Detection, partial purification and characterization of bacteriocin produced by *Lactobacillus brevis* FPTLB3 isolated from freshwater fish

Bacteriocin from *Lb. brevis* FPTLB3

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Abstract *Lactobacillus brevis* FPTLB3 was isolated from freshwater fish, capable of producing bacteriocin that had broad spectrum of inhibition (3200 AU/ml) against *Escherichia coli* MTCC 1563, *Enterococcus faecalis* MTCC 2729, *Lactobacillus casei* MTCC 1423, *Lactobacillus sakei* ATCC 15521 and *Staphylococcus aureus* ATCC 25923. The antimicrobial activity of crude supernatant fluid was stable after heating at 121 °C for 60 min and declined thereafter. Stability of antimicrobial activity was observed at pH range of 2.0 to 8.0. Its active principle was proteinaceous in nature since the bacteriocin was inactivated by proteolytic enzymes, but not by other non-proteolytic enzymes. Mitomycin C and UV light did not affect the activity of the bacteriocin, while chloroform extraction completely destroyed their activity. Exposure to surfactant resulted in an increase in titre, except Nonidet P-40, which led to total loss of activity. No bacteriocin adsorption was detected at pH 1 to 2, whereas 100% bacteriocin adsorption was found at pH 6.5. Based on Tricine SDS-PAGE the estimated molecular mass of bacteriocin was 54 kDa. No plasmid was found to present in the isolate.

Keywords Biopreservation · Lactic acid bacteria · *Lactobacillus brevis* · Bacteriocin · Antimicrobial activity

Food safety is an important issue of international concern. Consumers of this decade are interested in food without

chemical preservative and with additional health benefits. Pre-packed food items available in food market contain variety of chemical preservatives, which may alter chemical constituents, nutritional and organoleptic qualities of food thus may have serious adverse effects on health (Messi et al., 2003). Thus, biopreservation of food has emerged as an attractive and safe approach. Among biopreservatives, bacteriocins have received increasing attention due to their unique properties of inhibiting food-borne pathogens and spoilage causing microorganisms. Bacteriocins produced by lactic acid bacteria (LAB) have been evaluated for the preservation of milk, meat and vegetables due to their unique characteristics and their potential use as natural food preservatives.

Bacteriocins are proteins or peptides having bactericidal activity that can control the growth of food spoilage bacteria. These substances are produced by different types of Gram positive and Gram negative microorganisms, most importantly lactic acid bacteria with variable molecular weights, biochemical properties and mode of action (Bhattacharyya and Bhattacharjee 2007). They usually exhibit antagonistic activity against microorganisms closely related to the bacteriocin producing strain. Several of these bacteriocins are bactericidal against pathogenic and food spoilage bacteria (Caplice and Fitzgerald 1999). For this reason, bacteriocins are now widely used for preservation of food in commercial preparations.

Many works have been done on LAB and/or bacteriocins but very few on *Lactobacillus brevis*. *Lb. brevis* KB290, a plant derived probiotic lactic acid bacterium reportedly improves gut health, stimulates immune function and suggested that it was safe for human consumption (Yakabe et al. 2009). Kishi et al. (1996) demonstrated that *Lactobacillus brevis* increases the production of interferon-alpha. Interferon-alpha is mainly involved in immune response against viral

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infection. Banach et al. (2002) suggested that feeding on *Lactobacillus brevis* 1E-1 may provide a healthier intestinal microflora during lactation. Nisin producing LAB has inhibitory effect on various food-borne pathogens and may be used as natural preservatives in food products (Hazarika et al. 2007). The crude supernatant of *Lactobacillus delbruekii* subsp. *lactis* isolated from fresh water fish processing waste indicated this bacterium to be antagonistic towards several pathogens including *L. monocytogenes*, *Bacillus cereus*, *Yersinia enterocolytica* and *E. coli* (Sudeepa et al. 2007). In the present study, a bacteriocin produced from *Lactobacillus brevis*, isolated from fresh water fish, was partially purified and characterized.

Materials and methods

Bacterial strain and culture media

Lactic acid bacteria screened for bacteriocin production and bacteriocin producing strain *Lactobacillus brevis* FPTLB3 were isolated from fresh water fish, mrigala (*Cirrhinus mrigala*). Bacterial strains used as indicator organisms are shown in Table 1 along with their specific growth medium.

Screening of bacteriocin-producing lactic acid bacteria

Ten grams of fish gut was added to 90 ml of sterile peptone (0.1%) water and then thoroughly homogenized for 1 min in a Laboratory Blender Stomacher. From appropriate 10-fold dilutions, 100 µl was spread on modified deMan Rogosa Sharpe (MRSm) agar (containing 0.2% w/v glucose and supplemented with 50 µl/ml of cyclohexamide, Himedia) plates and incubated anaerobically at 30 °C for 24 h. After anaerobic incubation, the plates that provided separated bacterial colonies were overlaid with 10 ml of nutrient soft (0.7%) agar (Himedia) containing approximately 10^6 cells of *Staphylococcus aureus* ATCC 25923. The plates were anaerobically incubated at the optimal growth temperature for the indicator organism. The plates are then checked for inhibition zones around colonies of isolated lactic acid bacteria (Rattanachaikunsopon and Phumkhachorn 2006).

Spectrum of antimicrobial activity

The spectrum of antimicrobial activity of isolated lactobacilli was detected by two methods, the agar-spot deferred test and the well diffusion assay (Hernandez et al. 2005). In the first case, colonies of the strains to be tested for bacteriocin production were grown on the surface of MRSm containing 1.5% agar for 24 h at 30 °C. The indicator strains, as described in Table 1, were inoculated (4% v/v) into 7 ml of soft agar medium (containing 0.7% agar) specific for each strain, at a final concentration of 10^6 CFU/ml. The soft media were poured on the plate where growth of the producers occurred and the plates were incubated anaerobically, at the optimal growth temperature for the indicator strains, for 24 h. Inhibition was recorded positive in presence of a detectable clearing zone around the colony of the producer strain.

For the well diffusion assay, isolated strains were grown in 2 ml of MRSm broth, under anaerobic conditions in order to avoid H_2O_2 formation, up to stationary phase (48 h at 30 °C). Cultures were centrifuged at 8000 X g for 10 min; the supernatants were collected, adjusted to pH 6.5, sterilized by microfiltration (0.22 µm pore size filter paper, HiMedia). The solution thus obtained, designated as crude supernatant fluid (CSF), was stored at −20 °C until further use. A 50 µl of the CSF was placed into the well (6 mm diameter) of the seeded plates. After pre-diffusion at 4 °C for 1 h, the plates were incubated under the conditions described above for the various indicator strains. The antibacterial activity was determined by measuring the diameter of the inhibition zone around the wells and expressed as arbitrary units per ml (AU/ml). One AU was defined as the reciprocal of the highest serial twofold dilution producing a distinct inhibition on the indicator lawn (Hernandez et al. 2005).

$$\text{AU/ml} = \text{reciprocal of highest twofold dilution} \times 100$$

Identification of bacteriocin producing lactic acid bacteria

Bacteriocin producing lactic acid bacteria were identified on the basis of Gram staining, catalase reaction and the

Table 1 Inhibition of various indicator organisms by CSF produced by *Lactobacillus brevis* FPTLB3 in agar well diffusion assay

Indicator organisms	Specific medium	Incubation temperature (°C)	Zone diameter (mm) ^a
<i>Lactobacillus casei</i> MTCC 1423	MRSm	30	7±0.3
<i>Lactobacillus sakei</i> ATCC 15521	MRSm	30	8±0.3
<i>Staphylococcus aureus</i> ATCC 25923	Baird Parker	37	5±0.0
<i>Escherichia coli</i> MTCC 1563	Tergitol 7	37	8±0.3
<i>Enterococcus faecalis</i> MTCC 2729	BHI ^b	37	10±0.5

^a results are mean of three determination ($n=3$) with S.D

^b brain heart infusion agar

fermentation pattern of carbohydrates using the API 50CHL test strips and API web (Biomérieux, France).

Molecular mass determination

The isolated strain was grown in MRSm broth for 18 h at 30 °C. Cells were harvested (8000 X g, 10 min, 4 °C) and the bacteriocin precipitated from the supernatant with 80% saturated ammonium sulphate. The precipitate was resuspended in one tenth volume of 25 mM ammonium acetate buffer (pH 6.5), desalted by using a 1000 Da cut off dialysis membrane (HiMedia). The molecular mass of bacteriocin produced by *Lb. brevis* FPTLB3 was estimated in a Tricine SDS-PAGE system as described by Schagger and Von Jagow (1987), using 4% acrylamide in the stacking concentration gel and 16% acrylamide in the separation gel.

Electrophoresis was performed in vertical gels (80 mm×70 mm×0.75 mm) in a Mini-protean II cell (HiMedia) at 20 mA for 3 h. After electrophoresis, the gel was cut in two vertical parts. One part was fixed and stained with Coomassie brilliant blue R-250. The other part was assayed for antimicrobial activity according to Bhunia et al. (1987) with modifications. Briefly, the gel was fixed for 30 min (25% isopropanol, 10% acetic acid), rinsed with distilled water (1 h initial rinsed followed by two washes of 5 min), and overlaid with 25 ml of soft (0.7%) nutrient agar seeded with 10⁶ CFU/ml of indicator *S. aureus* ATCC 25923. After incubation at 37 °C for 24 h the gel was examined for the presence of an inhibitory zone. Molecular Mass Markers for Peptides (HiMedia) were used for mass standards.

Bacteriocin production

MRS broth was inoculated with an eight hour old culture (2% v/v) of strain FPTLB3. Incubation was done at 30 °C, without agitation. Sample was taken at 1 h intervals to determine the optical density at 600 nm (OD₆₀₀), pH of the culture and the antimicrobial activity (AU/ml) of the bacteriocin produced by the strain against *S. aureus* ATCC 25923, as described previously (Todorov and Dicks 2005).

Adsorption study

The effect of pH on adsorption of bacteriocin onto producer cells was evaluated as reported by Yang et al. (1992). After 18 h of growth at 30 °C, the culture broth was adjusted to pH 6.0 and centrifuged (20000 X g) at 4 °C for 15 min. It is then washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were resuspended in 10 ml of 100 mM NaCl (pH 2.0), agitated for 1 h at 4 °C and then harvested (20000 X g) at 4 °C for 15 min. The cell free supernatant was neutralized to pH 7.0 with sterile 1 M NaOH and tested for activity.

Cell lysis

Twenty ml bacteriocin was added to a 100 ml-culture of *Lactobacillus sakei* ATCC 15521 in early exponential phase (OD₆₀₀=0.06). The optical density of the culture was determined every 1 h interval for 9 h (Todorov et al. 1999).

Plasmid isolation

The procedure of Ivanova et al. (1998) was used. The cells of a 200 ml 18 h culture were collected and washed in 10 mM Tris–HCl, pH 8.2 and resuspended in 5 ml 20 mM Tris–HCl, pH 8.2. Then 300 mg lysozyme was added, followed by the addition of 24% polyethylene glycol (PEG 20 000) and 50 ml diethylpyrocarbonate (DEPC). After 1 h incubation at 37 °C and centrifugation, the pellet was resuspended in 9 ml 100 mM Tris–HCl, 10 mM Na₂ EDTA, pH 8.5. Cell lysis was achieved by the addition of 1 ml 10% SDS and incubation at 37 °C for 15 min. The lysate was adjusted to pH 12.2 with 5 M NaOH and stirred for 15 min. at room temperature, followed by the addition of 2 ml of 2 M Tris–HCl, pH 7 and 1.2 ml of 5 M NaCl. The deproteinization was carried out once with phenol, saturated with 3% NaCl and once with chloroform. The plasmid DNA was precipitated with two volumes of cold ethanol and dissolved in 200 µl of 10 mM Tris–HCl; 10 mM Na₂ EDTA, pH 8.0 and treated with RNase A (100 µg/ml) (All from HiMedia).

Characterization of CSF produced by *Lb. brevis* FPTLB3

The CSF of *Lb. brevis* FPTLB3 was characterized with respect to thermal and pH stability, susceptibility to denaturation by enzymes, stability during storage.

Heat resistance

CSF of *Lb. brevis* FPTLB3 (400 µl) was exposed to various heat treatments: 40, 60, 80, 100 and 121 °C. Aliquot volumes of each fraction were then removed after 0, 30, 60 or 90 min and assayed for bacteriocin activity

pH sensitivity

CSF of *Lb. brevis* FPTLB3 (400 µl) were adjusted to pH 2, 4, 6, 8, 10, and 12 with hydrochloric acid (1 N HCl) and sodium hydroxide (1 N NaOH), incubated for 4 h at room temperature and similarly assayed (Rattanachaikunsopon and Phumkhachorn 2006).

Stability of CSF during storage

CSF of *Lb. brevis* FPTLB3 was stored at –20, 4 and 37 °C. At different time intervals, samples were taken from the

stored material to determine bacteriocin activity (Corsetti et al. 2004).

Enzyme treatments

Sensitivity of the CSF to enzymes was tested by treatment with proteinase K, protease B, chymotrypsin, trypsin, papain, pepsin, pronase E, lysozyme, lipase, catalase and α -amylase at a final concentration of 1 mg/ml in phosphate buffer (pH 6.5). The supernatants were incubated with these enzymes at 37 °C for 2 h after which remaining activity was determined (Corsetti et al. 2004).

Effect of mitomycin C on antimicrobial activity

Mitomycin C was added at a final concentration of 1.0 μ g/ml to CSF of *Lb. brevis* FPTLB3 and incubation was carried out at 30 °C. Samples were removed at 60, 120, 240 and 360 min. and analysed by the well diffusion method (Wanda and Bonita 1991).

Extraction with organic solvents

Various organic solvents including iso-amyl alcohol, chloroform, n-propanol, hexane, Di – ethyl ether, petroleum ether were added to CSF of *Lb. brevis* FPTLB3 in 1:1 ratio. After thorough mixing, phase separation was achieved by centrifugation (2307 X g, 10 min). When propanol was used for the extraction, 50 g/l NaCl was added to the mixture in order to obtain phase separation. The organic phase and the aqueous phase were collected and solvent removed by evaporation at 45 °C. The residue from the organic phase was resuspended in an amount of saline (8.5 g/l NaCl) equal to the starting volume of the original supernatant fluid. Bacteriocin activity of both preparations was then determined (Hernandez et al. 2005; Corsetti et al. 2004).

Effect of surfactant on antimicrobial activity

This was carried out by incorporating non-ionic (triton X100, tween 20, tween 80, nonidet P40), anionic (sodium dodecyl sulphate, deoxycholic acid) and dipolar ionic (N-hexadecyl-N, N-dimethyl-3-ammonio-1-propane sulphonate, N-lauroylsarcosine) surfactants. The surfactants were added to CSF of *Lb. brevis* FPTLB3 at a concentration of 0.1 ml or 0.01 g of surfactant per ml of bacteriocin solutions. These preparations were incubated at 30 °C for 60 min. and similarly assayed for bacteriocin activity (Hernandez et al. 2005).

Statistical analysis

All the experiments were carried out in triplicate and the results are shown as mean $\pm$ standard deviation (SD). The

significance of differences amongst treatments after each day of storage during storage stability study was determined by analysis of variance using Statistical Package for Social Sciences (SPSS) program for Windows, version 6.1.2. All other calculations were performed using Microsoft Excel, version 5, statistical functions (Microsoft Corp., Redmond, WA, USA). Differences were considered significant at the $P < 0.05$ level.

Results and discussion

Inhibitory activity and spectrum

Lactic acid bacteria isolated from mrigala were initially screened for bacteriocin production against *Staphylococcus aureus* ATCC 25923. FPTLB3 strain was found to possess antibacterial activity both by the agar spot method and by the well diffusion method. It inhibited various bacteria with a spectrum of activity seen toward both gram positive and gram negative bacteria. During the well diffusion agar test (Table 1), CSF of FPTLB3 inhibited the growth of *Escherichia coli* MTCC 1563, *Enterococcus faecalis* MTCC 2729, *Lactobacillus casei* MTCC 1423, *Lactobacillus sakei* ATCC 15521 and *Staphylococcus aureus* ATCC 25923. The Gram-positive, catalase negative FPTLB3 was identified as *Lactobacillus brevis* FPTLB3 by API 50 CHL test kit. The CSF produced by FPTLB3 showed an antimicrobial activity of 3200 AU/ml against all indicator organisms.

This wide activity spectrum of *Lb. brevis* FPTLB3 differs from others, most of which inhibit only closely related strains. Villani et al. (1995) reported a bacteriocin active against *Listeria*, but not against *E. coli*. Activity against *E. coli* and other Gram-negative bacteria was rarely reported for bacteriocin producers (Skytta et al. 1993). Antibacterial efficacy of bacteriocin from *Pediococcus acidilactici* and *P. pentosaceus* and nisin was evaluated individually and in combination against several gram positive and gram negative food spoilage and pathogenic organisms like *Clostridium sporogenes*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* in liquid medium (Jamuna and Jeevaratnam 2009). In the present study the bacteriocin like substance appeared to be active against such food borne pathogens as *Escherichia coli* MTCC 1563, *Enterococcus faecalis* MTCC 2729 and *Staphylococcus aureus* ATCC 25923. It is also active against *Lactobacillus casei* MTCC 1423 and *Lactobacillus sakei* ATCC 15521. Similar results were reported by Ogunbanwo et al. (2003). The application of such a broad spectrum bacteriocin or the producer strain could result in improvement of the starter cultures and fish, meat or dairy product preservation. The use of bacteriocin-producing

starters is a convenient alternative to the direct addition of purified bacteriocin as food preservatives, which is supposed to be much cheaper.

Molecular mass determination

The partially purified bacteriocin was analysed by the tricine SDS-PAGE for determination of the molecular weight. One part of the gel was assayed for antimicrobial activity using 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 54 kDa, formed in the other part of the stained SDS-PAGE gel (Fig. 1). From the results obtained through SDS-PAGE, it may be concluded that the antibacterial bacteriocin produced by *Lb. brevis* FPTLB3 was a peptide with a molecular mass of 54 kDa. Similar molecular mass (53.5 kDa) for *Lb. brevis* BH2 was reported by Kim et al. (2007).

Bacteriocin production

Detectable level of bacteriocin was recorded after 3 h of growth in MRS broth, indicating that the peptides were primary metabolites. Production of bacteriocin by FPTLB3 increased throughout logarithmic growth (i.e. the first 15 h), but stabilized at 3200 AU/ml during the following 14 h of slow growth (Fig. 2a). The optical density at 600 nm increased upto 9 after 29 h of incubation. The pH of the culture media decreased from 6.5 to 3.5 during 29 h of experimental period (Fig. 2b).

From the results obtained in the present study, it may be concluded that, maximum level of the bacteriocin is

produced towards the end of logarithmic growth. The activity level of bacteriocin remained at 3200 AU/ml for the duration of incubation, suggesting that they are not affected by changes in culture conditions (e.g. lowering of pH from 6.5 to 3.5). The results also suggest that, broth medium of *Lb. brevis* FPTLB3 must be incubated at least 18 h at 30 °C to get optimum activity. Similar results were reported for plantaricin Y (Chin et al. 2001) and bacteriocin produced by *Lactobacillus pentosus* ST151BR (Todorov and Dicks 2004). As observed for sakacin K and a bacteriocin produced by *Enterococcus faecium* RZS C5, extended growth does not necessarily lead to increased production of activity levels (Leroy and De Vuyst 2002).

Adsorption study

Adsorption of *Lb. brevis* FPTLB3 bacteriocin onto producing cells was strongly influenced by the pH of the suspending environment. The bacteriocin adsorption increased from 50% to 100% as the pH increased from 4 to 6.5 and decreased thereafter at higher pH. No bacteriocin adsorption was detected at pH 1 to 2, suggesting that the bacteriocins did not adhere to the surface of the producer cells. This could be explained by the relatively low pH of the culture during growth.

Under the conditions tested the activity of CSF of the producer strain obtained after treatment of cells at pH 2.0 was nil, indicating that the inhibitory compound is completely released in the medium during growth and no significant absorption takes place following adjustment of the pH of the broth 6.5 before harvesting the cells by centrifugation. Similar results were reported by Todorov and Dicks (2006). Yang et al. (1992) reported that the highest percentage of bacteriocin adsorption onto producing cells occurred at pH 6 and 6.5 for pediocin AcH, 6.5 for nisin, 5.5 and above for sakacin A, and 5.5 for leuconocin Lem 1, while no bacteriocin adsorption was detected as pH below 1.5 for pediocin AcH, below 3 for nisin, below 2 for sakacin A, below 3 and above 8 for leuconocin Lem 1. Rattanachaiakunsopon and Phumkhachorn (2006) reported no bacteriocin adsorption at or below pH 3; highest adsorption was at pH 7 and decreased thereafter.

Cell lysis

The mode of action of the bacteriocin of *Lb. brevis* FPTLB3 as studied here may be considered as bacteriostatic. Addition of the CSF (3200 AU/ml), produced by FPTLB3, to early logarithmic-phase cells of *Lactobacillus sakei* ATCC 15521 (3 h-old; approx OD₆₀₀=0.06) resulted in a significant ($P<0.05$) growth inhibition after 1 h, followed by complete growth inhibition for the remaining 6 h (Fig. 3). Addition of same activity of CSF to stationary

Fig. 1 Tricine SDS-PAGE of bacteriocin produced by *Lactobacillus brevis* FPTLB3. Coomassie brilliant blue R-250 stained gel, Lane 1: molecular mass markers and Lane 2: bacteriocin

Fig. 2 **a** Bacteriocin production (AU/ml)^a by *Lactobacillus brevis* FPTLB3 with time. **b** The increasing optical density (OD₆₀₀) value^a of *Lactobacillus brevis* FPTLB3, showing decreasing pH value^a with time. ^aResults are mean of three determination (n=3) with S.D

phase cells of *Lactobacillus sakei* ATCC 15521, resulted in no inhibition suggesting that the older cells of *Lactobacillus sakei* ATCC 15521 became resistant to the bacteriocin or that the bacteriocin was destroyed by extracellular proteolytic

enzymes. A higher concentration could be necessary in order to achieve a bactericidal effect. Similar observation was reported by Todorov and Dicks (2004). Further experiments on the mode of action of the purified substance not only on growing cells but on buffered test cells will be needed.

Fig. 3 Effect of CSF produced^a by *Lactobacillus brevis* FPTLB3 on indicator organism. ^aResults are mean of three determination (n=3) with S.D

Plasmid isolation

The bacterial isolate does not appear to harbor any plasmid. In most cases the genes encoding bacteriocins are harbored on plasmids (Huot et al. 1996). However, for a number of bacteriocins the genes have been located on the genome (Ivanova et al. 1998; Todorov et al. 1999). Since no plasmid was isolated from the strain *Lb. brevis* FPTLB3, it may be concluded that the genes encoding the bacteriocin is located on its genome.

Characterization of the inhibitory agent

During characterization of CSF of *Lb. brevis* FPTLB3, antimicrobial activity was determined using *E. faecalis* MTCC 2729 as indicator organism. The inhibitory com-

pound (bacteriocin) produced by the isolated strain was considered to be heat stable. The activity of the CSF produced by *Lb. brevis* FPTLB3 remained constant (3200 AU/ml) after heating at 121 °C for 60 min, but declined thereafter (Fig. 4). In respect to the temperature sensitivity the studied bacteriocin exhibited strong heat stability as compared to all so far reported bacteriocins of *Lb. brevis*. Temperature stability is important if the bacteriocins are to be used as a food preservative, because many procedures of food preparation involve a heating step. Ogunbanwo et al. (2003) and Corsetti et al. (2004) reported similar loss of activity of antibacterial substances produced by *Lactobacillus* spp. after heat treatment at 121 °C for different time duration.

It was observed that CSF from FPTLB3 strain was stable at pH 2 to 8 for 4 h at 37 °C, but only partially inactivated in media above pH 10. In the present study, effect of time and temperature of storage on antimicrobial activity was also carried out. Antimicrobial activity was fully stable after storage for 60 days at −20 °C and 20 days at 4 °C. During further storage periods, at −20 °C and 4 °C the stability decreased. For storage at 37 °C stability decreased on the 20th day of storage (Fig. 5). The CSF was stable over a wide pH range, which is a common feature of many bacteriocins. Abdel-Bar et al. (1987) reported the highest activity and stability of bacteriocin produced by *Lactobacillus* spp. were at pH 2.2 and 4.0 respectively. Borpuzari et al. (2007) reported that *Lactobacillus acidophilus* E30 inhibited the growth of enteropathogenic *E. coli*, was resistant to drastic acidity and could not grow at pH 2. Hazarika et al. (2007) reported that nisin producing LAB exerted maximum inhibitory effect against verotoxic *E. coli* (VTEC) in beef gravy at pH 5.4. Ogunbanwo et al. (2003), Corsetti et al. (2004), Rattanachaikunsopon and Phumkhachorn (2006), Todorov and Dicks (2006) also reported the similar findings. The wide range pH tolerance indicates that such bacteriocin

Fig. 4 Effect of time and temperature on activity^a of CSF. ^aResults are mean of three determination (n=3) with S.D

Fig. 5 Effect of storage time and temperature on antimicrobial activity^a of CSF. ^aResults are mean of three determination (n=3) with S.D

may be useful in acidic as well as non acidic foods. The storage study of CSF suggests that cold temperature may be the most appropriate preservation technique. Ogunbanwo et al. (2003) reported the similar findings while working with *L. brevis* OG1 and *L. plantarum* F1. Corsetti et al. (2004) also reported the similar results while working with Sourdough lactic acid bacteria.

Results from enzyme inactivation studies demonstrated that antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes like proteinase K, protease B, trypsin, chymotrypsin, papain, pepsin, pronase E whereas treatment with lipase, catalase, lysozyme, α-amylase, mitomycin C did not affect the activity of antimicrobial substance produced by the test isolates, confirming its protein status. The sensitivity of the found substance to proteolytic enzymes is a proof of its proteinaceous nature, which allows considering it a bacteriocin. The other enzymes tested in our study several amylases and lipase did not cause any inactivation. This could be explained with the lack of lipid

Table 2 Influence of organic solvents in the extraction of bacteriocin produced by the test isolates

Organic solvent	Organic phase ^a	Aqueous phase ^a
Control	—	10±0.5
I – amylalcohol	8±0.3	8±0.3
Choloform	—	—
N – propanol	8±0.3	4±0.3
Di – ethylether	—	6±0.3
Hexane	—	9±0.5
Petroleum ether	—	5±0.3

Zone of inhibition (mm)

Control = bacteriocin without addition of organic solvent

^a results are mean of three determination (n=3) with S.D

or carbohydrate moiety in the studied bacteriocin. Besides, antimicrobial activity in this study was not due to hydrogen peroxide or acidity, as activity was not lost after treatment with catalase or peroxidase or adjustment of pH to 7.0. Similar results were reported by Todorov and Dicks (2004), Corsetti et al. (2004), Rattanachaikunsopon and Phumkhachorn (2006), Hernandez et al. (2005) and many other researchers

Various organic solvents were tested for the extraction of bacteriocin produced by the test isolates. It was observed that extraction with polar solvents such as hexane, di-ethyl ether, and petroleum ether did not result in the removal of bacteriocin produced at the aqueous phase to the organic phase, while chloroform extraction completely destroyed the bacteriocins activity. However, when different alcohols such as n-propanol and Iso-amyl alcohol were used in the extraction procedure, bacteriocin was removed from the aqueous phase and recovered from the organic phase (Table 2). Extraction of bacteriocin in this study using organic solvents indicated that bacteriocin was removed from the aqueous phase and could be recovered from the organic phase. This suggests that at least part of the bacteriocin molecule has a hydrophobic character, and shares this property with most other bacteriocins (Klaenhammer 1993).

The effect of dissociating agents on antimicrobial activity was studied in the present work. Exposure to surfactants resulted in an increase in titre (by at least one to two fold dilutions) rather than any decrease in activity with the exception of nonidet P-40, which led to total loss of activity. N-lauroyl sarcosine and deoxycholic acid had little or no effect on antimicrobial activity. The increase might be due to the effect of surfactant on the permeability of the cell membrane (Graciella et al. 1995).

Conclusion

Finally it may be concluded that the *Lactobacillus brevis* FPTLB3 isolated from fresh water fish *Cirrhinus mrigala* produced bacteriocin like substances that has spectrum of antimicrobial activity against *Escherichia coli* MTCC 1563, *Enterococcus faecalis* MTCC 2729, *Lactobacillus casei* MTCC 1423, *Lactobacillus sakei* ATCC 15521 and *Staphylococcus aureus* ATCC 25923. Partially purified bacteriocin is characterized by high heat stability, wide pH tolerance and sensitivity to various enzymes. Molecular weight of the antimicrobial substance is about 54 kDa. The strong antagonistic effect of CSF against the food borne pathogens indicated its usefulness in the preservation of different food items enhancing their shelf life. Hence, the study concluded that the ability of bacteriocin-like inhibitory substance, produced by the test isolate, in inhibiting a wide-range of bacteria may be of potential interest for food

safety and preservation providing future scope for application as food preservative.

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