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Production of Nisin Z by *Lactococcus lactis* Isolated from Dahi

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Abstract *Lactococcus lactis* CM1, an isolate from homemade "Dahi," a traditional fermented milk from India, used maltose as carbon source to produce a high level of bacteriocin. The bacterial cell mass and the bacteriocin production correlated with the initial pH of the medium and were highest when the initial pH was 11.0. The level of bacteriocin reached its peak at the late log phase with concomitant reduction of culture pH to 4.2, regardless of the initial pH of the medium. A combination of maltose and an initial medium pH of 11 resulted in the highest bacteriocin production. The antibacterial spectrum of the bacteriocin was closely similar to that of nisin and it inhibited a number of food spoilage and pathogenic bacteria. Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis, the compound migrated close to the position of nisin (3.5 kDa). However, it had higher stability than nisin at a wide range of pH and temperature. PCR amplification using nisin gene-specific primers and sequencing of the amplified DNA revealed the structural gene for the bacteriocin to be identical to that of *nisZ*.

Keywords *Lactococcus lactis* · Nisin · Inhibitory spectrum

Introduction

Lactic acid bacteria (LAB) are of great economic value for fermentation. They are extensively used in the production of various fermented foods like yogurt, cheese, buttermilk, and sausage. Many LAB produce bacteriocins, which are extracellular bioactive proteins with antibacterial activity towards species related to producer bacteria and a wide variety of Gram-positive food-borne pathogens and spoilage microorganisms [1, 2]. The

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bacteriocins produced by LAB have an important role as biopreservatives due to increasing consumer awareness of the potential risks derived not only from food-borne pathogens, but also from the chemical preservatives used to control them. Among the bacteriocins, nisin is the most extensively studied bacteriocin and is a classical example of a biopreservative.

The bacteriocin nisin, produced by certain strains of *Lactococcus lactis*, is nontoxic [3]. It is approved for use in the USA, the UK, and the European Union [4], and is currently recognized as a generally-regarded-as-safe (GRAS) food additive and preservative mainly for processed foods in approximately 50 countries worldwide. Nisin is a ribosomally synthesized, low-molecular-weight, heat-stable polycyclic peptide with 34 amino acid residues and has two natural variants, nisin A and Z. Nisin Z differs from nisin A by a single substitution at position 27, with asparagine (nisin Z) in place of histidine (nisin A) [5]. This structural modification of nisin Z imparts higher solubility and diffusion characteristics, which are important for food application [6]. Although *L. lactis* has been reported to grow at pH 9 [7], in general, LAB, including *L. lactis*, are grown in media of pH 6 to 7 for the production of bacteriocin. However, in these cultures, as the pH decreases to a certain minimum due to lactic acid accumulation, the growth of the organism, as well as of bacteriocin, stops [8].

The search for new strains of LAB able to grow at high pH for the production of increased levels of bacteriocin or for new or natural variants of bacteriocin is of immense significance to increase the current range of biopreservative applications. In this regard, many nisin-producing strains of *L. lactis* have been isolated from both dairy and nondairy traditional fermented foods [9–12]. However, the characterization of nisin from isolates of LAB from "Dahi," a traditional fermented milk from India (a product similar to yogurt), has not been done [13–15]. In this paper, we report high production of nisin Z by *L. lactis* strain CM1 isolated from Dahi under optimized conditions and compare it with that of nisin production by the standard strain *L. lactis*, American Type Culture Collection (ATCC) 11454. The biochemical properties and the antibacterial spectrum of the bacteriocin produced by the strain CM1 have also been investigated.

Materials and Methods

Cultures and Growth Conditions

Bacterial strains used as indicators are *Lactobacillus plantarum* NCDO 955, *Pediococcus acidilactici* LB42, *Leuconostoc mesenteroides* Ly, and *Enterococcus faecalis* MB1. These are sensitive to most bacteriocins and are able to grow at pH 4.0 and above. B. Ray, Department of Animal Science, University of Wyoming, USA, provided these strains along with *P. acidilactici* F^+. Lactic acid bacteria were grown in tryptone–glucose–yeast extract (all at 1%, TGE) medium [16] at 37°C. *Listeria* sp. was grown at 37°C in brain heart infusion (Hi Media, Mumbai, India), *Salmonella* sp., *Vibrio* sp., *Staphylococcus* sp., *Bacillus* sp., *Geobacillus* sp., *Enterobacter* sp., and *Escherichia coli* in Luria Bertani broth and *Clostridium* sp. were grown in reinforced clostridial medium. *Geobacillus* was grown at 60°C. A nisin-producing strain, *L. lactis* ATCC 11454, was used for comparative studies. The effect of different carbon sources on growth and bacteriocin production by *L. lactis* was studied in tryptone–yeast extract (TE; both at 1%) medium (Table 1). All the strains with ATCC and Microbial Type Culture Collection (MTCC) numbers used in this study (Table 2) were procured from MTCC, Institute of Microbial Technolgy, Chandigarh, India. *Geobacillus thermoleovorans*, *Vibrio cholerae*, *Staphylococcus aureus*, strains of *E. coli*,

Table 1 Influence of carbon source on growth (OD_{650}) and bacteriocin production (AU/ml) of *L. lactis* CM1 and *L. lactis* ATCC 11454 at 37°C.

Substrate	CM1		ATCC 11454	
	OD_{650}	AU/ml	OD_{650}	AU/ml
Glucose	1.12	2,000	0.96	2,000
Xylose	1.1	2,400	0.22	400
Sucrose	1.42	2,400	1.02	3,000
Lactose	1.12	2,000	0.18	200
Galactose	1.1	2,000	0.94	3,000
Maltose	1.2	3,000	0.80	2,000
Mannose	1.14	2,000	1.02	2,000
Trehalose	1.74	2,800	1.30	3,000
Cellobiose	1.8	1,000	1.02	2,000

Each carbon source was added at 1% to tryptone–yeast extract medium, pH 6.5. Fermentation was carried out for 7 h, except for growth and bacteriocin yield of ATCC 11454 on cellobiose and galactose for 24 h.

and *Pseudomonas putida* were from our laboratory collection. All the cultures were stored at −20°C with 10% dimethyl sulfoxide.

Isolation of Bacteriocin-Producing Strain

Samples of homemade Dahi prepared by fermenting cow milk at 37°C for 24 h by its natural microflora were serially diluted, and an aliquot of each dilution was plated on TGE-agar

Table 2 Antibacterial spectrum of bacteriocin of *L. lactis* CM1 and *L. lactis* ATCC 11454.

Target strain	*L. lactis* CM1	*L. lactis* ATCC 11454
Lactobacillius acidophilus ATCC 4356	−	−
Lactobacillius delbruecki ATCC 4797	+	+
Lactobacillius plantarum NCDO 955	+	+
Lactobacillius rhamnosus ATCC 7469	+	+
Lactococcus lactis ATCC 19257	+	+
Pediococcus acidilactici LB 42	+	+
Pediococcus acidilactici F^+	+	−
Bacillus cereus MTCC 1272	+	+
Geobacillus thermoleovorans	−	−
Clostridium perfringens ATCC 3624	+	+
Enterococcus faecalis MB1	+	+
Leuconostoc mesenteroides Ly	+	+
Leuconostoc mesenteroides ATCC 10830	+	+
Listeria monocytogenes ATCC 19111	+	+
Staphylococcus aureus	+	+
Escherichia coli EPEC	−	−
Escherichia coli ETEC	−	−
Enterobactor aerogenes ATCC 13048	−	−
Pseudomonas putida	−	−
Salmonella typhimurium ATCC 23565	−	−
Vibrio cholerae	−	−

Lactococcus lactis ATCC 11454 was used as positive control. Plus signs represent zone of inhibition (15 mm or more in diameter).

medium. The plates were incubated at 37°C. The colonies were examined for bacteriocin activity by the spot-on-the-lawn assay method against the four indicator strains [17]. The lawn was made by overlaying approximately 10^6 cells in TGE soft agar from each colony onto the TGE plate. The isolates showing zone of growth inhibition of all four indicator strains were streaked several times on TGE plates to obtain pure cultures and were further examined for bacteriocin production in liquid medium by a method described previously [16]. Cell-free culture supernatant of each isolate was divided into two parts: one part was boiled and neutralized and the other part was only boiled but not neutralized. Both samples were spotted on a TGE agar plate overlaid with the indicator strain, *L. plantarum* NCDO 955. Following incubation at 37°C, the plates were observed for zone of growth inhibition. For a comparison, the culture supernatants of *P. acidilactici* F^+ and *L. lactis* ATCC 11454 were used during the isolation procedure. The experiment was replicated thrice.

Identification of Bacteriocin-Producing Strain

The bacteriocin-producing isolate was identified by its colony morphology, Gram-staining, and biochemical tests [18, 19], as well as by 16S rRNA gene sequencing [20]. The isolate was examined for acid production from 49 compounds as sole carbon source using API 50 CH Test kit (BioMerieux, Crappone, France) according to the manufacturer's instructions.

DNA Manipulation, PCR, and DNA Sequencing

Genomic DNA of the bacteriocin-producing strain was isolated by lysozyme–proteinase K procedure [21]. Approximately 50 ng of the DNA was used as template for PCR amplification of 16S rRNA or of nisin gene in a total volume of 50 μl containing 1.25 U of *pfu* polymerase (Fermentas, Hanover, MD, USA), 40 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, and 2 mM $MgCl_2$. The 16S rRNA gene was amplified in 35 cycles with a thermocycler (Perkin Elmer, Waltham, MA, USA); each cycle consisted of a denaturing step at 95°C for 1 min, a primer annealing step at 60°C for 1 min, and an extension step at 72°C for 5 min. Bacteria-specific universal primers used for amplification of 16S rRNA gene were the forward primer 27F (5′-AGAGTTTGATCATGGCTC-3′) and the reverse primer 1327R (5′-CTAGCGATTCCGACTTCA-3′) [20]. The primer pair amplifies a 1.3-kb fragment of DNA. The PCR amplification conditions for nisin gene were similar to that for 16S rRNA gene, except that the primer annealing temperature was 53°C. Two sets of primers were used for nisin gene amplification. These were (1) PNIS1 (5′-CGCGAGCATA ATAAACGGCT-3′) and PNIS2 (5′-GGATAGTATCCATGTCTGAAC-3′), complementary to regions 80 bp upstream and 29 bp downstream of the coding region of the *nisA* and *nisZ* genes, respectively [5], and (2) NS1 (5′-TAGATACAATGATTTCGTTC-3′) and NS2 (5′-AGCTCACTACTATTATGGT-3′) complementary to nucleotide positions 106–125 upstream and positions 919–938 downstream of the start codon of *nis A* and *nis B* genes, respectively [22]. PCR products were checked by electrophoresis on 1.5% agarose gel. A 100-bp DNA ladder was used as the molecular marker (Fermentas). PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced from both ends with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) using the same oligonucleotide primers used for PCR. All the primers were synthesized by Genei, Bangalore, India.

Production and Assay of Bacteriocin

Kinetics of growth and bacteriocin production was studied at 37°C in TGE medium for 10 h from a 2% inoculum of an overnight culture of *L. lactis* CM1. Bacterial growth was

measured following the OD of the culture at 650 nm, whereas bacteriocin activity in the culture supernatant was measured by the extinction-to-the-dilution method [16]. The culture broth was withdrawn periodically and treated at 100°C for 10 min. An aliquot of the treated sample was serially diluted (1:2 to 1:50) with deionized water, and 5 μl from each dilution was spotted on the TGE agar plate overlaid with soft agar (0.7%) containing 10^6 cells of an overnight grown *L. plantarum* NCDO 955. The plates were incubated at 37°C and examined for any zone of growth inhibition. The activity unit per milliliter (AU/ml) was defined as the reciprocal of the highest dilution that produced a definite zone of inhibition. For a comparison, a growth kinetics study of *L. lactis* ATCC 11454 with CM1 was performed in parallel. All the experiments were performed in triplicate.

To study bacteriocin production using different carbohydrates, CM1 was grown overnight in TGE medium at 37°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice with saline, and resuspended in TE broth to its original density. This was used to inoculate fresh TE medium supplemented with different carbohydrates to a final concentration of 1%. The pH of each medium was adjusted to 6.5 before inoculation. Following incubation at 37°C, the growth and bacteriocin activity were measured as described. A nisin-producing strain *L. lactis* ATCC 11454 was also grown for comparison. The experiment was done in triplicate.

In a separate experiment, bacteriocin production was studied in TGE medium adjusted to various pH values ranging from 5.0 to 11.0. The strain *L. lactis* CM1 grown overnight in TGE medium at 37°C was harvested by centrifugation at 10,000 rpm for 10 min, washed with saline, and finally resuspended in TGE medium to its original density. The media with different pH values were inoculated with this inoculum at 2%. The fermentation was carried out at 37°C for 24 h. The activity of bacteriocin produced was assessed. The *L. lactis* ATCC 11454 was also grown under the same conditions for comparison. Following inoculation, the initial pH of the media was checked in a duplicate set. The experiment was replicated thrice.

Stability of Bacteriocin

To determine the thermostability of the bacteriocin at different pH values, it was precipitated at 70% saturation of ammonium sulfate, dialyzed, and adjusted to the desired pH in the range of 2.0 to 12.0 using 1 M NaOH or 1 M HCl. The preparations were then incubated either at 37°C for 5 h, at 100°C for 1 h, or at 121°C for 15 min, and then readjusted to pH 6.0. All the samples were then assayed to determine the residual activity using the indicator strain, *L. plantarum* NCDO 955. Untreated sample or buffer alone served as the control. Nisin A produced by *L. lactis* ATCC 11454 was also treated similarly, and its stability was determined for a comparison. For stability determination, the preparations contained 2,000 AU/ml of the bacteriocin activity. The effect of sodium dodecyl sulfate (SDS), urea, Tween 80 or Triton X-100 at 1%, and EDTA (10 mM) or mercaptoethanol (50 mM) on the activity of bacteriocin was studied at 37°C for 5 h. Bacteriocin preparation was mixed with each of the chemicals, and after incubation, the samples were diluted 10-fold and a 5-μl aliquot was spotted on a TGE agar plate overlaid with *L. plantarum* NCDO 955. The plates were incubated at 37°C for 24 h and observed for any zone of growth inhibition. Untreated samples and the chemicals served as the control. In a separate experiment, the culture supernatants adjusted to pH 6.5 were treated with protease, namely, trypsin, papain, or proteinase K, at 37°C for 2 h, and the residual activity was determined to assess the stability of the bacteriocin to these treatments. The untreated culture supernatant served as the control. Nisin A of *L. lactis* ATCC 11454 was used for

comparison under the same experimental conditions. The experiments were done in triplicate.

Antibacterial Activity Spectrum

The strain CM1 was grown in TGE medium at 37°C for 24 h. Semipurified bacteriocin was prepared by 70% ammonium sulfate precipitation of the heat-treated cell-free culture supernatant and was examined against Gram-positive and Gram-negative bacteria (Table 2). A 50-μl aliquot of the bacteriocin preparation corresponding to 2,000 AU/ml was spotted on the lawn of the target bacteria prepared using 10^6 of the overnight grown cells. In a parallel experiment, similarly prepared nisin from the culture supernatant of *L. lactis* ATCC 11454 was used for a comparison. The plates were incubated at 37°C for 24 h, and at 60°C for *Geobacillus* sp. Following incubation, the plates were observed for any zone of growth inhibition. Each assay was replicated thrice.

Detection of Bacteriocin Activity and Molecular Size Approximation by SDS Polyacrylamide Gel Electrophoresis

Bacteriocin activity was directly detected on polyacrylamide gel (15%) as described previously [23]. The isolate was grown in a medium containing glucose (1%) and yeast extract (1%), but without tryptone. Bacteriocins were partially purified from the culture supernatants by CM-cellulose chromatography and were electrophoresed at 200 V for 3 h. After electrophoresis, the gel was cut vertically into two halves. One half was stained with silver nitrate to visualize the protein bands [24]. The other half was washed in sterile deionized water for 3 h at room temperature to remove SDS and then overlaid with soft agar containing 10^6 cells of an overnight grown culture of *L. plantarum* NCDO 955. After incubation at 30°C for 16 h, it was examined for zone of growth inhibition. The protein band corresponding to the antibacterial activity was identified. Low-range standard peptides were used as molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for size approximation.

Results

Out of a total of 616 colonies isolated from Dahi sources, the isolate CM1 produced a zone of growth inhibition against all the four bacteriocin-sensitive indicator strains. The boiled culture supernatant of the isolate, whether neutralized or not, exhibited similar inhibitory activity. The inhibitory activity was destroyed by treatment with proteases, namely, trypsin, papain, and proteinase K, indicating that the isolate released a heat-stable proteinaceous substance into the growth medium.

Scanning electron microscopy demonstrated the cells of the isolate CM1 to be coccoid and 1 μm in diameter. The bacterium is Gram-positive, catalase-negative, VP-positive, and able to hydrolyze arginine. The isolate could grow at 10°C but could not at 45°C or at 6.5% NaCl. It did not produce gas from glucose. The results indicated that the strain belonged to the genus *Lactococcus* [18]. The isolate fermented ribose, mannitol, arbutin, salicin, cellobiose, maltose, lactose, esculin, trehalose, and β-gentibiose, but not sorbitol, melibiose, inulin, melezitose, turanose, tagatose, or raffinose, similar to *L. lactis* subsp. *lactis* [25]. In contrast to *L. lactis* subsp. *lactis* ATCC 19435, the isolate, however, utilized L-arabinose, sucrose, amygdalin, and D-xylose. On the basis of acid production from 49 carbohydrates,

the isolate was tentatively identified as *L. lactis* [19]. The strain was further characterized by sequencing of its PCR-amplified 16S rRNA gene. Comparison of the 16S rRNA gene sequence of the strain CM1 with those in the database showed 99% homology to those of *L. lactis*, and having the highest level of identity with that of *L. lactis* subsp. *lactis*.

The kinetics of growth and bacteriocin production of the strain CM1 was studied in media of various pH ranging from 5.0 to 11.0 and at temperatures ranging from 25° to 40°C. The optimum temperature for growth and bacteriocin production of the strain was a broad one from 30° to 37°C. However, at 40°C, both bacteriocin production and growth were adversely affected. At 37°C, bacteriocin production was detected at the early log phase of growth and the level increased in parallel to growth of the bacterium and reached its maximum at the late log phase, and thereafter, it remained essentially constant. Concomitant to growth, the pH of the culture gradually declined to 4.2. Although bacteriocin production and cell mass increased with an increase in the initial pH of the growth medium, the final pH of the cultures was always lowered to 4.2 regardless of the initial pH. The highest level of bacteriocin production by the strain was assessed to be 2,000 AU/ml in the TGE medium, with an initial pH of 6.5 and was equal to that of *L. lactis* ATCC 11454. Although growth of the strain CM1 was recorded to be highest (1.8 OD_{650}) with cellobiose, the bacteriocin production was lowest with this carbon source. On the other hand, the highest level of bacteriocin (3,000 AU/ml) of the strain CM1 was produced with maltose, whereas trehalose was the next preferred carbon source for the bacteriocin production (Table 1). As compared, the strain ATCC 11454 produced the highest level of bacteriocin with sucrose, galactose, and trehalose. Xylose and lactose supported only a little growth and bacteriocin production of the strain ATCC 11454 in contrast to the strain CM1, where a high level of growth and bacteriocin production was observed with these three carbon sources (Table 1).

The effect of culture pH adjustment on growth and bacteriocin production of *L. lactis* strain CM1 was studied. In TGE medium, pH 6.5, where glucose served as the carbon source, the strain CM1 attended only an OD_{650} of 1.12, and the bacteriocin production was 2,000 AU/ml (Fig. 1) when the medium pH was down to 4.2. However, if the medium pH was adjusted back to pH 6.5 with the addition of alkali at this time, further growth and bacteriocin production was observed. Figure 1 shows that the addition of alkali to the culture at the fifth, sixth, and seventh hours back to pH 6.5 allowed further growth and bacteriocin production. However, at the eighth hour, such pH adjustment yielded no further

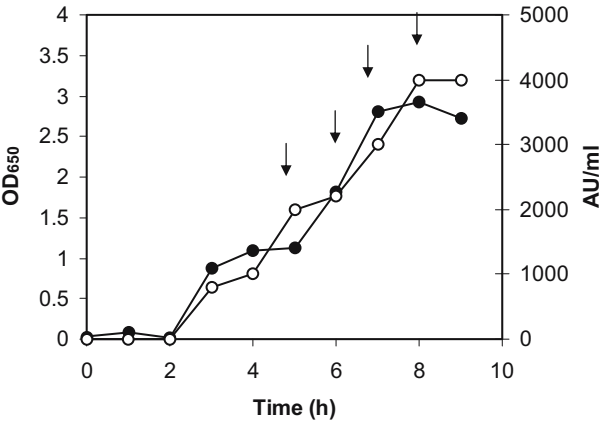

Fig. 1 Effect of pH adjustment on the production of cell mass (*closed circles*) and bacteriocin (*open circles*) of *L. lactis* CM1 in TGE medium at 37°C. The initial pH of the medium was 6.5. *Arrows* indicate the time points (5, 6, 7, and 8 h) when alkali was added to adjust the pH back to 6.5

growth or bacteriocin production, and the final growth attended was almost 3 OD_{650} and the bacteriocin production was almost 4,000 AU/ml (Fig. 1).

The effect of the initial medium pH on growth and bacteriocin production of *L. lactis* strains CM1 and ATCC 11454 was studied. For this, the pH of the TGE medium was initially adjusted to different values ranging from 6.5 to 11.0, and the growth and bacteriocin production were assessed (Fig. 2). It was found that both growth and bacteriocin production of the strains increased almost linearly up to pH 9. Further increase in growth and bacteriocin production was noted for the strain CM1 at initial medium pH values of 10 and 11; the strain ATCC 11454, however, failed to grow and thereby to produce bacteriocin at an initial medium pH of 10. At an initial medium pH of 11, the growth of the strain CM1 almost reached an OD_{650} of 2.5, and the level of bacteriocin was almost 5,000 AU/ml (Fig. 2).

In a separate experiment, the glucose of the TGE medium was replaced with maltose as the carbon source, the initial pH of the medium was either adjusted to 6.5 or to 11.0, and the kinetics of growth and bacteriocin production of *L. lactis* strain CM1 were studied (Fig. 3). It was observed that, in the medium with an initial pH of 6.5, the strain grew to an OD_{650} of 1.2, and the bacteriocin production of the strain was about 3,000 AU/ml. These values compared to be only about one-third to those attended in the medium with an initial pH of 11 where the growth of the strain was higher than 3 OD_{650} and the baceriocin production was almost 9,000 AU/ml (Fig. 3).

At 37°C, the activity of the bacteriocin produced by the strain CM1 was found to remain stable for at least 5 h at pH values between 2.0 to 9.0 and lost only half of the activity at pH 10.0 and 90% at pH 12.0 (Fig. 4). The activity of nisin A produced by the strain ATCC 11454, however, was found to be stable in the pH range of 2.0 to 6.0, and it lost 50% of its activity at pH 7.0 and 100% at pH 12.0 (data not shown). At an acidic pH (2.0 to 4.0), the activity of bacteriocin produced by the strain CM1 remained stable even at a high temperature of 121°C. Treatment of the bacteriocin obtained from the strain CM1 for 1 h at 100°C caused a loss of only 20% of its activity at pH 6.0 (Fig. 4), in contrast to the loss of 80% of nisin A activity of ATCC 11454. The antibacterial activity was fully retained following exposure to SDS, Tween 80, Triton X100, urea, EDTA, and mercaptoethanol for 5 h at 37°C.

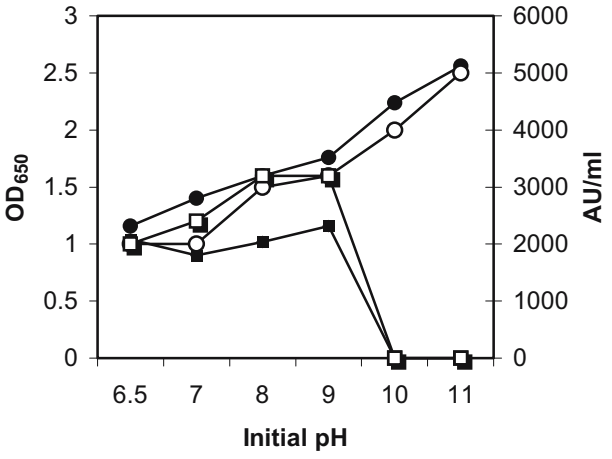

Fig. 2 Effect of initial medium pH on growth (*closed circles*, *L. lactis* CM1; *closed squares*, *L. lactis* ATCC 11454) and bacteriocin activity (*open circles*, *L. lactis* CM1; *open squares*, *L. lactis* ATCC 11454)

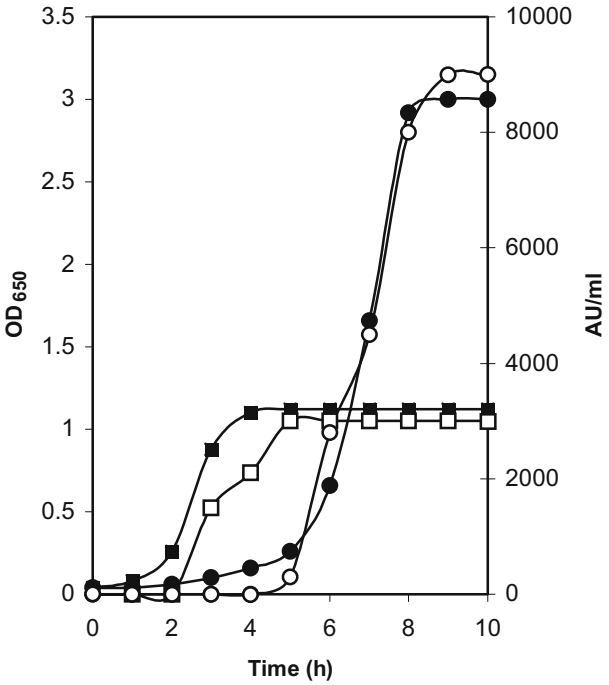

Fig. 3 Growth and bacteriocin activity of *L. lactis* CM1 in maltose containing medium with different initial pH. Growth was recorded in media with an initial pH of 6.5 (*closed squares*) and 11.0 (*closed circles*) and bacteriocin activity in media with an initial pH of 6.5 (*open squares*) and 11.0 (*open circles*)

The bacteriocin exhibited a broad antibacterial spectrum against several food spoilage and pathogenic bacteria, as well as related species of LAB. However, it was not effective against Gram-negative bacteria, but proved to be active against *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium perfringens*, *E. faecalis*, *S. aureus*, and several species of LAB (Table 2). Additionally, *P. acidilactici* F^+ resistant to nisin A of ATCC 11454 was killed by the bacteriocin produced by CM1.

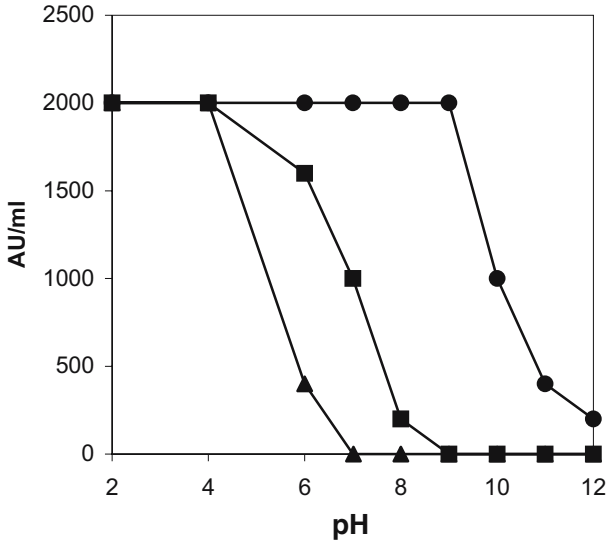

Fig. 4 Effect of temperature on the stability of the bacteriocin of *L. lactis* CM1. *Circles*, 37°C; *squares*, 100°C; *triangles*, 121°C

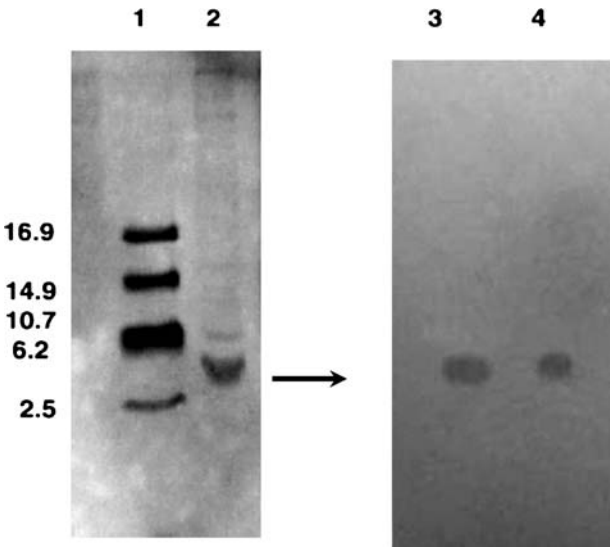

Fig. 5 Antimicrobial activity of partially purified bacteriocin of *L. lactis* CM1 following SDS polyacrylamide gel electrophoresis. *Lane 1*, low-molecular-weight protein markers; *lane 2*, silver-stained band of bacteriocin; *lane 3*, zone of inhibition of *L. plantarum* NCDO 955 by the nisin Z of *L. lactis* CM1; *lane 4*, zone of inhibition of *L. plantarum* NCDO 955 by the nisin A of *L. lactis* subsp. *lactis* ATCC 11454

The bacteriocin produced by the strain CM1 was found to be a short peptide (about 3.5 kDa), as judged by SDS polyacrylamide gel electrophoresis (Fig. 5). The molecular weight was judged by comparing the distance of the zone of growth inhibition of the indicator strain, *L. plantarum* NCDO 955 overlaid onto the gel from the point of application with the distances traversed by standard proteins.

The presence of nisin gene in the strain CM1 was identified by PCR amplification using two sets of nisin gene-specific primers and sequencing of the amplified products. The PCR product with each set of primers upon agarose gel electrophoresis showed a single band of expected size and were 330- and 835-bp fragments, respectively, for the sets of PNIS and NS primer pairs. The DNA sequence of the PCR amplicons was found to be identical to the *nisZ* sequences in the database. The result indicates that the bacteriocin produced by *L. lactis* CM1 is nisin Z. However, the sequence corresponding to the *nisB* gene, which is involved in dehydration of serine and threonine causing posttranslational modification of nisin [26], differed by one nucleotide out of 678 nucleotides sequenced in both orientations. It was found that this change in one nucleotide added a different codon for the same amino acid.

Discussion

Nisin is produced by certain strains of *L. lactis*. The success of the practical application of nisin as a biopreservative would rely on overcoming the problems associated with its low level of production and, thereby, would result in its high cost. A number of attempts were made to produce high levels of bacteriocin using different cumbersome processes. These involved either the addition of alkali to culture following cessation of growth, extraction of lactate, or removal of lactate by microfiltration or through assimilation of lactate by the yeast *Kluyveromyces marxianus* [27, 28]. Readjustment of culture pH was found to extend the growth period and, thereby, enhance the yield of bacteriocin, as has also been found in the present investigation. However, the strain *L. lactis* CM1 isolated from Dahi in the

present investigation yields appreciably high bacteriocin activity in a medium with an initial pH of 11.0. This is in contrast to the strain *L. lactis* ATCC 11454, which attained its highest growth and bacteriocin production in a medium with an initial pH ranging from 8 to 9 (Fig. 2). However, the strain failed to grow and thereby to produce bacteriocin in medium with an initial pH of 10.0, implying that the growth of an organism at a high pH is strain-specific. Cheigh et al. reported that the strain *L. lactis* subsp. *lactis* A164 isolated from kimchi produced a nisin-like bacteriocin optimally at pH 6.0 [29]. In TGE medium with an initial pH 6.5, the bacteriocin yield of the strain CM1 was improved when alkali was added to the culture following cessation of growth and bacteriocin production. Nevertheless, the yield was compared to be lower than in cultures started with an initial pH of 11.0 with maltose as the carbon source. We previously reported that *L. lactis* strain W8 could also grow and produce bacteriocin in a medium with an initial pH of 11.0 [30]. The strain W8 was distinguishable from the strain CM1 based on 16S rDNA sequences, biochemical properties, antibacterial activities, and the stability of the bacteriocins. Unlike the strain W8, the strain CM1 utilizes L-arabinose but not gluconate and is VP-negative. However, both strains W8 and CM1 exhibit similar growth patterns on lactose, but the β-galactosidase activity of W8 is almost 10-fold that of CM1 (unpublished observation). No other LAB so far is reported to grow and produce bacteriocin at pH 11.0.

A number of studies have shown that nisin biosynthesis is related to carbon metabolism [31]. Sucrose has been used in the growth media to obtain a maximum stimulating effect on nisin production by *L. lactis* ATCC 11454 [32, 33]. In the present study, we found that the strain 11454 produced the highest level of nisin (3,000 AU/ml) using galactose and trehalose, along with sucrose as carbon source in the medium with a pH of 6.5. The bacteriocin activity of 3,000 AU/ml was also produced by the strain CM1 using maltose as the carbon source, as compared to 2,000, 2,800, and 2,400 AU/ml with galactose, trehalose, and sucrose, respectively, as carbon source. Thus, it appears that nisin biosynthesis and carbon source metabolism of the strain CM1 could be different from that of the strain *L. lactis* ATCC 11454.

The stability of bacteriocin is an important factor for isolation and application for food preservation. The level of bacteriocin produced by the strain CM1 at the end of log phase remained essentially stable, whereas bacteriocin from most producer strains significantly loose their activity due to either protein degradation, adsorption to cell surface, protein aggregation, or complex formation [34, 35].

Nizin Z produced by the strain *L. lactis* CM1 shows greater pH tolerance and thermostability than those reported for other bacteriocins, including nisin of *L. lactis* [36]. Nisin is unstable and inactivated at high pH [37]. The antibacterial spectrum of the bacteriocin, nisin Z, produced by the strain *L. lactis* CM1 is a wide one and is similar to that of nisin. However, *P. acidilactici* F^+ resistant to nisin of *L. lactis* ATCC 11454 was sensitive to bacteriocin of the strain CM1. *Geobacillus thermoleovorans* and *P. putida* were resistant to the bacteriocin of CM1 but were sensitive to that of the strain W8 [30]. It was reported that both nisin A and nisin Z retained their antibacterial activity after treatment with tyrpsin [38, 39], whereas nisin Z of CM1 was sensitive to trypsin. The data indicate that the bacteriocin produced by CM1 is similar but not identical to any previously reported nisin. Park et al. [40] also reported a difference in proteolytic sensitivity of nisin Z of *L. lactis* subsp. *lactis* K231 that was also unstable to proteolytic enzymes in contrast to previous observations [38].

The bacteriocin produced by the strain *L. lactis* CM1 did not inhibit the nisin-producing strain *L. lactis* 11454 or vice versa. Because bacteriocin-producing organisms have self-immunity against their own bacteriocin or similar ones produced by others, it is concluded

that bacteriocin of CM1 and ATCC 11454, despite other differences, are recognized as similar for self-immunity. Because the bacteriocin of CM1 has greater pH stability at high temperature and has a wide antibacterial spectrum, the opportunity exists to examine its potentiality for its application in foods as a natural preservative. *Lactococcus lactis* is considered as a GRAS microorganism, and thus, has a potential to be used as a nisin-producing safe starter.

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