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ISOLATION, PURIFICATION AND CHARACTERIZATION OF A BACTERIOCIN WITH BROAD SPECTRUM ACTIVITY FROM *Lactococcus lactis* JC10 FROM PERISHABLE PAPAYA FRUIT

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ABSTRACT

Food-grade bacteria capable of producing bacteriocin with preservation abilities have been isolated from perishable papaya. Characterization of the bacteria isolated on MRS agar, by using 16S rRNA sequence analysis, exhibited antagonistic effect towards the growth of a wide range of Gram + and Gram - bacteria including pathogens also. Extracellularly produced bacteriocin was purified by ion-exchange chromatography on a Fast Protein Liquid Chromatography (FPLC) system equipped with a Mono-Q column. The purity rate and molecular mass of 21 kDa of this compound were determined using SDS-PAGE. Activity units (AU) of bacteriocin were increased in each step of purification, reaching up to 9500 AU/mL. The increase in the activity units directly affected the antimicrobial activity of purified bacteriocin, resulting in an increase of the inhibition zones against indicator bacteria. It withstood very high temperature, up to 115.6 °C, for 10 min but lost activity after autoclaving (121°C), wider pH range, from 3.0 to 10.0, complete inactivation in the presence of proteolytic enzymes (protease and α chymotrypsin) and storage stability up to 6 months.

Keywords: Bacteriocin, *Lactococcus lactis* JC10, purification, biopreservative, antimicrobial activity

INTRODUCTION

The ability of Lactic Acid Bacteria (LAB) to inhibit the growth of other bacteria has been known for many years. LAB have been defined as "generally recognized as safe" (GRAS) microorganisms .The inhibitory activity of LAB may be attributed to their acidification of the medium, competition for substrates and the production of antimicrobial compounds such as Hydrogen peroxide, Diacetyl and antimicrobial peptides known as Bacteriocins (**Parente *et al.*, 1999**).

Bacteriocin is one of the antagonistic compounds found to posses major applications in food industries as safe food preservative. The bacteriocins produced by LAB have been classified into four groups: class I, small, heat-stable, lanthionine-containing peptides (≤ 5 KDa); class II, small, heat-stable, lanthionine-containing peptides (≤ 10 KDa); class III, large, heat-labile proteins (≥ 30 KDa); and class IV, complex proteins composed of one or more chemical moieties, either lipid or carbohydrate. Class I and class II bacteriocins are the most likely candidates for use as biopreservatives in the food industry. Now a days, food safety is an important issue of international concern. Consumers of this decade are interested in food without harmful chemical preservatives and with additional health benefits. Packaged food items available in market contain variety of chemical preservatives that may alter chemical constituents, Nutritional as well as organoleptic qualities of food thereby causing serious adverse health effects (**Messi *et al.*, 2003**). Thus biopreservation of food has emerged as an attractive and safe approach. Among biopreservatives, bacteriocins have received increased attention especially due to their low toxicity, as they are degradable by digestive enzymes (**Cleveland *et al.*,2001).** Formulations containing bacteriocins such as Nisaplin (Nisin) and ALTA 2351(Pediocin PA-1) have been added to food to increase shelf life and increase food safety (**Mills *et al.*, 2011**).

Many researchers suggested that useful criteria for antagonistic activity of bacteriocins as: 1) the presence of an essential, biologically active protein or peptide moiety, 2) inhibitory activity against closely related bacterial species and 3) a bacteriocin's mode of action .

In an extensive suvey of bacteriocin producers, it was observed that about 43% (out of 162 strains) Lactococcal strains tested were capable of producing bacteriocin. On the other hand Nisin is the only bacteriocin from *Lactococcus lactis* that has been studied in detail. The inhibitory spectra of the different lactococcal bacteriocins vary but they are generally narrower than that of Nisin (**Geis *et al.*,1983**). Schnell et al 1988 stated that many of the Lactococcal bacteriocins described are very different from Nisin and does not belong to the lantibiotic family of bacteriocins.

The objective of this present study was to describe a novel bacteriocin produced by *Lactococcus lactis* JC10 isolated in our laboratory from papaya fruit. This bacteriocin having potential antibacterial activity including pathogens, thermal stability and small production time can be used in food industry as an alternative for chemical preservatives. Further, purification and characterization of this bacteriocin was studied in detail for their potential application as food preservative in future.

MATERIAL AND METHODS

Bacterial strains and culture conditions

The following food spoilage bacterial strains were employed in the screening for potent bacteriocinogenic strain: *Lactobacillus plantarum* 2083, *Lactobacillus plantarum* 2592, *Lactobacillus casei* 2737 (Collected from NCIM, Pune, India). MRS medium (Hi-Media, India) was used for sub culturing and strain maintenance for these three indicator strains. These strains were maintained by sub culture aerobically at static condition (37°C for 24 hrs).

The following spoilage and pathogenic bacterial strains were considered in the screening for antagonistic activity : *E.coli* NCIM 2065, *Pseudomonas putida* NCIM 2650, *Klebsiella pneumoniae* NCIM 2707, , *Bacillus megaterium* NCIM 2034, *Bacillus subtilis* NCIM 2545, *Vibrio fischeri* NCIM 5269, *Enterobacter aerogens* NCIM 2340, *Micrococcus luteus* NCIM 2169, *Staphylococcus aureus* NCIM 2127, *Proteus vulgaris* NCIM 2027, *Alcaligenes viscosus* NCIM 2446, *Leuconostoc mesenteroides* NCIM 2073 (collected from NCIM, Pune), *Salmonella typhi* MTCC3216, *Shigella flexneri* MTCC1457, *Bacillus thuringiensis MTCC6941* (collected from MTCC, Chandigarh).All these strains were maintained in Nutrient medium (Hi-Media, India). Strains were stored as lyophilized frozen stocks at -20 °C when strains are not in regular use.

Screening and isolation of potent lactic acid bacteria

Food samples (acidic) (158) were collected from local market in sterilized zip-lock packet. A little fraction of each sample was vortexed in sterilized distilled water (5 ml) in a tube. A fixed amount of sample and indicator culture were mixed with molten soft agar medium and spread on MRS hard agar plate. Then

the plates were incubated overnight at incubator at 37°C for a selecting appearance of producer colony of LAB with a hollow zone around it. The producer colony was randomly picked and purified by streaking onto MRS agar plate. The purified colonies were primarily identified by Gram staining and catalase tests. Only gram positive, catalase- negative colonies were presumed to be LAB. The pure single colony was inoculated in MRS broth overnight at 37°C for bacteriocin production. 1.5 ml of broth culture was centrifuged at 10,000 rpm for 10 minutes (at 4°C). Cell free supernatant (CFS) was collected in a fresh sterilized eppendorf. Crude CFS was adjusted at pH 7.0 by 1 (N) NaOH (Merck, Germany) to rule out acid inhibition. Inhibitory activity from hydrogen peroxide was ruled out by the addition of catalase (250 IU/ml). In the meantime indicator cultures were mixed with molten MRS soft agar medium and spread on MRS hard agar plates and kept for solidification for approximately 15 minutes. 5 µl neutralized CFS was tested on different indicator cultures. Plates were incubated for 24 hrs, at 37°C to monitor any kind of inhibition zone against all the indicator cultures. Degree of antagonism was determined by measuring the inhibition zone diameter against respective indicator culture using an agar well diffusion assay.

Characterization of the antimicrobial compound

Chemical nature of the antimicrobial compound was determined by application of several proteolytic enzymes (Pepsin, Trypsin, Proteinase K, Protease, α-chymotrypsin) on cell free supernatant. 1 mg/ml stock of each proteolytic enzyme was mixed with crude CFS in 1:1 (v/v) ratio and incubated for 30 minutes at 37°C. With treated CFS, spot on lawn assay was performed on indicator culture *Lactobacillus plantarum* 2083. Results were observed on the next day to determine the chemical characteristics of the antimicrobial compound. Each and every experiment was done in triplet with proper control related with the experiment.

Characterization and identification of isolated strain

Morphological study

Morphology was determined by gram staining procedure initially. The strain isolated was inoculated into sugar broth tubes to find out the fermentation capability of the organism in different sugars. Catalase activity was tested by spotting colonies with 3% Hydrogen peroxide.
The isolated viable bacterium was examined with scanning electron microscopy (SEM) later on. Isolated LAB strain used in scanning electron microscopy study was obtained from 24 hrs old culture in MRS media (37°C). The O.D was adjusted to 0.1 and 1.5 ml of cell suspension was taken in sterilized eppendorf and centrifuged at 6000 rpm for 10 minutes. Supernatant was discarded, cell pellet was dissolved in 0.1 M phosphate buffer (pH 6.9) and washed twice with 0.1 M phosphate buffer and stored at 4°C. After centrifuging the eppendorf (6000 rpm, 10 minutes), cell pellet was fixed with 5% (v/v) glutaraldehyde (EM grade, Merck, Germany) for 2 hours at 4°C. Fixative was prepared in sterilized sodium cacodylate buffer (pH 7.4). The fixed pellet was dehydrated in a graded ethanol series (30% to 100%), incubating in each grade for 10 minutes. The dried preparation was coated with gold/palladium (60:40) with thickness below 10 nm using deposit thickness monitor. It was then observed under electron microscope (FEI Quanta- 200 MK2) with a magnification of 12000X.

16s rRNA sequence analysis

The strain was identified according to Sneath et al. (**Sneath *et al*.,1986**) and Thomas et al. (**Thomas *et al*., 1980**). Preliminary identification was based on its morphological and biochemical characteristics in addition to its carbohydrate fermentation profile following Bergey's Manual of Systematic Bacteriology (**Garrity *et al*., 2005**). Finally it was characterized according to its 16S rDNA sequence analysis. The 16S rRNA gene of the strain *Lactococcus lactis* JC10 was amplified by the method described earlier (Das et al, 1996). Primers used for the amplification of 16S rRNA were 5'-GAG TTT GAT CCT GGC TCA G-3' (forward primer) and 5'-AGA AAG GAG GTG ATC CAG CC-3' (reverse primer). Genomic DNA amplification was performed with a Thermal Cycler, Model PCT-200 (M.J. Research, Waltham, MA, USA) with the following temperature conditions: initial denaturation step at 94°C for 4 min; followed by 30 cycles of 62°C for 1 min, 72°C for 1.5 min and 94°C for 1 min and final extension at 72°C for 7 min. The PCR product was purified by using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Gel-purified 16S rRNA was sequenced using a CEQ Dye terminator cycle sequencing kit in an automated DNA sequencer Model CEQ 8000 (Beckman Coulter, Fullerton, CA, USA) (**Panday *et al*., 2010**). Nucleotide sequences thus obtained were assembled using the sequence alignment editor program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Subsequently these sequences were blasted against the GenBank database (http://ncbi.nlm.nih.gov/BLAST) (Altschul et al, 1997). Evolutionary distance to other strains of *Lactococcus* was computed by neighbor joining method (**Saitou *et al*. 1987**). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and phylogenetic tree was constructed by software MEGA5 (**Tamura *et al*., 2007**).

Effect of thermal treatments on bacteriocin activity

Thermal stability test was performed with the neutralized CFS of the isolated LAB strain. The effect of temperature on stability of bacteriocin was determined in the CFS subjected to treatment at 100°C for 1 hr, 100°C for 2 hrs, 100°C for 2 hrs, 108°C for 10min, 115.6°C for 20 min and 121°C for 15 min. Immediately after each treatment, samples were cooled under refrigeration and residual activity was determined by spot on lawn assay on indicator strain *Lactobacillus plantarum* 2083. A positive control, freshly produced neutralized CFS was tested in parallel.
The effect of extended storage at low temperature (-20°C and 0°C) on bacteriocin stability was also determined by placing CFS in a deep freeze for 270 days. Residual activity was determined by the spot on lawn assay, as previously described. In all instances, a positive control, consisting of freshly prepared neutralized CFS was tested in parallel.

Effect of pH on bacteriocin activity

About 5ml aliquot of purified bacteriocin was taken in test tubes and pH values of the contents were adjusted from 2 to 10 individually using either dilute NaOH or HCl (1M NaOH or 1M HCl solution). After allowing the samples to stand at room temperature for 2 hours, antimicrobial activity was assayed.

Effect of organic solvent on bacteriocin activity

To study the effect of different solvents on bacteriocin, neutralized CFS was mixed with 1:1 (v/v) Ethanol, Isopropanol, Acetone, Methanol, Butanol. It was kept at room temperature for 30 min. After evaporation of solvents, 5 µl of solvent treated CFS was spotted on lawn of indicator organisms with appropriate control. Activity of the antimicrobial compound was similarly evaluated in presence of β mercaptoethanol.

Antimicrobial spectrum of the bacteriocin

Neutralized CFS was tested against several gm+ and gm- bacteria. Activity of bacteriocin was tested by constructing soft agar lawn of each test microorganism on MRS plate separately and spot of neutralized CFS (5 µl) was given. Each plate was incubated (37°C, 24 hrs) and examined for inhibition zone to be appeared on the next day.

Extraction of crude bacteriocin

Two liter culture of *L. lactis* JC10 was grown in MRS broth media (pH 6.8) at 37 °C at static condition for 5 hours. Cell free supernatant obtained after centrifuging the broth culture at 10000 rpm for 15 min at 4 °C was used as crude bacteriocin.

Concentrating bacteriocin sample

Diluted solution of crude bacteriocin was concentrated 10 times by lyophilization at 4°C.

Ammonium sulfate precipitation

Concentrated crude bacteriocin was subjected to ammonium sulfate fractionation. Ammonium sulfate was gently added to the supernatant maintained at 4 °C to obtain 40% saturation, and the mixture was stirred for 4 h at 4 °C. It was then centrifuged for 30 min at 20000×g at 4 °C; the resulting pellet was resuspended in required amount of 0.1 M phosphate buffer (pH 6.9). It was considered as partially purified bacteriocin.

Determination of protein content

Due to the presence of tween 80 in MRS medium, protein determination by Lowry method gave a false estimation of protein content in the medium. To avoid interference of detergent, protein content of medium was determined by modified Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) (**Lowry *et al*., 1951**) using Bio-Rad RCDC protein assay kit (detergent compatible) as per manufacturer instruction. Bovine serum albumin was used as a standard. Specific activity was defined as the ratio of bacteriocin activity (AU/mL) to protein concentration (mg/mL).

Bacteriocin production and purification

The supernatant fluid of a 5 hr *Lactococcus lactis* JC10 culture was collected by centrifugation at 10000 rpm for 10 minute and filtered through a membrane filter (0.45 µm pore size, Millipore). The proteins were precipitated with 40%

ammonium sulphate overnight at 4°C. The precipitate was recovered and exhaustively dialyzed against phosphate buffer 0.1M pH 6.9 which was crude bacteriocin. This was further purified by FPLC anion exchange chromatography. The sample was absorbed on a Mono Q 5/50 GL (AKTAFPLC, GE Life Sciences) column, previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). Elution was accomplished with an increasing concentration of 20mM Tris-HCl, pH 8.0 + 1 M NaCl (buffer B). Fractions were collected and assayed for antimicrobial activity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The antimicrobial preparations and FPLC purified sample were examined on 10% SDS-PAGE (**Laemmli, 1970**). Samples and molecular weight standards 1mg/ml were dissolved in sample buffer and loaded onto the gel. After electrophoresis at 40 milliamps for approximately 4 hours, the gel was stained with coomassie brilliant blue dye.

RESULTS

Identification of isolated strain

Microbial characteristics of *Lactococcus lactis* JC10 was found as gram-positive,spherical shaped, non-endospore forming, catalase negative bacterium (Tab 1). It fermented a wide array of sugars. It could utilize glucose, sucrose, mannitol, lactose etc producing organic acid only but no CO_2 gas. Acid production was maximum in the fermentative degradation of lactose. Methyl red test, V-P test, Starch degradation test, Citrate production test and Gelatin liquefaction test showed negative results.

Table 1 Morphological and biochemical characteristics of *Lactococcus lactis* JC10

Morphological		**Biochemical**	
Size & shape	Spherical	Test	Result
Arrangements	Discrete	Glucose fermentation	A++, G-
Gram reaction	Positive	Sucrose fermentation	A++, G-
Endospore formation	No	Mannitol fermentation	A++, G-
		Lactose fermentation	A+++, G-
		Methyl red test	_
		V-P test	_
		Starch degradation	_
		Citrate production	_
		Gelatin liquefaction	_
		Indole test	_
		Catalase test	_

Legend: A- acid,G -gas

Morphologically cells structure was evidenced from SEM image (Figure 1). The cells are found to be spherical or ovoid shape, within the size range of 1.2 µm to 1.5µm , occuring in pairs or short chains.

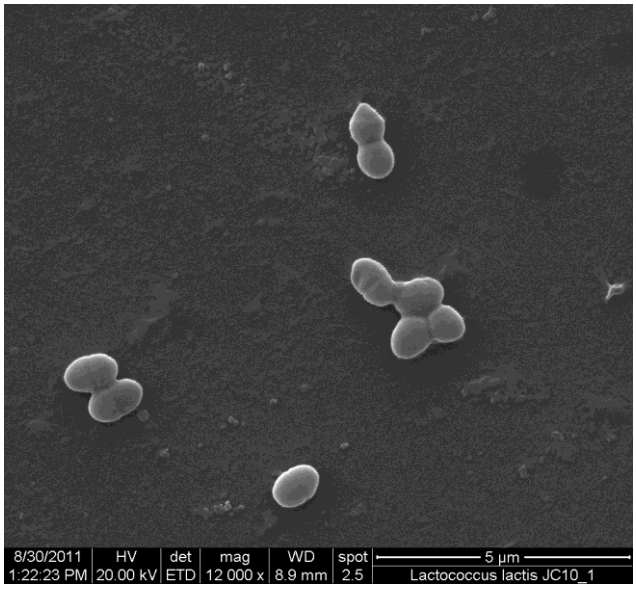

Figure 1 Electron micrograph of bacteriocin producing *Lactococcus lactis* JC10

The result of the biochemical identification was further confirmed by amplification of the 16s rRNA genes. PCR amplification and sequencing of its 16s rDNA gene was edited to a total length of 1438 bp. BLAST search showed sequence homology with *Lactococcus lactis*. Phylogenetic tree based on different species of *Lactococcus* was constructed using neighbor joining method (Figure 2).

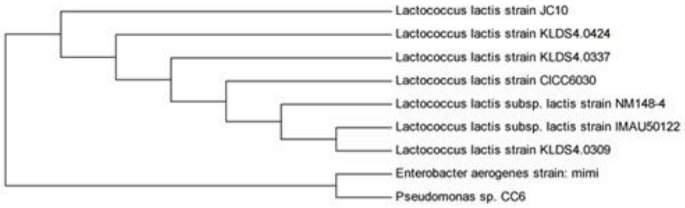

Figure 2 Phylogenetic relationship of 16s rRNA sequence of *Lactococcus lactis* JC10 with other reference strains of *Lactococcus*.

Result indicated that the strain JC10 was very closely (greater than 97% similarity) related with *Lactococcus lactis* strain. It is known that sequence similarity >97% is acceptable level for microbial identification and the microbial strain shall be considered as same species (**Janda *et al*., 2007**). Based on information from phenotypical, physiological and molecular testing, the strain was identified as *Lactococcus lactis*. Further based on microscopic and biochemical techniques this LAB stain was named as *Lactococcus lactis* JC10.

Bacteriocin purification and molecular weight determination

The supernatant fluid of a 5 hr *Lactococcus lactis* JC10 culture was collected by centrifugation at 10000 rpm for 10 minute and filtered through a membrane filter (0.45 µm pore size, Millipore). The proteins were precipitated with 40% ammonium sulphate overnight at 4°C (**Keen, 1966**). The precipitate was recovered and exhaustively dialyzed against deionized water and this was crude bacteriocin. The dialysate formed an insoluble portion which contained most of the antimicrobial activity (**Joerger *et al*., 1986**). This was purified by FPLC anion exchange chromatography (Figure 3).

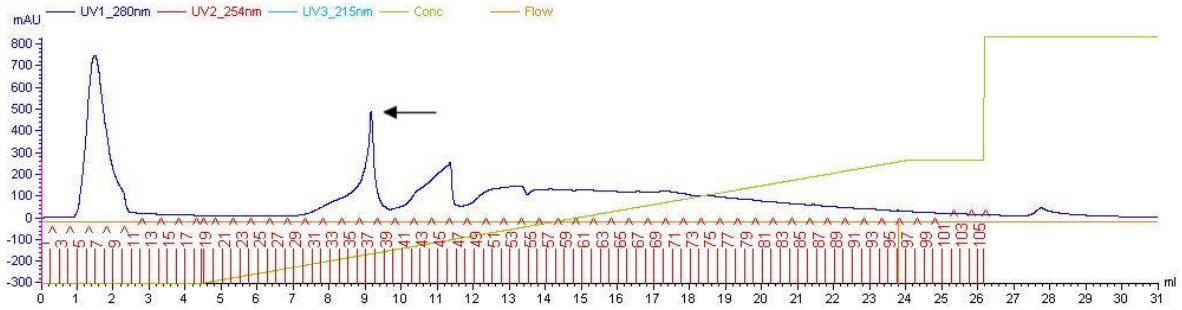

Figure 3 Fast Protein Liquid Chromatogram of purified bacteriocin produced from *Lactococcus lactis* JC10. Fractions (36-38) showed highest bacteriocin activity is indicated by arrow.

The sample was absorbed on a Mono Q 5/50 GL (AKTA FPLC, GE Life Sciences) column, previously equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer A). Elution was accomplished with an increasing concentration of 20mM Tris-HCl, pH 8.0 + 1 M NaCl (buffer B). Fractions were collected and assayed for antimicrobial activity. Pooled fractions from 34 to 38 revealed the highest bacteriocin activity which yielded (at recovery of 22.07% and a 3.75 fold purification as indicated in (Tab 2)) a titre of 9500 AU/ml and a specific activity of 2794 AU/mg protein. The results of the purification procedure were summarized in Table 2.

Table 2 Purification of bacteriocin from *Lactococcus lactis* JC10

Purification stage	Volume (ml)	Activity (AU/ml)	Total activity (10^4)	Protein (mg/ml)	Specific activity (AU/mg)	Purification factor	Recovery (%)
Culture supernatant	500	6500	325	15.4	422	1	100
Ammonium sulfate precipitation (0-40%)	20	7600	15.2	10.2	745	1.76	66.23
Purified bacteriocin	10	9500	9.5	3.4	2794	3.75	22.07

SDS-PAGE analysis of the bacteriocin obtained by FPLC revealed a single band with a molecular mass of approximately 21 kD (Figure 4) . The bacteriocins of lactic acid bacteria belonging to class III have molecular weight >10 KD . So the higher molecular mass of bacteriocin of *Lactococcus lactis* JC10 (21KD) suggested that it might belong to class III bacteriocin group.

FPLC purified bacteriocin showed larger zone of inhibition than control (Figure5) indicating a 3.75 fold purification.

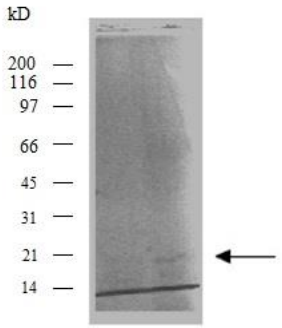

Figure 4 SDS PAGE of FPLC fraction. FPLC pufified fraction showing zone of inhibition on MRS plate in SDS PAGE shows 21 kD band indicated by arrow.

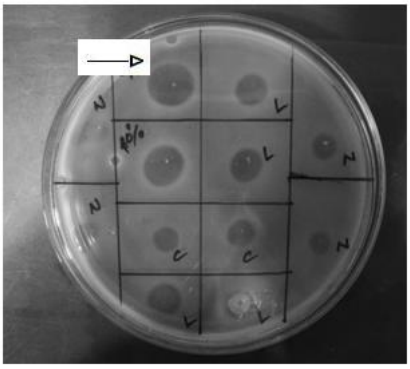

Figure 5 Zone of inhibition of bacteriocin. FPLC purified fraction is marked by arrow.

Antimicrobial spectrum

The antimicrobial activity of bacteriocin was tested against a number of Gram + and Gram - bacteria including pathogens (Tab 3). Table 3 shows sensitivity of the various strains to the bacteriocin produced by *Lactococcus lactis* JC10 as measured with the Spot-on-lawn method.

Table 3 Antimicrobial spectrum of bacteriocin produced by *Lactococcus lactis* JC10.

Name of microorganisms	NCIM/MTCC No.	Zone of inhibition (mean of three trials) (mm)
E.coli	NCIM 2065	14
Pseudomonas putida	NCIM 2650	12
Klebsiella pneumoniae	NCIM 2707	12
Salmonella typhi	MTCC 3216	16
Shigella flexneri	MTCC 1457	14.5
Bacillus thuringiensis	MTCC 6941	12
Bacillus megaterium	NCIM 2034	12
Bacillus subtilis	NCIM 2545	14
Vibrio fischeri	NCIM 5269	13.5
Enterobacter aerogenes	NCIM 2340	15
Micrococcus luteus	NCIM 2169	12
Staphylococcus aureus	NCIM 2127	16
Proteus vulgaris	NCIM 2027	0
Alcaligenes viscosus	NCIM 2446	0
Leuconostoc mesenteroides	NCIM 2073	11.5
Lactobacillus plantarum	MTCC 2083	13
Lactobacillus plantarum	MTCC 2592	14
Lactobacillus casei	MTCC 2737	13

The bacteriocin obtained from *L. lactis* JC10 showed very strong inhibitory activity against *E.coli*, *Shigella flexneri*, *Enterobacter aerogenes* etc which are Gram negative bacteria. It is known that generally bacteriocins of lactic acid bacteria do not inhibit the growth of Gram Negative bacteria. This unusal result indicates that this bacteriocin has a broad antimicrobial spectrum of activity.

It was not active against *Proteus vulgaris* and *Alcaligenes viscosus*.

The largest zone of inhibition(16 mm) was obtained against *Staphylococcus aureus* NCIM 2127 and *Salmonella typhi* MTCC 3216. This bacteriocin is reported to be active against other pathogens also.

Effect of temperature, solvents, enzymes and pH on bacteriocin activity

Bactericin from *Lactococcus lactis* JC10 has shown remarkable temperature stability. Figure 6 shows the effect of temperature on bacteriocin activity in terms of inhibition zones. It has been found to be thermostable in nature. It was equally stable in wide range of temperatures ranging from -20°C to 115.6°C as compared with control (Figure 6).

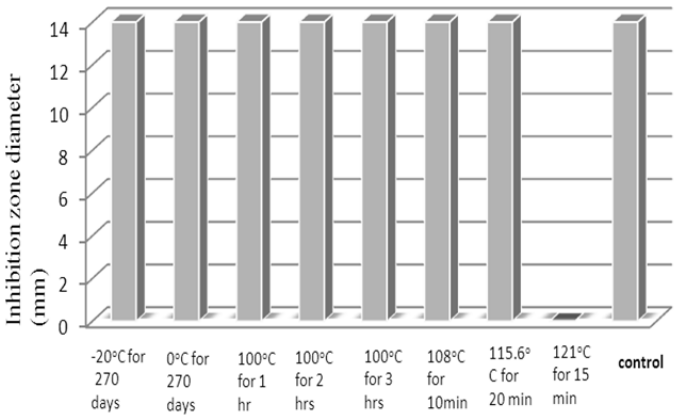

Figure 6 Thermal stability of bacteriocin

It was stable for more than 6 months in refrigerated condition. Full stability was recorded after autoclaving the bacteriocin at 10 lb pressure for 20 min (115.6 °C). But it lost its activity after autoclaving at 15 lb pressure for 15 min (121°C).
This Bacteriocin was found to be stable in presence of different organic solvents (Tab 4).

Table 4 Effect of different solvents on bacteriocin produced by *Lactococcus lactis* JC10

Organic chemicals	Inhibitory zone diameter on MRS plate (mm)
Ethanol	11
Isopropanol	10.5
Acetone	11
Methanol	11
Butanol	10
2 mercaptoethanol	0

Restoration of complete function of bacteriocin in presence of several organic solvents broadens its application in several natural environments. The antimicrobial activity of the compound was totally lost in presence of 2 mercaptoethanol.
Treatment with proteolytic enzymes like protease and α-chymotrypsin nullify the antimicrobial property of neutralized CFS showing no inhibition zone on Spot-on-lawn assay whereas catalase, pepsin, trypsin and proteinase K showed zone of inhibitions of varied diameter showing that the activity was not affected by these enzymes (Tab 5).

Table 5 Effect of different enzymes on purified bacteriocin

Enzymes added	Inhibitory zone diameter on MRS plate (mm)	Activity (AU/mL)
Catalase (Sigma No. C9322)	12	3200
Pepsin (Sigma No.P6887)	11	3200
Trypsin (Sigma No. T6567)	12	3200
Proteinase K (Sigma No.P2308)	10	3200
Protease (Sigma No.P2714)	0	-
α-chymotrypsin (Sigma No.C4129)	0	-

This protease sensitivity assay demonstrated that this antimicrobial substance was proteinaceous in nature.
The residual activities of the purified bacteriocin from *Lactococcus lactis* JC10 revealed that the bacteriocin retained its total activity in the pH range of 3-10 with no change in AU/ml .This indicates that the bacteriocin is pH stable and it could retain its antimicrobial activity when there was a shift to acidic or basic range. Stability of bacteriocin at different pH range is a limiting factor for recommending its use in food items.

DISCUSSION

Within the last several years, studies of bacteriocin have attracted significant attention because of their potential use in many fields. Our research has focused on a search for new bacteriocin produced by generally recognized as safe (GRAS) bacterial strains. The results, presented here indicates that our isolate *Lactococcus lactis* JC10 have shown to produce maximum level of bacteriocin (6400 AU/ml) only within 5 hours of incubation. There is no such report of food grade LAB to produce highest level of bacteriocin within such short time span. This might be very useful in commercial bacteriocin production or generating a high yielding starter culture for manufacturing fermentative food products.
The bacteriocin studied was thermostable as heating at temperature higher than 100°C (115.6°C) did not destroy its inhibitory activity. Several studies have been reported that the bacteriocin treated at 100°C for 120 min and 121°C for 15 min were stable (**Do *et al*., 2001**; **Pilar *et al*., 2008**). Pediocin SJ-1 (**Schved *et al*.,1993)** was not affected by heat treatment for 30 min at 100°C. These examples clearly indicate that bacteriocins posses thermostable property. The heat stability of bacteriocin discussed here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products. Nevertheless, it exhibited activity at low temperatures and the protein maintains its initial activity i. e. same zone diameter of inhibition when it was kept at -20°C for six months. This noteworthy temperature stability (-20 °C to 115.6°C) of this bacteriocin make this bacteriocin more fitted candidate for commercialization. However more studies on these aspects are needed.
The inhibitory activity of this antagonistic substance was completely lost after treatment with either protease or α chymotrypsin, thereby revealing its proteinaceous nature. Other bacteriocins are also susceptible to these same enzymes.
The bacteriocin activity was totally lost by organic solvent 2-mercaptoethanol, which indicates that this bacteriocin might contain S-S linkage which is known to contribute to protein thermal stability. Although the reason for bacteriocin heat stability could be due to its complex nature.
Several protocols and chromatographic methods have been proposed for the analytical purification of bacteriocins. Chromatographic methods, such as ion-exchange or size-exclusion (gel filtration, are usually applied after an initial concentration step by salt precipitation (Ammonium sulphate precipitation) (**Muriana *et al*., 1991**). This bacteriocin of interest was purified by a sequential concentration by lyophilization, salting out followed by ion exchange chromatography protocol using FPLC with Mono-Q column. The substance was bound to the Mono-Q matrix, indicating that the protein is anionic in nature. Because it is anionic, it is likely that its mode of action is different from class 1 and 2 bacteriocins, which are membrane active peptides and proteins. Further SDS PAGE analysis showed that the fraction showing antimicrobial activity was of high molecular mass (21 KD).
Though in general, bacteriocins posses narrow spectrum antimicrobial activity (**Riley *et al*., 2002),** this bacteriocin from *Lactococcus lactis* JC10, on virtue of its broad spectrum antimicrobial activity, can be useful regarding biological food preservation. To get rid of hazards of chemical preservatives, bacteriocins could be employed.
Bacteriocins could also be utilized to develop new novel approaches for controlling food-borne bacterial disease-causing agents with extensively observed increased antibiotic resistance among all bacterial groups (**Gyles, 2008; Nathan *et al*., 2005**). Hence, application of purified bacteriocin (from *L.lactis* JC10) to preserve foods can be exercised. Apart from this, with respect to medical applications, isoloated LAB srain (*L.lactis* JC10) and bacteriocin produced by it, might play a role during in vivo interactions occurring in the human gastrointestinal tract contributing to gut health. Further research is needed to unravel the precise role of LAB and bacteriocins in this process.

CONCLUSION

The study revealed that bacteriocin from *Lactococcus lactis* JC10 isolated from papaya fruit posseses a wide spectrum of inhibitory activity against a number of pathogens. Bacteriocin with such thermostability and pH stability has a potential for application as a biopreservative in differenet thermally processed food products as such or in combination with other preservation methods. More studies are necessary to better describe the protein and elucidate its mode of action and genetic characteristics.

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