Purification and characterization of a broad spectrum bacteriocin produced by a selected *Lactococcus lactis* strain 63 isolated from Indian dairy products

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Abstract *Lactococcus lactis* subsp. *lactis* strain 63 was isolated from Indian dairy products, produced a bacteriocin with broad spectrum inhibition against several of food pathogens like *Listeria monocytogenes* and *Bacillus cereus* as well as Gram negative bacteria viz., *E. coli*, *Yersinia*, *Citrobacter*, *Proteus*, *Enterobacter*, *Klebsiella* and *Serratia* strains. Bacteriocin production was higher in GM-17 and MRS as compared to TYGE broth and enriched skim milk broth and reached the maximum level during the early stationary phase. The bacteriocin was purified by performing ammonium sulfate precipitation. The bacteriocin was able to survive 90 °C/10 min but not 100 °C/10 min. Complete inactivation of bacteriocin was observed after autoclaving. The bacteriocin maintained its activity over a wide range of pH (3–9). The antimicrobial compound produced by the isolate 63, was sensitive to papain, pepsin, trypsin and amylase but was resistant to detergents like SDS and urea. Tween 20, Tween-80 as well as Triton X-100 enhanced its activity. Since the treatment with proteolytic enzymes resulted in loss of activity, this shows that the proteinaceous nature of the antimicrobial substance. Tentative molecular weight of the bacteriocin was

found to be between 3.5 and 5 kDa by Tricine SDS-PAGE. Finally, we confirmed the presence of gene for nisin, and the sequence thus obtained, was identical to the sequences previously described for nisin Z. *Lactococcus lactis* subsp. *lactis* 63 or its bacteriocin, which has a wide inhibitory spectrum, has the potential for use as a starter or protective culture in the manufacture of fermented products.

Keywords Lactic acid bacteria · Biopreservation · Bacteriocin · Antimicrobial activity · *Lactococcus lactis*

Introduction

Food safety, quality and health are the major issues that act as a driving force to uncover the newer and innovative ideas in the field of food biopreservation technology. Metabolites of Lactic acid bacteria (LAB) are currently one of the hot topics in the field of biopreservation. LAB occur naturally in many of the food sources, and besides its usage for centuries in food fermentation without any adverse health effects. These bacteria, therefore, have been given the Generally Regarded as Safe (GRAS) status. Due to the safe nature of LAB, coupled with the recent scientific understanding of their antimicrobial principles and enhanced health effects, they are being seen as potential alternatives to chemical preservatives (Balciunas et al. 2013). The biopreservative principle of LAB is mainly attributed to the production of a variety of antimicrobial compounds such as organic acids, phenolic compounds, diacetyl, hydrogen peroxide and bacteriocins that confer these bacteria a competitive advantage over the other bacteria (Jordan et al. 2014). Amongst the group of antimicrobial compounds from LAB, bacteriocins have

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received considerable attention owing to their potential for industrial applications in food biopreservation.

Bacteriocins are ribosomally synthesized antimicrobial peptides that are produced by many bacteria and which kill or inhibit the growth of other bacteria, either in the same species (narrow spectrum of activity) or across genera (broad spectrum of activity) (Cotter et al. 2013). Many bacteriocin producing LAB strains have been isolated from milk, plant and fermented products of dairy, cereals or meat origin and many of which have been identified and characterized (Ivanova et al. 2000). Biopreservation strategies based on bacteriocins have also been tested for many products such as meat, fish, dairy, cereals etc. However despite a long list of bacteriocins discovered till date, only two LAB bacteriocins, namely nisin and pediocin PA-1, are commercially available (Balciunas et al. 2013). Among the bacteriocins of LAB, nisin has gained special attention as a natural antimicrobial food additive and recently its potential as therapeutic agent against some medically relevant microbes and some diseases like mastitis and cancer has also been beefed up by several reports (Shin et al. 2015a). Nisin is widely used in the food industry as a natural biopreservative for different types of foods such as pasteurized and processed cheese. It is a cationic peptide of 3.5 kDa, belonging to a group of cationic peptides produced by *Lactococcus lactis* subspecies *lactis* antimicrobials and is classified as Type A (I) lantibiotics (Breukink et al. 1999).

A decade ago, different researchers exploring the antimicrobial spectrum of nisin, have shown that nisin is predominantly active against Gram-positive bacteria and to act against Gram-negative bacteria, such as *Escherichia coli* or *Salmonella* species, it should be used in conjunction with chelating agent like EDTA or physical treatments like sub-lethal heat, osmotic shock and freezing etc., which damage the outer membrane of cell (Breukink et al. 1999). However, it has been shown that purified nisin Z and some bioengineered nisin variants have antagonistic activity against Gram-negative bacteria too (Yuan et al. 2004; Kuwano et al. 2005; Cotter et al. 2013; Shin et al. 2015b). Apart from nisin, peptides having similar sequences like nisin have also been documented in literature those inhibit the growth of Gram-negative microbes (Aslam et al. 2012). However, establishment of the fact that nisin Z or other similar variants (from Lactococci) have antagonistic effect on Gram-negative bacteria requires more studies in this direction and should be critically reviewed. Hence the present study was carried out to isolate and characterize a bacteriocinogenic culture of *Lactococcus* having antimicrobial activity against Gram-positive as well as Gram-negative pathogens of food relevance.

Materials and methods

Lactococci isolation and culture conditions

Bacterial strains used in this study, their source and the media employed are listed in Table 1. Before use, cultures were grown by successive transfer in their respective media and incubated at suitable temperature. LAB indicators were routinely propagated in de Man Ragosa Sharpe (MRS) broth (Himedia) and Non-LAB strains were grown in BHI broth (Himedia) or nutrient broth (Himedia) at their respective temperature of incubation. A total of 30 samples of Indian dairy products like cream, dahi, yogurt, cultured milk and flavored milks were used as source for the isolation of lactococci. Serially diluted samples in 0.9% saline were plated on GM-17 medium (M-17 medium supplemented with 0.5% glucose) and incubated aerobically at 30 °C for 48 h, and then several colonies were randomly picked. Preliminary identification of lactococci based on Gram reaction and catalase test were carried out, subsequent to which the presumptive lactococci were screened for their bacteriocinogenic potential by the method given by Gupta et al. 2010 against a sensitive strain *Pediococcus acidilactici* LB 42. After incubation at 37 °C the plates were examined for zones of inhibition.

Taxonomic identification

Out of total 145 isolates, 13 showed considerable antibacterial activity. These isolates were subjected to phenotypic and genotypic identification. Preliminary tests included Gram-staining, catalase test and biochemical characteristics such as the ability of the strain to grow in the presence of 6.5% (w/v) NaCl, at 10 and 45 °C, ability to hydrolyze esculin and arginine and lastly, carbohydrate fermentation profile.

The identification of the isolates was further confirmed by molecular techniques like species specific PCR. Briefly, total DNA from the isolates was obtained using NucleoSpin Tissue DNA extraction kit (Machery-Nagel, Germany), applying the protocol for GPB using lysozyme (Sigma-Aldrich). PCR amplification was carried out as described previously (Ward et al. 1998) using specific primers pair Y1 and Y2 (Supplementary 1 Table B) with slight modification in PCR thermal cycle program as follows: initial denaturation 94 °C/4 min, 30 cycles of 94 °C/20 s, 55 °C/30 s, 72 °C/30 s; 72 °C/5 min.

Characterization of the inhibitory substance

In order to know the biochemical nature of the inhibitory substance, several treatments were given to the

Table 1 Bacterial indicators used in this study

Bacterial species	Origin	Growth medium	Activity (mm)
<i>Pediococcus acidilactici</i>	LB-42, USA	MRS ^a , 37 °C	++
<i>Enterococcus faecalis</i>	NCDC 114, Karnal	MRS, 37 °C	++
<i>Listeria monocytogenes</i>	ATCC 15303, USA	BHI ^b , 37 °C	+
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	BHI, 37 °C	++
<i>Bacillus cereus</i>	NCDC 66, Karnal	NB ^c , 37 °C	++
<i>Bacillus subtilis</i>	NCDC 70, Karnal	NB, 37 °C	++
<i>Serratia marcescens</i>	ATCC 13880, USA	BHI, 37 °C	+
<i>Klebsiella pneumonia</i>	ATCC 43864, USA	BHI, 37 °C	+++
<i>Citrobacter freundii</i>	ATCC 43864, USA	BHI, 37 °C	++
<i>Escherichia coli</i>	ATCC 25922, USA	BHI, 37 °C	+
<i>Salmonella abony</i>	NCTC 6017, ENGLAND	BHI, 37 °C	++
<i>Yersinia enterocolitica</i>	ATCC 130715, USA	BHI, 37 °C	++
<i>Proteus vulgaris</i>	ATCC 33420, USA	BHI, 37 °C	++
<i>Enterobacter aerogenes</i>	ATCC 13048, USA	BHI, 37 °C	++

*Agar well assay for *L. lactis* ssp *lactis* 63; +++ = > 15 mm; ++ = 10–15 mm; + = < 10 mm

^aMRS = de Man, Rogosa, Sharpe

^bBHI = Brain heart infusion

^cNB = Nutrient Broth

antimicrobial substance. The effect of enzymes on the antimicrobial activity of the CFCS was tested to know whether the inhibitory substance was proteinaceous in nature or not. It was done by mixing 1 ml of the CFCS with chymotrypsin, pepsin, papain, protease and lysozyme (Sigma, New York, USA) each at 20 mg/ml concentration and incubated at 37 °C for 30 min. The enzymatic reactions were stopped by boiling for 5 min and the antimicrobial activity was assessed by the spot-on-lawn test with untreated bacteriocin and enzyme solutions as controls. The effect of pH on the antimicrobial activity of the CFCS was tested adjusting the pH of the CFCS from 1.0 up to 11.0 (with increments of one pH unit) with 1 M NaOH or 1 M HCl (Himedia, India). The bacteriocin activity was assayed after 30, 60 and 90 min of incubation at 37 °C. To evaluate thermostability, 1 ml aliquots of CFCS were subjected to different heat treatments viz. 80, 90, 100 and 121 °C/15 min (autoclaving), then cooled on ice immediately and assayed for the residual activities after every 30 min up to 2 h. The effect of surfactants like Sodium Dodecyl Sulphate (SDS), Tween 80, Tween 20 and Tritone X100 (Hi Media, India) on antimicrobial activity was determined by adding these chemical agents at 1% (w/v) concentration in CFCS, incubating at 37 °C for 5 h and then testing for residual activity. For all tests the antimicrobial activity was determined by the spot-on-the-lawn test, using *P. acidilactici* LB 42 as an indicator strain.

Optimization of the cultivation conditions for maximal bacteriocin production by *Lactococcus lactis* 63

For achieving the maximum bacteriocin titre, four different types of media were taken as follows: de Man Rogosa Sharpe broth, GM-17 broth, Tryptone Glucose Yeast Extract broth and Enriched Skim Milk broth (12% skim milk supplemented with yeast extract @ 1.5% and Tween 80). The pH of the broth was kept constant at 6 because pH range of 5.8–6 has been considered to be the optimum pH for bacteriocin production (Yang and Ray 1994; Cheigh et al. 2002). Broth was inoculated @1% and antibacterial activity of CFCS was determined at every two hour intervals up to 24 h by spot-on-lawn method using *P. acidilactici* LB 42 as an indicator strain.

Partial purification of bacteriocin

The crude bacteriocin was purified by growing *L. lactis* 63 till stationary phase at 30 °C in GM-17 broth (initial pH 6). The cell free culture supernatant was obtained centrifugation at 10,000 rpm for 20 min at 4 °C and the pH of the cell free culture supernatant was adjusted to 6.5 by the addition of 1 N NaOH (Himedia), and then passed through a sterile filter (0.45 µm, Merck, Germany). The purification process was optimized by precipitating small aliquots of CFCS with ammonium sulphate from 30 to 80% saturation levels overnight at 4 °C. Highest activity was observed at 60% ammonium sulphate saturation. Finally CFCS from 1

L broth was precipitated at 60% ammonium sulphate saturation overnight at 4 °C. The mixture was centrifuged (10,000 rpm at 4 °C for 30 min), and the surface pellicles and bottom pellets were harvested and resuspended in sodium phosphate buffer (50 mM, pH 6.5). This precipitate was subjected to dialysis through a membrane (Benzoylated Dialysis tubing, MWCO < 1kDA, Sigma Aldrich) against distilled water to remove salt. The increase in the volume of precipitate was compensated by concentrating it to the original volume in a lyophilizer after freezing. Bacteriocin activity (AU ml⁻¹) and protein content (Lowry et al. 1951) were determined to calculate the specific activity.

Bacteriocin activity

The inhibitory spectrum of activity for strain 63 was assessed using the agar-well assay (Gupta et al. 2010) against the strains listed in Table 1. TGYE agar plates (1.5% agar) were overlaid with TGYE soft agar (0.75%) seeded with actively growing cells of the test organisms. After that 6 mm wells were punched with a stainless steel borer and 100 µl of the cell free culture supernatant (CFCS) of an overnight grown culture of isolates in GM-17 broth, obtained after centrifugation at 7000 rpm for 10 min at 4 °C, was added to the wells. For Gram-negative test strains, 100 µl of partially purified bacteriocin was used. Plates were pre-incubated for 3–4 h at 4 °C for proper diffusion of CFCS through agar and subsequently incubated under the conditions essential for the growth of the test organisms. Zone of clearance around wells were observed to evaluate the sensitivity of the strains in question.

Molecular weight approximation

Molecular weight of the partially purified bacteriocin sample was determined by SDS PAGE as described by Schagger et al. (2006) on 16% slab gels. In first half portion of the gel, marker and bacteriocin sample were loaded and in the second half of the only bacteriocin sample was loaded. After electrophoresis, one half of the gel was stripped off, fixed and stained with Coomassie Blue R 250. The second half of the gel containing only bacteriocin samples was washed with sterile chilled deionized water for 4 h. The wash water was replaced every 30 min with the fresh one for effective cleaning of the gel. The washed gel was transferred to a dried TGE agar plate. The gel was overlaid with 7 ml of TGE soft agar (0.8% agar) previously seeded with 30 µl of an active culture of indicator strain *P. acidilactici* LB 42. The plate was incubated overnight at 37 °C and examined for the zone of inhibition after 24 h. The stained gel was superimposed on the gel used to detect

the bacteriocin activity. Approximate molecular weight of the activity band was estimated by comparing the center of the zone of the inhibition with the molecular weight markers.

PCR detection for the presence of bacteriocin gene in *L. lactis* subsp. *lactis* 63

PCR was run to detect genes responsible for coding of nisin A/Z (De Vos et al. 1993). Total DNA of strain *L. lactis* 63 was isolated using DNA extraction kit (MN, Germany) according to the manufacturer's protocol. Oligonucleotide primers used for PCR were NisF and NisR, which are complementary to regions 80 bp upstream and 29 bp downstream of the coding regions of the *nisA* and *nisZ* genes, respectively. The product of amplification was purified by using a ChargeSwitch® PCR Clean-Up kit (Invitrogen Bioservices), and sequence of the nisin gene was determined by Sanger sequencing (1st Base Laboratories, Malaysia). BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to determine the homology to sequences listed in GenBank. 16S rDNA sequencing was also performed for one strain 63 (highest bacteriocin producer) using the universal primer pair 27F and 1492R (Supplementary 1 Table B) (Suzuki and Giovannoni 1996). PCR product purification and sequencing was done as explained above. A search for homology of the DNA sequence was made using the BLAST algorithm (NCBI, USA).

Results and discussion

Isolation and identification of Lactococci

A total of 145 presumptive lactococci isolates (based on Gram staining and catalase reaction) were screened initially for antibacterial activity. Thirteen isolates showing considerable antibacterial activity were further subjected to phenotypic identification (Supplementary 1 Table A). All the 13 isolates were negative for phenotypic test used for enterococci differentiation from lactococci, such as growth at 45 °C and in presence of 6.5% NaCl and esculin hydrolysis, which indicates that isolates did not belong to the genus *Enterococcus* (Chuard and Reller 1998). All the isolates were able to hydrolyse arginine and showed growth at 40 °C. All the isolates were also able to ferment lactose, glucose, fructose, adnitol, xylose, salicine, ribose and maltose but were unable to produce acid from arabinose, trehalose, glycerol, inulin, mannitol, melibiose, raffinose and sorbitol. The ability of *L. lactis* isolates to ferment lactose, glucose, fructose, salicine, maltose, ribose, cellobiose, xylose and inability to utilize sugars such as

glycerol, sorbitol, trehalose, melezitose, raffinose and dulcitol have also been reported by many other studies (Teixeira et al. 1996). However, results obtained for the fermentation of sugars such as melibiose, raffinose, xylose, maltose, ribose and trehalose were not in concordance with the earlier reports for lactococcal isolates (Fernández et al. 2011). Hence the phenotype tests conducted above was not able to reliably identify the isolates as *Lactococcus* spp. Phenotypic tests based on biochemical characters have many times been observed to give ambiguous results; hence DNA sequence based methods are now the current trend for appropriate bacterial identification (Fernández et al. 2011). Therefore, we amplified a 348-bp region of 16S rRNA gene of all the fourteen isolates, using species-specific primer pair (Y1 and Y2) (Fig. 1) as previously described for the identification of lactococci (Ward et al. 1998). All the isolates showed the presence of 348 bp region of the 16S rRNA gene with the *Lactococcus* specific Y1 and Y2 primers. Accordingly, we confirmed all the isolates to be *Lactococcus lactis*.

Bacteriocin nature of antimicrobial substance

From the results obtained after initial antibacterial activity assay, we selected isolate 63 (showing relatively higher activity) for elucidation of the antimicrobial substance it produced. In order to determine the nature and the stability of the antimicrobial component of isolate 63 the culture supernatant was subjected to different enzymes, pH, temperature and surfactants. Bacteriocins are proteinaceous in nature and this property has been utilized to ascertain whether the antimicrobial substance is protein in nature (bacteriocin) or not. Proteolytic enzymes such as pepsin, papain, trypsin, α -chymotrypsin and protease negatively affected the activity of antimicrobial substance of the isolate 63, although no such effect was observed for lysozyme (Fig. 2a). The antimicrobial substance in the culture supernatant can thus be considered to be a bacteriocin since proteinaceous nature of the antimicrobial component was revealed on enzymatic action also observed for most other LAB bacteriocins (Furtado et al. 2014).

Many studies conducted have proven that bacteriocins especially lantibiotics derived from *Lactococcus* spp. are

fairly stable at 100 °C for 15 min to 2 h, but a loss of 25–75% of activity has been observed at 121 °C due to the presence of thermostable amino acids like lanthionine and β -methyl-lanthionine (Akkoc et al. 2011; Balciunas et al. 2013; Furtado et al. 2014). Similarly Akkoc et al. 2011 also reported a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* MA23 to be stable at temperature of 100 °C for 15 min. Another lantibiotic from *Lactococcus* strain, lacticin NK24 was stable to a heat treatment of 90 °C for 30 min and inhibitory activity was detected during treatments of up to 100 °C for 30 min. However, lacticin NK24 lost its inhibitory activity when heated at 121 °C for 15 min (Lee and Paik 2001). In our study too, the bacteriocin from isolate 63 was found to be fairly stable up to 90 °C/10 min, however when heated to 100 °C/10 min, the activity was reduced to 10% and complete loss of activity was observed when it was autoclaved (Fig. 2).

With respect to effect of pH, the lantibiotics have been observed to be stable over a wide pH range due to the acidic nature of its molecule (Balciunas et al. 2013). Lantibiotic lacticin NK24 was reported to be stable in the pH range 2.0–9.0 (Lee and Paik 2001). Akkoc et al. 2011 reported that bacteriocin from *L. lactis* MA23 was active in a pH range of 2–7. The estimation of the residual activity of the pH adjusted culture broths of the lactococcal strain 63 revealed that the bacteriocin was stable at pH range 3–9 (Fig. 2).

One of the key characteristic of lantibiotics is its amphipathic nature since due of the presence of three cationic amino acids lysine and N-terminal hydrophobic residues like leucine, isoleucine and proline (Bauer and Dicks 2005). Because of the amphipathic nature of bacteriocins, treatment with surfactants often leads to enhancement of activity due to emulsifying nature of bacteriocin molecule. Keren et al. 2004 reported that Tween 80 increased the inhibitory action of lacticin RM eightfold, either by enhancing the activity of lacticin RM or by increasing the sensitivity of the indicator strain. Similarly, production of bacteriocin from *Lb. sakei* 2a, has been reported to increase by the addition of Tween 20 and glucose (Malheiros et al. 2015) whereas, lactococcin BZ, produced by *L. lactis* ssp. *lactis* BZ, on the other hand maintained its activity in the presence of SDS, urea, tween 80, and triton X-100 (Sahingil et al. 2011).

Though the activity of the bacteriocin 63 increased on treatment with Tween 80, Tween 20 and Triton X-100, but no change in its activity was observed in presence of anionic detergent SDS (Fig. 2). Surfactants such as Tween 80 may have an enhancing effect on bacteriocin activity which may be attributed to the splitting of protein polymers to monomers.

The above treatments with different enzyme, heating and different pH provide sufficient evidence that the

Fig. 1 PCR amplified product of 16S rRNA gene of bacteriocinogenic isolates of Lactococci. Lane M—100 bp Marker; Lane 1- *L. lactis* subsp. *lactis* NCDC 90; Lane 2 *L. lactis* subsp. *cremoris* NCDC 60; Lane 4–16- Lactococci isolates

Fig. 2 Characterization of bacteriocin produced by *L. lactis* subsp. *lactis* 63. **a** Effect of proteolytic enzymes on bacteriocin activity. **b** Heat stability of bacteriocin 63. **c** Effect of pH on bacteriocin activity. **d** Effect of surfactants on bacteriocin activity

antimicrobial substance produced by isolate 63 is a bacteriocin of Lantibiotic class. Further characterization on the basis of purification and antimicrobial activity was also carried out.

Production of bacteriocins

Bacteriocin production from the selected lactococcal strain 63 started at the end of the log phase (9–12 h of growth), however, highest activity (20,000 AU/ml) was observed only after 22 h of bacterial growth (Supplementary 1 Fig I). Cheigh et al. 2002 reported maximum bacteriocin activity of 1.31×10^5 AU ml⁻¹ by strain *Lactococcus lactis* subsp. *lactis* A164 at early stationary growth phase (20 h). Tafreshi et al. 2010 also reported maximum bacteriocin production from *Lactococcus lactis* subsp. *lactis* ATCC 11454 at the end of the log phase. The production of bacteriocins by *Lactococcus* strains during the early stationary phase is also supported by another study by Banerjee et al. (2013) who reported highest bacteriocin titre from *Lactobacillus brevis* FPTLB3 during 19–29 h of growth. However, bacteriocin activity started decreasing after 24 h of bacterial growth. This is a common phenomenon that is observed towards the end of the fermentation period and has been attributed to many factors such

as proteolytic degradation of the bacteriocin, instability at low pH, bacteriocins self-aggregation or adsorption of bacteriocins to bacterial cell surface (Zamfir et al. 2016).

Many factors such as media composition, growth rate, temperature of incubation, initial pH, aeration etc. have been reported to influence bacteriocin production by *L. lactis* strains (Guerra and Pastrana 2002; Zamfir et al. 2016). In this study we tried to check the influence of different media (composition) on bacteriocin production by isolate 63 keeping all the other factors at optimum level. Amongst the four different media tested, higher bacteriocin production was observed both in MRS as well as in GM-17 medium (maximum 20,000 activity unit/ml) compared to TYGE and enriched skim milk broth. It has been documented that in general, higher cell densities are recovered by growing the cells under optimum environment for 16 h, which in turn leads to higher bacteriocin production (Yang and Ray 1994). This may explain the higher bacteriocin production in commercial growth media as compared to TYGE and enriched skim milk broth, since commercial media such as MRS and M17 have been reported to be the most suitable media for the growth of lactococci (Cheigh et al. 2002). These commercial media are also relatively richer in some of the amino acids such as serine, threonine and cysteine that have been reported to strongly stimulate

nisin production, hence favoring better nisin biosynthesis (Guerra and Pastrana 2002). Amongst the 4 tested media, lowest activity was observed in enriched skim milk broth, since food components like proteins and traces of fat in skim milk can interact with the bacteriocins due to its amphipathic nature and lower its activity (Zamfir et al. 2016). Also lack of essential nutrient such as free amino acids (required for bacteriocins production) may also be one of the reasons for poor bacteriocin production in skim milk broth media.

Antimicrobial spectrum of *L. lactis* subsp. *lactis* 63

The neutralized CFCS from 20 to 22 h old culture of strain 63 inhibited the growth of all the Gram positive indicator bacteria which included *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis* and *Enterococcus faecalis* (Table 1), however, no effect was observed against any of the Gram-negative bacteria. The antibacterial effect of CFCS against tested Gram-negative bacteria was observed only after partial purification by ammonium sulphate precipitation of the CFCS (Fig. 3). The partially purified bacteriocin inhibited all Gram-negative bacteria described in Table 1. This shows that inhibition of Gram-negative bacteria by bacteriocins from lactococci requires either relatively higher concentration or more purified form of the bacteriocin. Earlier it has been stated that insensitivity of nisin against Gram-negative bacteria is due to the presence of protective outer membrane (OM) which covers the cytoplasmic membrane and peptidoglycan layer of Gram-negative bacteria. The outer leaflet of OM is a heterocyclic anionic polymer which forms a tight hydrophilic layer which excludes the attacking N-terminal hydrophobic domain of nisin and hence it cannot reach its target (Helander and Mattila-Sandholm 2000). However, many reports have suggested that purified form of some of the lantibiotics such as nisin Z, bioengineered variants of nisin, epidermin, mutacin and enterocin could inhibit these Gram-negative bacteria (Yuan et al. 2004; Cotter et al. 2013). Kuwano et al. 2005 observed that

unpurified nisinZ failed to inhibit *E. coli* but showed an MIC of 600 nM when purified form was used. A recent study by Shin et al. 2015b has also shown that highly pure form of nisinZ (nisinZP > 95% purity) could exhibit antimicrobial activity against cariogenic Gram-negative bacteria like *Fusobacterium*, *Aggregatibacter*, *Porphyromonas*, *Prevotella* and *Treponema*.

Reports regarding the mechanism of antibacterial activity of nisin Z against Gram-negative bacteria are scanty. In an attempt to reveal the mechanism of action of purified nisin Z against *E. coli*, Kuwano et al. (2005) mentioned that purified nisin Z was able to cause permeabilization of the cytoplasmic membrane of *E. coli* more efficiently as compared to unpurified form. The hinge region of Nisin has also been indicated to play a crucial role in its activity against Gram-negative bacteria (Yuan et al. 2004). The study showed that alteration in the hinge region by introducing a positively charged amino acid such as lysine at this site plays an important role in the activation against several Gram-negative bacteria.

Purification of the bacteriocins

Purification of bacteriocin is a common practice in order to increase its activity by selectively increasing its quantity in the CFCS and excluding other proteins from the CFCS. Due to the cationic nature of the peptide, ammonium sulphate precipitation followed by chromatographic techniques has quite often been employed to purify the bacteriocins. Here we tried to partially purify the bacteriocin by Ammonium sulphate precipitation followed by its dialysis. Recovery percentage, specific activity and degree of purification are summarized in supplementary 1 Table C. *L. lactis* 63 was grown in 1 L GM-17 broth for 22 h and the bacteriocin in the CFCS was concentrated using ammonium sulfate (60% saturation). A total bacteriocin activity of 2×10^7 AU was obtained in CFCS and 1.63×10^7 AU was obtained after ammonium sulfate precipitation, which accounted for ~ 82% recovery and 16 fold purification. Similarly Choi et al. 2000 observed 98% recovery for

Fig. 3 Antimicrobial Activity of *L. lactis* ssp. *lactis* 63 (KU359424). (A) Unneutralized partially purified bacteriocin, (B) neutralized partially purified bacteriocins and (C) Control against indicators *S.*

marcescens ATCC 13880 (1), *K. pneumoniae* ATCC 27736 (2), *E. coli* ATCC 25922 (3) and *C. freundii* ATCC 43864 (4)

bacteriocin from *L. lactis* ssp. *lactis* A164 (isolated from Kimchi) after ammonium sulphate precipitation and ten-fold purification was achieved. Saraiva et al. 2014 found 20% recovery and 10 fold increases in specific activity after ammonium sulphate precipitate. For the removal of salt the precipitate was dialyzed against distilled water and concentrated to original volume.

Tricine SDS-PAGE determination of molecular weight

The partially purified bacteriocin sample was run on Tricine–SDS-PAGE. One part of the gel with molecular weight markers and bacteriocin sample was Coomassie blue R-250 stained and the other part of the gel was used to determine the bacteriocin activity. It may be observed from the gel stained with the dye showed several bands but did not show any protein band for bacteriocin corresponding to in situ activity in the other half of the gel (Fig. 4), which can be attributed to the lower concentration of the antimicrobial protein ($< 0.2 \mu\text{g}/\text{band}$) in sample taken for Tricine–SDS-PAGE since a minimum of $\sim 0.2 \mu\text{g}$ of protein are only suitable for Coomassie staining of the protein bands (Schägger 2006). However, the unstained other part of the gel overlaid with the indicator strain *P. acidilactici* LB 42 gave clear zone of inhibition. Superimposing of stained gel over the gel used to detect the bacteriocin activity revealed that the bacteriocin has a molecular weight of around 3.5–5 kDa. Many other studies have also reported the molecular weight of bacteriocins from lactococci to be 2–6 kDa (Ribeiro et al. 2016). Choi et al. 2000 also found that the bacteriocin from *L. lactis* ssp. *lactis* A164 gave the same molecular weight of 3.5 kDa as that of nisin by Tricine SDS PAGE. Our results

also indicate that the antimicrobial substance produced by *L. lactis* 63 is similar to nisin or its variants. However, the molecular weight of bacteriocin reported in our study is an approximation.

Molecular analysis of bacteriocin encoding gene

To confirm the identity of antimicrobial substance as nisin, we amplified the gene corresponding to nisA/nisZ region. Oligonucleotides for nisin family of bacteriocins enabled amplification of a DNA fragment of the expected size (350 bp). The PCR fragment was sequenced and analyzed, the deduced amino acid sequence being identical to nisin Z (Class I lantibiotic peptides). The sequence of this bacteriocin revealed a peptide of 34 amino acids with an asparagine at 27 position (ITSISLCTPGCKT-GALMGCNMKTATCNCSEIHVSK) as shown in Fig. 5. The 16S rDNA amplification of the isolate 63 (highest bacteriocin producer) presented 99% identity with the 16S rDNA sequences reported for *L. lactis* subsp. *lactis* in the GenBank database, such as strain IL1403 (accession number: NR_103918). The near-full length (1411 bp) 16S rDNA sequence has been deposited in GenBank database under accession number KU359424.

Conclusion

Results reported in the presented work indicate that the *L. lactis* 63, is a nisin Z-producing strain, having potential for its application in reducing the number of Gram-positive as well as Gram-negative food borne pathogens. The bacteriocinogenic isolate can be used as starter, co-starter or protective adjunct cultures in the manufacturing of various fermented dairy products whereas the concentrated form of bacteriocin can be used in the packaging material. Since the bacteriocin produced showed a potent inhibitory spectrum of activity against Gram-negative food-born pathogen, further laboratory-scale studies in food products spiked with pathogens are required. This will indicate how this strain or its bacteriocin will behave in controlling the food borne pathogens and spoilage bacteria in these products. Hence, the study concludes that the ability of bacteriocin produced by the test isolate in inhibiting a wide-range of bacteria may be of potential interest in food industry for the preservation as well as safety of foods.

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Fig. 4 Determination of molecular weight of partially purified bacteriocin of strain 63 by SDS-PAGE. **a.** Gel stained with brilliant blue R; M-Marker (size in kb); band of bacteriocin produced by *L. lactis* 63 (**b**). Unstained gel depicting the in situ bacteriocin activity

CLUSTAL format alignment by MAFFT (v7.310)

AM410671.1	-----attataaggaggcactcaaaatgagtacaa	30
X61144.1	atgatttcggttcgaaggaactacaaaataaattataaggaggcactcaaaatgagtacaa	60
63	----ttcgttcgaaggaactacaaaataaattataaggaggcactcaaaatgagtacaa	55
	*****	
AM410671.1	aagattttaacttggatttgggtatctgtttcgaaaaagattcaggtgcatcaccacgca	90
X61144.1	aagattttaacttggatttgggtatctgtttcgaaaaagattcaggtgcatcaccacgca	120
63	aagattttaacttggatttgggtatctgtttcgaaaaagattcaggtgcatcaccacgca	115
	*****	
	I T S I S L C T P G C K T G A L M G C N	
AM410671.1	ttacaagtatttcgctatgtacaccggttgtaaaacaggagctctgatgggttgtaaca	150
X61144.1	ttacaagtatttcgctatgtacaccggttgtaaaacaggagctctgatgggttgtaaca	180
63	ttacaagtatttcgctatgtacaccggttgtaaaacaggagctctgatgggttgtaaca	175
	*****	
	M K T A T C H C S I H V S K	
AM410671.1	tgaaaacagcaacttgtcattgttagtattcacgtaagcaaataaccaaatacaaggatag	210
X61144.1	tgaaaacagcaacttgtcattgttagtattcacgtaagcaaataaccaaatacaaggatag	240
63	tgaaaacagcaacttgtcattgttagtattcacgtaagcaaataaccaaatacaaggatag	235
	*****	
	N	
AM410671.1	tattttgttagttcagacatg-----	231
X61144.1	tattttgttagttcagacatggatactatcctatttttataagttattta	290
63	tattttgttagttcagacatggatact-----	263
	*****	

Fig. 5 Nucleotide sequences of DNA containing nisin genes (structural nis genes) of *L. lactis* subsp. *lactis* 63 compared with the sequences of Nisin A (Gene Bank: AM410671) and nisin Z (Gene Bank: X61144) and predicted amino acid sequences of the nisin DNA coding region

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