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Full Length Research Paper

BACILLUS LICHENIFORMIS IITRHR2: A novel source of antimicrobial proteinaceous food substance

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Spoilage of food due to contamination by bacteria present in the environment is a major problem which affects human health and efforts to identify antibacterial components from natural sources has gained momentum. In this regard we isolated a new strain of *BACILLUS LICHENIFORMIS* IITRHR2 (accession number FJ447354) which produced bacteriocin like inhibitory substance (BLIS). Partially purified BLIS showed a major peak on HPLC with antimicrobial activity. This proteinaceous substance (~1.2 kDa) was thermo-stable and pH resistant but lost activity when subjected to proteinase treatment. BLIS inhibited growth of 16 bacteria including environmental and clinical contaminants of food products and potentiated the activity of nisin. In conclusion, the proteinaceous substance isolated from *B. LICHENIFORMIS* IITRHR2 showed potential to combat contamination of food products by bacteria present in the environment.

Key words: Antimicrobial activity, *Bacillus licheniformis* IITRHR2, BLIS, nisin.

INTRODUCTION

Environmental contamination of food is a common problem faced in this fast paced life style where dietary requirements are fulfilled mostly by refrigerated or preserved food. Although, foods that are natural and require minimal processing to maintain hygiene are preferred by consumers. Alternative treatments which can safeguard nutritional properties without reducing food safety against contamination are replacing traditional methods of chemical preservatives. Many ofthe naturally occurring contaminants in food are of microbiological origin and consist of harmful bacteria and their toxins. In food industry, thermal, non-thermal and natural antimicrobial substances are the prime modes of preservation (Bendicho et al., 2002). Natural antimicrobial compounds have been exploited as preservatives to extend the shelf life, ameliorate food spoilage and decrease transmission of food borne pathogens. Lactic acid bacteria (LAB) have been used as starter culture in dairy products as an effective method for extending shelf life by simple fermentation. LAB's including *Lactococcus lactis subsp. lactis* (Sharma et al., 2010) *Lactobacillus* and *Streptococci* have been extensively researched for bacteriocin production. Non-LAB has also been reported to produce bacteriocin with distinct diversity in their inhibitory actions. Among them, *Bacilli* are most investigated for their ability to produce bacteriocin like inhibitory substances. *Bacillus (B.) thuringiensis* (Sutyak et al., 2008), *Bacillus subtilis* (Stein, 2005) and *B. amyliquefaceiens* (Lisboa et al., 2006) have been reported to produce bacteriocins. Nisin secreted by *L. lactis* subsp. *Lactis* is the only commercialized approved

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Abbreviations: AU, Arbitrary unit; **BLIS,** bacteriocin like inhibitory substance; **BSA,** bovine serum albumin; **CFU,** colony forming unit; **HPLC,** high performance liquid chromatography; **LAB,** lactic acid bacteria; **MTCC,** microbial type culture collection; **NCDC,** national collection for dairy culture; **PBS,** phosphate buffer saline; **TFA,** tri-fluro acetic acid; **US-FDA,** United States-food and drug association.

bacteriocin by US-FDA.

Effective in the microbial control of a number of dairy products, it is extensively used in cheese manufacturing at low pH (Sobrino-Lopez and Martin-Belloso, 2008). Some limitations in its usage include heterogeneous distribution in dairy product matrices and change in the flavor of food. In order to overcome these challenges faced by food industry, combined use of nisin with other antimicrobial compounds is being explored now-a-days against food contamination. The objective of the present study was to characterize a bacteriocin like inhibitory substance (BLIS) from non-LAB, which can be used to inhibit the growth of microbial contaminants. A new strain of *B. licheniformis* IITRHR2 was isolated from cottage cheese (accession number FJ447354) and antimicrobial activity of BLIS isolated from it was assessed. The combined effect of BLIS and nisin was also investigated against *Listeria (L.) monocytogenes* MTCC839 in order to assess the capacity of this combination in preventing microbial contamination in food products.

MATERIALS AND METHODS

Media and chemical reagents

Nutrient agar 1.5%, nutrient broths, brain heart infusion (BHI) agar, BHI broth, sterile disc (6 mm) were purchased from Himedia Biosciences (Himedia Laboratories, Mumbai, India). All the chemicals used throughout the study were commercial products of the highest purity grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise mentioned. Membrane filters (0.22 and 0.45 µm) were purchased from Millipore (Bangalore, India). Solutions were prepared in de-ionized ultra-pure water (Direct Q5, Millipore, India).

Microorganisms

Microorganisms were procured from Microbial Type Culture Collection (Institute of Microbial Technology, Chandigarh, India) and National Collection of Dairy Cultures (National Dairy Research Institute, Karnal, India). Stock cultures were maintained in 20% (v/v) glycerol at -20°C. The microorganisms were propagated on appropriate media (Table 1).

Isolation of bacteriocin producing bacteria

The bacteriocin producing strain was selected among 69 bacterial strains isolated from fermented dairy products purchased from local market of Lucknow, India. Strains were isolated from homogenised fermented dairy products using serial dilution method. BLIS production was screened by disc diffusion assay against *L. monocytogenes* MTCC839 (used as indicator bacteria throughout the study). The strains showing growth inhibition against indicator organism were identified according to Bergey's Manual of Systemic Microbiology (Clauss and Berkeley, 1986). Carbohydrate fermentation profile was studied using rapid hicarbohydrate^{TM} kit (Himedia, Mumbai, India). 16S rRNA sequence based identification was also performed with the support of bacterial identification service (Bangalore Genei Services, Bangalore, India) to confirm its taxonomical identity.

Partial purification of BLIS

Bacterial strain was grown in BHI broth in a shaker incubator (Orbitek, Scigenics biotech, India) at 150 rpm, 30°C for 48 h. Cell-free culture supernatant was precipitated using ammonium sulphate (80% w/v) and stored at 4°C overnight with continuous stirring. The pellet was collected, suspended and dialysed in PBS (pH 7.4) for 48 h and passed through Sephadex G-50 column (0.8 x 30 cm). Fractions were collected and monitored for antimicrobial activity against indicator bacteria. The fraction with antimicrobial activity was filter sterilized and analyzed on reverse phase HPLC (Waters, Singapore) equipped with 515 HPLC pump, pump control module-II, automated gradient controller, UV detector (212 to 240 nm) and analytical HPLC column (C_{18}, 25 x 0.46 cm, 5 µm) at the flow rate of 1.0 ml/min.

After equilibration with solvent A [0.1% tri-fluoro acetic acid (TFA) in water], the peptides were eluted in increasing order of the solvent B (0.1% TFA in acetonitirile) as follows: 0 to 5 min (10% B), 5 to 30 min (10 to 50% B), 30 to 35 min (50 to 80% B), 35 to 40 min (80 to 90% B), 40 to 45 min (90% B), 45 to 50 min (90 to 100% B) (Kemperman-Kuipers et al., 2003). Protein was estimated using bicinchoninic acid (BCA) method taking BSA as standard (Stoscheck, 1990). Fractions positive for BLIS were pooled and stored at 4°C until further use.

Tricine SDS-PAGE electrophoresis

BLIS was separated by 16% tricine SDS-PAGE as described by Schagger and Von Jagow (1987). Ultra low molecular weight marker proteins (1 to 26.6 kDa) were used to determine the approximate molecular size of active protein band. After electrophoresis, one gel was stained with coomassie brilliant blue and another was kept for identification of bacteriocin band on an unstained gel and washed three times in 0.1% tween-80. Indicator bacteria (10^6 CFU/ml) suspended in BHI broth (1%) was overlaid on the gel placed on BHI agar plate and incubated overnight at 37°C to detect the growth inhibition indicating the actual band (Ivanova et al., 1998).

BLIS stability

The residual BLIS activity was determined against indicator bacteria using disc diffusion assay when treated with different enzymes, surfactants and solvents. BLIS (1500 AU/ml) was treated with proteinase K, pronase E, α-chymotrypsin and lipase A (2 mg/ml) for 2 h. Organic solvents (acetone, n-butanol, chloroform and toluene) were used at the working concentration of 50% v/v and surfactants (SDS, triton X-100, tween-20 and tween-80) were used at 10% v/v. Thermal stability was assessed by exposing BLIS to different temperatures (10 to 80°C) and 121°C/103.5 kpa/15 min (autoclave conditions). The level of BLIS activity was also monitored at varying pH (pH 3 to 10) levels in MRS broth.

Antimicrobial spectrum of BLIS

The antimicrobial activity of BLIS was tested using disc diffusion assay against 21 selected bacterial strains (Table 1). Exponential phase cultures of all bacteria were inoculated (10^6 CFU/ml) and spread to prepare uniform bacterial lawn on the nutrient agar medium. BLIS (20 µl) was added (10^{-2} dilution) to each sterile disc placed on the bacterial lawn and incubated for 24 h at 37°C (Ivanova et al., 1998). Plates were observed for clear inhibitory zones and measured using zone scale meter (Himedia Mumbai, India). Each test was repeated thrice and activity expressed in terms of AU/ml defined as the reciprocal of highest dilution (2^n)

Table 1. Antimicrobial activity of BLIS produced from *B. licheniformis* IITRHR2 against various microorganisms.

Microorganisms	Media[a]	Temp (^{O}C)	Inhibitory activity (AU/ml)[b]
Bacillus cereus MTCC1305	BHI	37	1966.7 ± 6
Bacillus subtilis MTCC736	BHI	37	1663.3 ± 5
Bifidobacterium bifidum NCDC235	MRS	30	696.6 ± 4.4
Enterococcus faecalis MTCC439	BHI	37	663.3 ± 2.6
Enterococcus faecalis NCDC114	BHI	37	803.3 ± 4.1
Lactobacillus casei NCDC017	MRS	30	690.0 ± 4.7
Lactobacillus lactis NCDC094	MRS	30	410.0 ± 3
Lactobacillus plantarum NCDC021	MRS	30	nil
Lactobacillus rhamnosus NCDC019	MRS	30	nil
Leuconostoc mesenteroides NCDC219	BHI	37	956.6 ± 2.4
Listeria monocytogenes MTCC387	BHI	37	2216.6 ± 4.3
Listeria monocytogenes MTCC1143	BHI	37	2300.0 ± 4.3
Pediococcus pentosaceus MTCC3817	MRS	30	nil
Pediococcus pentosaceus NCDC273	MRS	30	663.3 ± 4.6
Staphylococcus aureus MTCC737	BHI	37	nil
Staphylococcus thermophilus NCDC074	BHI	37	710.0 ± 4.3
Escherichia coli MTCC1687	NA	37	990.0 ± 3.8
*Pseudomonas aeruginos*a MTCC9027	BHI	37	690.0 ± 4.2
Salmonella typhi MTCC733	MH	37	nil
Shigella flexneri MTCC1457	MH	37	1266.6 ± 4.4
Shigella sonnei MTCC2957	MH	37	973.3 ± 4.2

[a] Media used in the bioassays were BHI: Brain Heart Infusion; MRS: deMan Rogosa Sharpe; MH: Mueller-Hinton, NA: Nutrient Agar. [b] Arbitrary Unit (AU/ml); Values are arithmetic mean±S.D of three determinations.

resulting in growth inhibition.

Combined activity of BLIS and nisin

To determine the bactericidal action of BLIS and nisin separately, and in combination, indicator bacteria was incubated with them in BHI medium. Briefly, BHI medium with an initial log phase culture (10^5 CFU/ml) of indicator bacteria was inoculated with a combination (1:1) of nisin (15 µg) and BLIS (15 µg). Separate treatments of nisin and BLIS were also performed simultaneously (He and Chen, 2006). All treated flasks were incubated at 30^{O}C and viable cell count was monitored at 30 min intervals. BHI medium inoculated with indicator bacteria served as control. Cells were harvested by centrifugation at 5000xg for 3 min, washed with PBS and viable cell count (CFU/ml) was determined as described earlier using serial dilution plate method on BHI agar.

RESULTS

Identification and characterization of bacteriocin producing strain

Out of the 69 bacteria isolated from fermented products, strain isolated from cottage cheese showed highest antimicrobial activity against *L. monocytogenes*, indicator contaminant bacteria. Preliminary characterization of the strain was done by examining its morphological and biochemical properties. It was found to be Gram-positive, rod shaped, and catalase positive showing ability to grow at high salt concentrations (2 to 7% NaCl). In addition, carbohydrate fermentation pattern identified the strain as *Bacillus*. Furthermore, 16S rRNA partial sequence of this strain confirmed its taxonomic identity as *B. licheniformis.* The sequence was submitted to NCBI (National Center for Biotechnology Information, MD, USA) data bank (Genbank Accession no. FJ447354) and strain was designated as IITRHR2. 16S rRNA sequence of strain IITRHR2 showed 99% homology with that of *B. licheniformis* strain PLLA-2 (DQ480087), 98% identity with that of *B. licheniformis* L1 (AB305265) and *B. licheniformis* strain DZ023 (DQ408586).

BLIS purification

The inhibitory activity, specific activity and fold purification of BLIS produced by *B. licheniformis* IITRHR2 during the three-step purification procedure are summarized in Table 2. BLIS was purified from 48 h culture incubated at 30°C in MRS at pH 6.0. The specific activity increased from 722 to 2857 AU/ml after passing through the column. The active fraction when analysed on HPLC showed two minor peaks with one major peak (Figure 1A). All the peaks were collected and monitored for antimicrobial activity. Peak at retention time 23.69 min was found to have inhibitory action on indicator bacteria.

Table 2. Purification of BLIS from *B. licheniformis* IITRHR2.

Purification step	Vol (ml)	Protein (mg)	Activity [a] (AU)	Specific Activity (AU/mg)	Purification (fold)
Culture supernatant	1000	24.7	540,000	218	1.0
Ammonium sulphate precipitation	100	38.9	28,100	722	3.3
Sephadex G-50 column	50	4.9	14,000	2857	13.0
HPLC fraction	0.1	0.3	64,00	18285	83.6

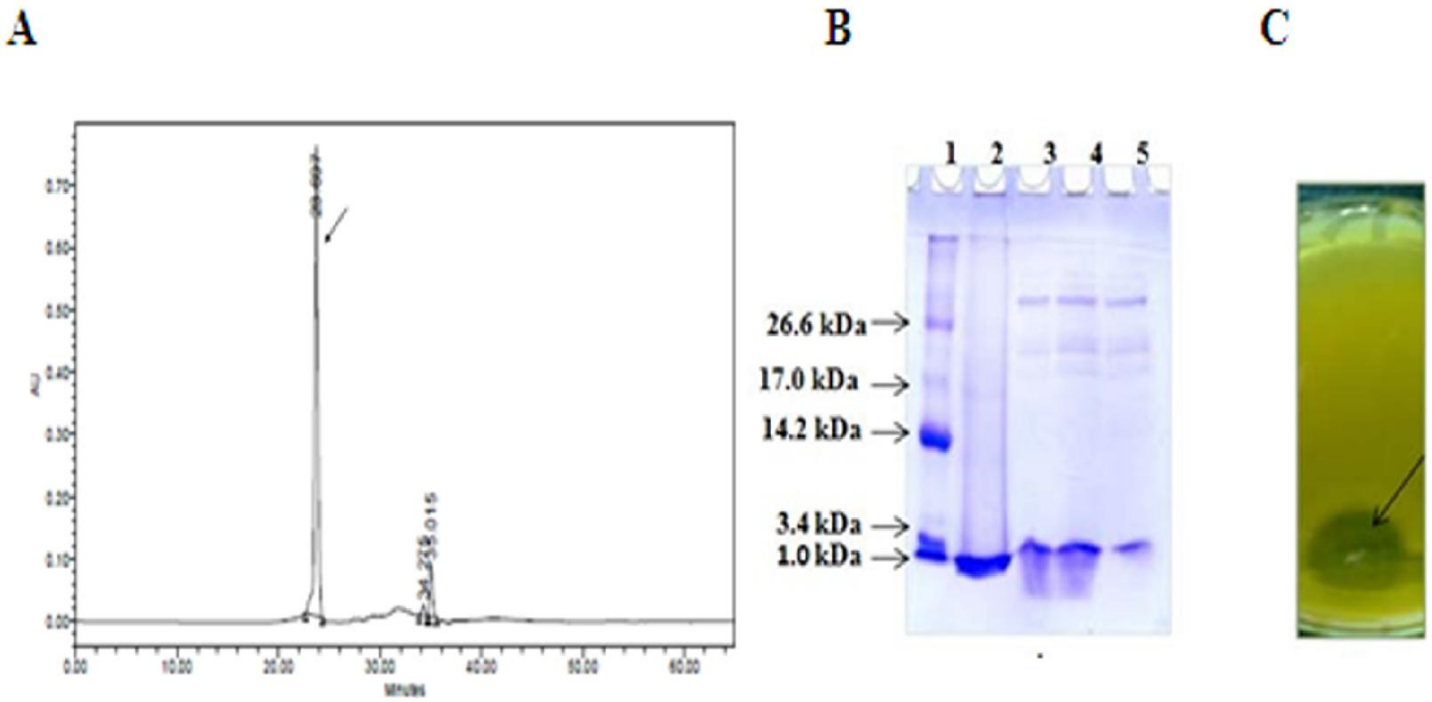

Figure 1. (A) HPLC chromatogram of active fractions of BLIS from *B. licheniformis* IITRHR2. Arrow indicates active peak. (B) Tricine-SDS-PAGE analysis of BLIS. Gel was stained with coomassie brilliant blue. Lane 1: marker; Lane 2: HPLC purified active fraction; Lane 3: Sephadex G-50 fraction, Lane 4; ammonium sulphate salted out fraction; Lane 5: culture supernatant. (C) The second gel was overlaid with *L. monocytogenes* MTCC389 to identify the band corresponding to antibacterial activity.

At this step, the purification fold was found to be 83.6 with specific activity of 18285 AU/ml. The ammonium sulfate (80%) precipitated fraction of BLIS, on tricine SDS-PAGE showed multiple bands due to the presence of BLIS along with broth proteins. BLIS preparation showed a prominent protein band at ~1.2 kDa (Figure 1B). Authenticity of the partially purified protein was confirmed by inhibitory zone observed with the gel half overlaid with *L. monocytogenes* MTCC837 (Figure 1C).

Characterization of BLIS

The partially purified BLIS was found to be sensitive to proteolytic enzymes that is, proteinase K and pronase E (Table 3). BLIS was more sensitive to triton x-100 as compared to other surfactants that is, tween-20, tween-80 and SDS but lost its activity when treated with TCA indicating proteinaceous nature. Reduced activity was observed on toluene treatment but it was resistant to lipase A, chloroform, n-butanol and acetone. At pH 6.0, the maximum reduction in observed residual activity was 49.4% at pH 3.0 (Figure 2A). BLIS was found to be resistant to temperature up to 80°C (Figure 2B) but showed decreased activity at higher temperatures indicating thermo-intolerance beyond 80°C.

Antimicrobial spectrum of BLIS

BLIS was tested for the presence of antimicrobial activity against 21 selected environmental and food contaminant

Table 3. Effect of different enzymes, surfactants and chemicals.

Treatment	Final concentration	Residual activity (%)
Enzymes (mg/ml)		
α-Chymotrypsin	2	82 ± 10
Proteinase K	2	74 ± 7
Pronase E	2	77 ± 4
α-amylase	2	82 ± 8
Lipase A	2	95 ± 2
Surfactant (%)		
SDS	10	81 ± 7
Triton X-100	10	76 ± 6
Tween 20	10	81 ± 5
Tween 80	10	88 ± 6
Chemicals (v/v)		
Acetone	50	96 ± 4
n-Butanol	50	93 ± 4
Chloroform	50	99 ± 1
Toluene	50	33 ± 8
TCA	20	21 ± 6

Values are mean±S.D. of three determinations in each case.

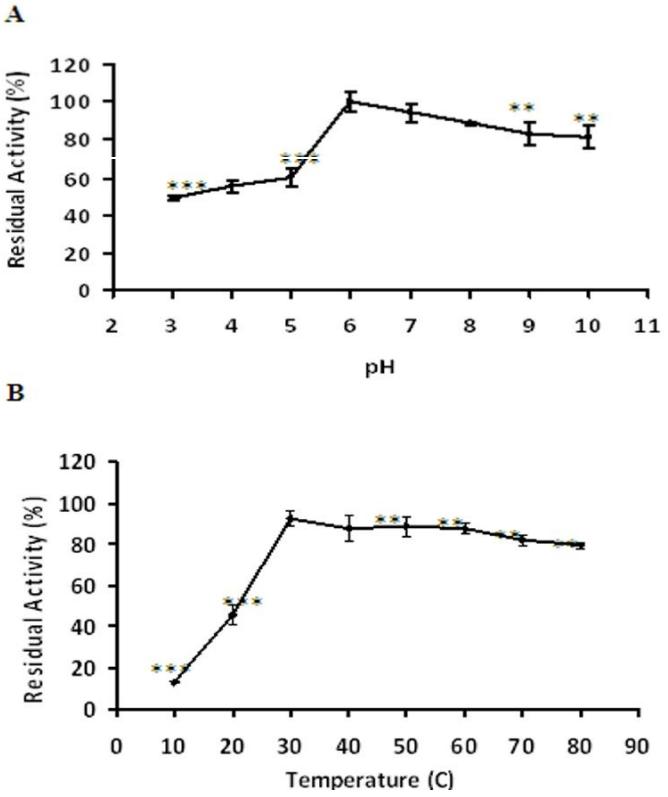

Figure 2. Residual activity of partially purified BLIS from *B. licheniformis* IITRHR2. (A) Effect of pH variation on the antimicrobial activity. (B) Effect of different temperatures on the antimicrobial activity of BLIS from *B. licheniformis* IITRHR2 indicator bacteria.

strains (Table 1), BLIS was found to have antimicrobial activity against 16 bacteria. It inhibited various gram positive bacterial strains such as *B. subtilis* MTCC736, *B. cereus* MTCC1305, *Streptococcus thermophilus* NCDC074, *Pediococcus pentosaceus* NCDC273, *Leuconostoc mesenteroides* NCDC219, *L. monocytogenes* MTCC837, *L. monocytogenes* MTCC1143, *Bifidobacterium bifidum* NCDC235, *Enterococcus faecalis* MTCC439 and *E. faecalis* NCDC114. Growth of gram negative bacteria was also inhibited by BLIS which included *Shigella flexneri* MTCC1457, *Shigella sonnei* MTCC2957, and *Pseudomonas aeruginosa* MTCC9027. BLIS was also tested against selected strains of *Lactobacilli* to check its growth inhibitory activity. It was observed that BLIS did not interfere with the growth of *Lactobacillus plantarum* NCDC 021 and *Lactobacillus rhamnosus* MTCC1408. Thus, the data suggests that the selected concentration of BLIS can be used to check growth of environmental pathogens.

Combined activity of nisin and BLIS

Combined use of different bacteriocins may be an attractive approach to avoid development of resistant strains. Combined activity of nisin and BLIS was monitored against indicator bacteria *L. monocytogenes* as evident from Figure 3A. After 60 min incubation nisin and BLIS used separately were found to be bactericidal showing decrease in cell count (each 4.0 log units)

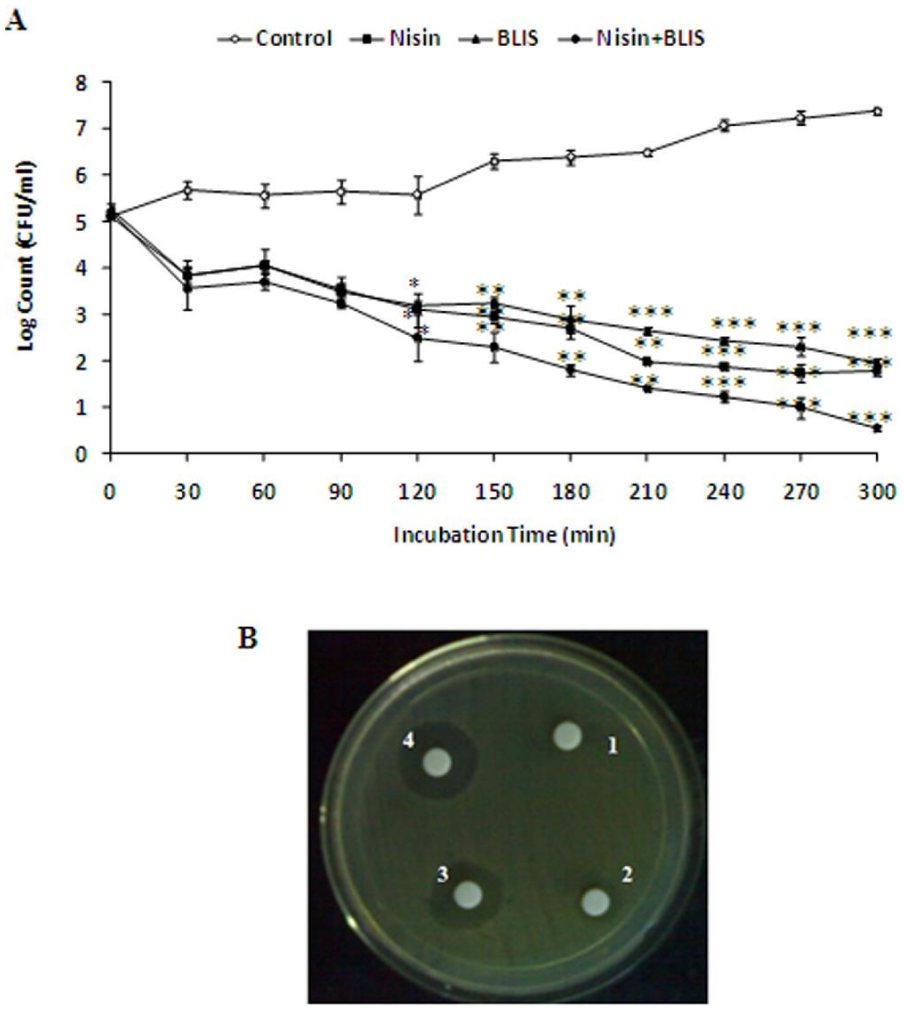

Figure 3. (A) Effect of nisin ( ), BLIS (■), nisin+BLIS in-combination (▲) compared to control (◊) on the viable cells of the indicator strain *L. monocytogenes* MTCC839. Values are mean±S.E. of three determinations in each case [*p<0.05, **p<0.01, ***p<0.001]. (B) Zone of growth inhibition against indicator bacteria. Disc 1: negative control (vehicle); disc 2: BLIS; disc 3: nisin and disc 4: nisin+BLIS in-combination on BHI plate.

whereas, the combined action showed further decrease in cell count (3.7 log units) as compared to control (without treatment). The combined action of nisin and BLIS after 2 h showed further reduction in viable cell count (2.5 log units) indicating enhanced activity in combination as compared to nisin and BLIS alone (3.1 and 3.2 log units). Combined effect of nisin and BLIS was also observed during *in-vitro* plate assay using disc diffusion method (Figure 3B). Nisin and BLIS in combination showed largest inhibition zone (17 mm) as compared to nisin (15 mm) and BLIS (10 mm) alone.

DISCUSSION

Bacteriocins are extracellularly released low molecular mass proteins (usually 30 to 60 amino acids) which have bactericidal / bacteriostatic effect on other bacteria including food-borne pathogens (Klaenhammer, 1988). Lichenin, bacillocin 490 and P40 produced by *B. licheniformis* strain 26 L-10/3RA, 490/5 and P40, respectively have been reported (Pattnaik et al., 2001; Martirani et al., 2002; Teixeira et al., 2009) showing an array of bacteriocins from non-LABs (Sutyak et al., 2008) having equal importance.

The genus *Bacillus* has many commercially important species including the ones producing proteinaceous antibiotics. Species such as *B. subtilis* and *B. licheniformis*, are "generally recognized as safe" (GRAS) bacteria and have been used in food and industry (Paik et al., 1997). In this study, *B. licheniformis* IITRHR2 was isolated from a fermented dairy product that is, cottage cheese. The strain was first identified as *Bacillus* on the basis of biochemical and carbohydrate fermentation pattern and taxonomical identity was confirmed as *B. licheniformis* using 16S rRNA gene sequence analysis. It

is established that pH and temperature optimization are essential to regulate bacteriocin production in MRS medium (Sharma et al., 2010). In our system, BLIS from *B. licheniformis* IITRHR2 was active at low pH and temperature. We also found strong stability pattern for temperature and pH stress which was comparable to previous reports of peptides from other strains of *B. licheniformis* (He et al., 2006, Teixeira et al., 2009). The data suggests that BLIS from *B. licheniformis* IITRHR2 is sensitive to proteolytic action (α-chymotrypsin, proteinase K and pronase E), and some inhibition was observed in the presence of carbohydrate degrading enzyme, α-amylase.

It showed resistance to chloroform, n-butanol, and acetone. BLIS was labile to toluene, triton x-100 and TCA whereas it was stable in the presence of other surfactants such as SDS, tween-20, tween-80. BLIS produced by *B. licheniformis* IITRHR2 exhibited pronounced inhibitory activity against various species of gram positive bacteria in our study which is in conformation with earlier reports (Teixeira et al., 2009). BLIS showed no antimicrobial activity against *L. plantarum* NCDC021 and *L. rhamnosus* NCDC019. The preliminary findings suggest that this property may be optimized and applied to get controlled viable population of fermenting starter culture. Combined use of different bacteriocins may be an attractive approach to avoid development of resistant strains. In our study we tried the use of combination of two bacteriocins which showed enhanced bacteriostatic mode of action against *L. monocytogenes.* Combined activity of BLIS with nisin can further enhance the stability at storage pH and temperature. Since, the growth inhibitory activity of BLIS was studied by us in liquid media only, difference in its activity in various matrices of food products cannot be ruled out at this stage.

These initial findings - indicate possible application of BLIS isolated from novel source that is, *B. licheniformis* IITRH2 as bio-preservative against microbial contamination.

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