacteriocin activity except Tween 20 and 80, which decreased the activity (Table 2). Bacteriocin was found more stable at -20°C and 4°C for 30days. Activity decreased at 32°C after 20 days (Fig. 4). However, increase in the concentration of metal ion tested had reciprocal effect on activity of bacteriocin. Metal ions such as Calcium Chloride, Zinc Sulphate, and Mercuric Cholride showed decresase in activity of bacteriocin from 0.5mg/ml. Copper Sulphate did not alter the activity (Fig. 5).

Table 2 Effect of surfactants on stability of bacteriocin

rable 2. Effect of surfactants of stability of bacteriocin							
Conc. Detergent	EDTA	SDS	TWEEN 20	TWEEN 80	HDTMEB		
(%)							
(Zone of inhibition in mm)							
0.2	11.33 ab	9.25 a	5.25 ab	7.72 ab	12.13 ab		
0.4	11. 50 ab	9.45 a	7.08 bc	8.86 bc	14.36 ab		
0.6	9.58 a	11.0 ab	4.97 ab	8.13 ab	14.46 b		
0.8	10.45 ab	12.4 b	4.70 ab	7.80 ab	12.03 ab		
1.0	13.83 b	10.9 ab	3.83 a	6.97 a	11.60 ab		
0.0	10.0 a	10.0 a	9.87 ⁰	9.93 ◦	9.93a		

Scheffe post hoc test: Means sharing different superscripts in a column significantly different (P<0.05). EDTA- Ethylene diamine tetra aceticacid, SDS – Sodium dodecyl sulfate, HDTMEB- Hexadecyl trimethylamonium bromide

#### Purification of bacteriocin

A significant increase in the yield and purification fold of the bacteriocin was observed during different purification stage (Table 3). The bacteriocin activity was

Table 3. Purification of bacteriocin from L. acidophilus

Purification stage	Bacteriocinactivity (§)	Protein concentration (†)	Specificactivity (*)	Purification factor (††)
Culture supernatant	1000	480	2.0	1.0
Ammonium sulphate	2000	360	5.5	2.7
Precipitation 80%				
Gel filtration	5000	160	31.25	5.6

§ Bacteriocin activity (AU ml-1); † Protein concentration (µg ml-1) Specific activity is the activity unit divided by the protein concentration

†† Purification factor is the increase in the initial specific activity



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Fig.4. Storage stability on bacteriocin activity Scheffe post hoc test: Means sharing different superscripts is significantly different (P<0.05).

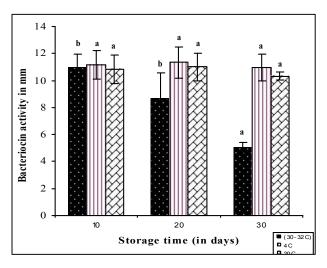


Fig. 5. Effect of metal ion on bacteriocin activity

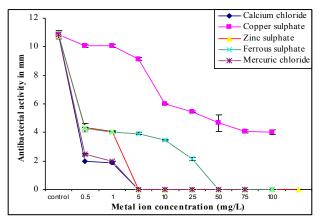


Fig. 6. Purified bacteriocin showing Homogeneity. Lane 1: Broader range Marker protein, Lane 2: Bacteriocin and Lane 3: Medium range Marker protein.

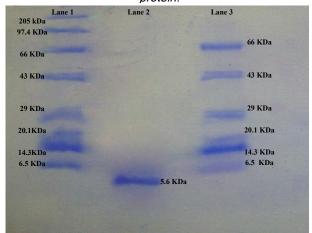


Fig. 7. Bacteriocin showing a single peak at retention time of 17.0 to 21.2

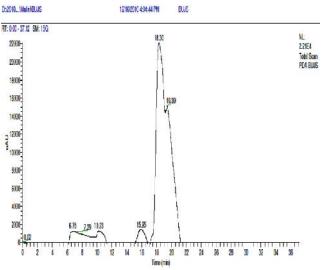


Fig. 8. Mass spectrum of bacteriocin was detected as  $(M+10H)^{10+}$  (m/z 583.28) ions

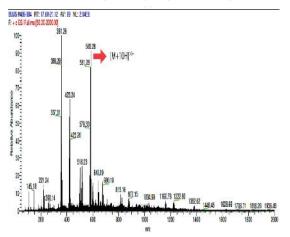
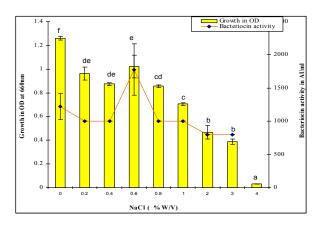


Fig. 9. Effect of Salt (NaCl) by L.acidophilus NCIM5426 Scheffe post hoc test: Means sharing different superscripts is significantly different (P<0.05).



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found at 80% of ammonium sulphate precipitation. Recovered active proteins were then fractionated by gel filtration. Fractions from 15 to 18 showed bacteriocin activity against *L. monocytogenes* and *S. aureus*. HPLC analysis of these active fractions showed a single peak at retention time of 17.6 to 21min indicating the homogeneity of protein.

Molecular weight

The active fraction showed a single band with the estimated molecular weight of 5.6 KDa in SDS PAGE (Fig. 6). Incubation of the electrophoresed gel on the agar seeded with *S. aureus* and *L. monocytogenes* confirmed inhibitory nature of the bacteriocin. *LC/MS* 

A peak at 17.6 - 21 min could be identified as a potent bacteriocin-derived peak (Fig. 7). The mass spectrum at this retention time showed that this molecule was detected as m/z 583.26 which correspond to (M + 10H)<sup>10+</sup>. Therefore, the calculated molecular mass of the bacteriocin was 5.8KDa (Fig 8). Salt tolerance

*L. acidophilus* NCIM5426 was able to grow and produce 50% of bacteriocin upto 3% of salt concentration. Growth was significantly (p <0.05), decreased as the concentration of salt increased (Fig. 9).

## Discussion

The present investigation highlights the isolation and characterization of bacteriocin from a strain of L. acidophilus NCIM5426 isolated from home made paneer. Out of 135 samples, 10% of milk and milk products (Good life, Slim, Nandini flavoured milk etc) did not show any isolates, indicating the sterility of the products which obviously due to the high temperature employed during sterilization process. Among the isolated LAB, 70% belonged to the genera Lactobacillus, 10% Lactococcus. 5% to Pediococcus and Bifidobacterium. It is generally documented that these genera are more predominant in milk samples (Esayas et al., 2008; Azadina et al., 2009; Forouhandeh et al., 2010).

Our initial study showed that 80% of LAB isolated by us was effective against pathogenic organisms tested. The gram positive bacteria (*L. monocytogenes, S. aureus*) were more sensitive against bacteriocin than the gram negative bacteria (*E. coli, S. typhi*). The sensitivity of gram positive bacteria can be attributed to the susceptible nature of the lipoteichoic acid in cellular envelop to bacteriocin. Experimental evidence in *Pediococcus acidilacticin* reveals that lipoteichoic acid is susceptible to bacteriocin (Bhunia *et al.*, 1991). *Lactobacillus acidophilus* NCIM5426 which showed maximum zone of inhibition (22-24 mm in diameter) towards pathogens tested is selected for the present study.

Lactic Acid Bacteria synthesizes bactericidal agents that vary in their spectra of activity (Fricourt *et al.,* 1994). Many of these agents are bacteriocins with a proteinaceous active moiety, while others are non protein

agents such as lactic acid, hydrogen peroxide and diacetyl (Mojgani *et al.*, 2009). In our investigation, we observed that the antibacterial activity by culture free supernatant was completely lost when exposed to trypsin, (0.25mg mL<sup>-1</sup>) revealing its proteinaceous nature and therefore identified as bacteriocin.

The phenomenon of heat stability of bacteriocin from LAB up to 121°C for 15min has been reported earlier in Lactocin RN 78 (Mojgani and Amirinia, 2007) and in L.brevis OGI (Ogunbanwo et al., 2003). Our findings are also in agreement with the above, by observing the activity of bacteriocin in L.acidophilus NCIM5426 after heat treatment at 121°C for 15min. Therefore, it could be grouped under heat stable low molecular weight bacteriocin. Ogunbanwo et al. (2003), Mojgani and Amirinia (2007) reported that bacteriocin from L. brevis OG1 and from L. brevis NM24 respectively exhibit highest activity in acidic pH range of 2.0 to 6.0. However, bacteriocin from L. acidophilus NCIM5426 showed stability at a broader pH value of 4.0 - 9.0. Exposure of the bacteriocin sample to surfactants like anionic and cationic resulted in an increase in bacteriocin titers. It gives indirect information about the structure of the active molecule. Ivanova et al. (2000) reported that anionic detergent, SDS is known to unfold proteins by complexing to the interior hydrophobic core of their native structure thus affecting their three dimensional conformation. In our findings, addition of SDS resulted in an increased bacteriocin effect, which might be due to the solubilization of insoluble aggregates. We observed that the addition of nonionic detergents such as Tween 20 and 80 reduced the bacteriocin activity from L. acidophilus NCIM5426 in contradictory to Sahar et al. (2007) who reported increase in activity with tween 20. Cationic detergent hexadecyl trimethylammonium bromide increased activity. Muriana and Klaenhammer (1991) suggested that the dispersion of the bacteriocin complex into active subunits ultimately results in more lethal hits and consequently enhanced the activity. In our study, there was a decrease in the activity of bacteriocin when the concentration of metal ion increased. Similar such observation was observed by Graciela et al. (1995) in L. casei. Activity of bacteriocin in L.acidophilus NCIM5426 was found to be more stable after storage for 30days at -20 °C and 4°C but decreased within 20 days of storage at 32°C, indicating that cold temperature is the most appropriate preservation technique.

Purification of bacteriocin from L.acidophilus NCIM5426 revealed homogeneity by ammonium sulphate followed by gel filtration chromatography. Its molecular weight was estimated to be 5.6kDa by SDS PAGE. Barefoot and kelhammer (1984) reported molecular weight of bacteriocin, lactacin B from L.acidophilus as 6.5 kDa. Mass spectrum at the retention time showed that the bacteriocin was detected as [M+10H]<sup>10+</sup> (m/z 583.26) and calculated mass is 5832.6 Daltons, which approximately corresponding to the molecular weight



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determined by SDS page *i.e* 5600.0 Daltons. Similar such variation in the mass of bacteriocin between SDS PAGE and LC/MS has been reported by Dimitrijevic et al., 2009. Zendo *et al.* (2008) used different method for high resolution of bacteriocin by LC/MS analysis, by precipitating with acetone. However, the structure has to be determined to derive the exact molecular weight of the bacteriocin in *L. acidophilus* NCIM5426.

Growth of *L. acidophilus* NCIM5426 was determined up to 3% of salt concentration. Addition of salt influence changes the cell morphology to longer, thicker cells (Olfat *et al.*, 2011). Olfat *et al.*, 2011 has reported that some of the Egyptian dairy products are generally produced under high salt concentration, which allows preservation for extended periods of products.

The Bacteriocin identified from *L. acidophilus* NCIM5426 isolated by us from home made paneer posses significant characters such as heat resistance, acid and alkaline tolerance and storage stability at room temperature for a longer period of time. Based on these characters, *L. acidophilus* NCIM5426 can be recommended as a bio-preservative in dairy industry. The stability of bacteriocin in low concentration (0-0.5mg L<sup>-1</sup>) of metal ion can also be considered as a significant factor since in recent days bacteriocin is incorporated as one of the ingredients in cosmetics.

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