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Isolation of Bacteriocin Producing *Lactococcus lactis* Strain from Dairy Products

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Abstract: Nisin is a bacteriocin produced by *Lactococcus lactis* strains and has been used internationally as food preservative. In this study bacteriocin producing bacteria were isolated from dairy products and milk by MRS agar. To identify the bacteriocin biochemical tests were carried out. Also the effect of pH, autoclaving temperature and proteases such as Pepsin and Proteinase K were studied. The results showed that the newly isolated bacteriocin has different stability in acidic pH in comparison to Nisin. Unlike Nisin it loses its activity in acidic pH. The molecular weight of isolated bacteriocin was determined by SDS-PAGE. It had the same molecular weight as Nisin. Also the inhibitory effects of new bacteriocin and Nisin on SDS-PAGE were compared. It had similar inhibitory effect as Nisin. For further identification, PCR was carried out with Nisin specific primers and control primers to 16S rRNA gene. Although, positive result was obtained for the control gene, no result was obtained with specific primer. The inhibitory effect of isolated bacteriocin on *Bacillus cereus* and *Listeria monocytogenes* was also studied. The growth of both strains was inhibited by the bacteriocin and more inhibition was observed on *Listeria monocytogenes* than *Bacillus cereus*. Finally, to optimize the production of bacteriocin the effect of pH and temperature were studied. The optimum conditions for bacteriocin producing bacterium were pH 6 and 37°C.

Key words: Bacteriocin, nisin, *Lactococcus lactis*, *Bacillus cereus*, *Listeria monocytogenes*

Introduction

Lactic acid bacteria (LAB) are a group of catalase negative gram-positive bacteria which have been widely used in food fermentation including dairy, meat, vegetable and bakery products (Marrug, 1991; Breit and Feleming, 1997). They are known to produce many different antibacterial substances including bacteriocins (Scott *et al.*, 1997; Olasupo *et al.*, 1999). Bacteriocins are antimicrobial protein compounds that are inhibitory towards several undesirable Gram-positive bacteria (Olasupo *et al.*, 1999; Nes *et al.*, 1996; Ko and Ahn, 2000; Ayad *et al.*, 2002). Nisin is a bacteriocin produced by *Lactococcus lactis* and has been used as a food preservative because of its lethal action and wide spectrum of activity (Olasupo *et al.*, 1999). Two Nisin variants, which differ only by one amino acid substitution at position 27, have also been isolated. Nisin A contains histidine and Nisin Z asparagine (Olasupo *et al.*, 1999; Noonpakdee, 2003). Usually, Nisin producer strains are isolated from dairy products, some from vegetables and very few from fermented meat. The use of bacteriocin-producing strains as starter cultures or protective culture's in the *in situ* control of food pathogens is

on of the possible ways to improve food safety (Olasupo *et al.*, 1999; Ko and Ahn, 2000; Noonpakdee *et al.*, 2003). The present study reports the detection and isolation of Nisin like producing *L. lactis* from dairy products.

Materials and Methods

All LAB isolates and indicator strains (*Lactobacillus delbrukii* PTCC1333) were grown in MRS broth (Merck) at 30°C. *Listeria monocytogenes* PTCC1164 and *Bacillus cereus* PTCC1015 were grown in Brain Heart infusion broth at 30°C.

Isolation of Organisms

Diary samples were collected from dairy producing centers in the city of Isfahan. Lactic Acid Bacteria (LAB) were isolated from dairy products as follow; sample of 10 g or 10 mL were homogenized with 90 mL of 0.85% sterile physiological saline and serially diluted in same diluent. The LABs were selectively isolated on MRS agar plates incubated anaerobically at 30°C for 3 days. The LABs were screened for antagonistic potential by agar spot test method described by Uhlman *et al.* (1992) against *Lactobacillus delbrukii* PTCC1333.

Bacteriocin Screening Assay

Isolates with antagonistic activity were selected for further identification and experimentation. The isolates were inoculated into MRS broth media and incubated at 30°C for 24 h. Culture supernatants were obtained by centrifugation at 13,000 rpm for 10 min then adjusted to pH 6.5 with 5 M NaOH and filtered through 0.45 µm filter. Inhibition was tested by spotting 20 µL of the supernatant onto soft agar lawn (0.6%) seeded with 10⁶ cfu mL⁻¹ of *Lactobacillus delbrukii* and incubated overnight. Cultures producing inhibitors were then purified by streaking from the broth and restreaking for single colony isolates. For a semiquantitative assay of bacteriocins two fold serial dilution of supernatant were tested using *L. delbrukii* PTCC1333 as indicator strain. The activity was expressed in arbitrary units (AU mL⁻¹). One AU was defined as the reciprocal of the highest serial two fold dilution which did not show inhibition of the indicator strain mL⁻¹ (Olasupo *et al.*, 1999; Ko and Ahn, 2000; Noonpakdee *et al.*, 2003).

Identification of the Bacteriocin Isolate

Only one isolate designated P1 produced bacteriocin in broth media. Strain P1 was therefore further characterized and identified on basis of Gram stain, catalase reaction, oxidase test, carbohydrate fermentation, salt tolerance (4 and 6.5% NaCl), gas production from glucose, growth at different temperatures (10, 40 and 45°C), pH (3.9 and 9.6) and arginine hydrolysis (Olasupo *et al.*, 1999; Noonpakdee *et al.*, 2003; Uhlman *et al.*, 1992; Moschetti *et al.*, 2001; Schillinger and Luck, 1987).

Effect of Enzymes, Heat and Ph on Antimicrobial Activity

The effects of various enzymes on bacteriocin activity was determined by incubating 200 µL of filter-sterilized cell-free supernatant with 20 µL of 1 mg mL⁻¹ proteinase K (pH 7) or Pepsin (pH 3). After 2 h of incubation at 37°C enzyme activity was terminated by heating at 100°C for 5 min, untreated samples were used as control. The residual activity was assayed against indicator strain *L. delbrukii* PTCC1333. The sensitivity of bacteriocin to different pH values was estimated by adjusting the pH supernatant sample between 2 and 10 using 5 M NaOH or 5 M HCl. After 2 h of

incubation at room temperature the residual activity was assayed. Samples treated with proteinase k before pH adjustment was used as control to correct inhibition due to pH. To study the effect of heat on bacteriocin activity, the cell free supernatant samples were heated 121°C for 15 min (Olasupo *et al.*, 1999; Uhlman *et al.*, 1992; Buncic *et al.*, 1997; Schillinger *et al.*, 1993).

Growth Inhibition of Selected Pathogenic Organisms

To study the effect of antimicrobial compound on *Listeria monocytogenes* PTCC1164 and *Bacillus cereus* PTCC1015, 2 mL of culture supernat (pH 6) was added to 10 mL 10^6 cfu mL⁻¹ growing cell from *Listeria monocytogenes* or *Bacillus cereus* in BHI broth medium and the absorbance was determined at appropriate intervals (Ivanova *et al.*, 2000).

Determination of Molecular Weight of Bacteriocin

The bacteriocin- producing strain was grown at 30°C for 24 h in MRS broth. Cells were then removed by centrifugation at 5,000 rpm for 30 min. Ammonium sulfate was added to achieve 75% saturation. After 24 h at 4°C the precipitate was collected. The precipitate was dissolved in sample buffer (15 mM Tris-HCl pH 6.8, 2.3% SDS, 10 mM 2-mercaptoethanol, 20% glycerol, 1% bromophenol blue) and was run on 20% polyacrylamide gel. After electrophoresis the gel was removed and cut in half, the half containing samples and molecular weight marker was stained with silver nitrate. The other half containing only samples was tested for antimicrobial activity. The gel was fixed immediately by treating 20% methanol and 10% acetic acid for 1 h then washed for 4 h in sterilized distilled water. The gel was then placed on to a petri dish and overlaid, with 7 mL of 0.6% agar containing 10^6 cfu mL⁻¹ of indicator strain. The plate incubated at 30°C for 24 h and analyzed for location of clear zone (Ko and Ahn, 2000).

PCR

Overnight MRS broth cultures of isolates were pelleted and suspended in sterile double-distilled water to a final concentration of 10^6 cell μ L⁻¹. The cells were lysed at 100°C for 10 min and then used directly as template in a 50 μ L PCR reaction in thermal cycler (master gradient cycler from ependorf originations). PCR Conditions consisted of 30 cycle of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min, with initial denaturation at 94°C for 5 min and final elongation at 72°C for 10 min. Nisin primer sequences used for PCR were as follow; NISF 5'-CGGCTCTGATTAAATTCTGAA-3'; NISR1. 5'-TTCTTACTAAAAACGGTTGAG-3'; NISR2 5'-GGAGTTGATCGTAAATAACTT-3'. Primers against 16s rRNA gene (Biomolecular of Germany) was used as positive control (Davis *et al.*, 1996).

Results

Fifty three LAB strains were isolated from milk and dairy products. When the isolates were subjected to bacteriocin assay, only one isolate was found to produce bacteriocin (Table 1). This bacteriocin producing strain was isolated from a traditional cheese and designated P1. The isolate produced inhibition zone against indicator strain *L. delbrukii* PTCC1333. Morphologically, the cells of strain P1 was coccid in shape and existed in short chains, the strain was gram-positive, catalase and oxidase-negative, non motile and did not produce gas from glucose. It hydrolyzed arginine, grew at 10-40°C, pH 3.9, 4% NaCl but did not grow at 45°C, pH 9.6 or in 6.5% NaCl. It produced lactic acid, fermented glucose, sucrose, lactose, ribose, melibiose, manitol, trehalose, surbitol, maltose, mannose, celobiose, raffinose and galactose but not arabinose, xylose and ramnose (data has not shown). Based

Table 1: Comparison of the effects of enzymes, heat and pH on the activity of bacteriocin produced by strain P1 and commercial Nisin

Treatments	Nisin activity (AU mL ⁻¹)	Bacteriocin activity (AU mL ⁻¹)
Control	25000	1600
Proteinase K	0	0
Pepsin	25000	1600
pH		
2	25000	0
4	25000	200
6	6000	1600
8	6000	0
10	3000	0
Autoclave temperature	6000	800

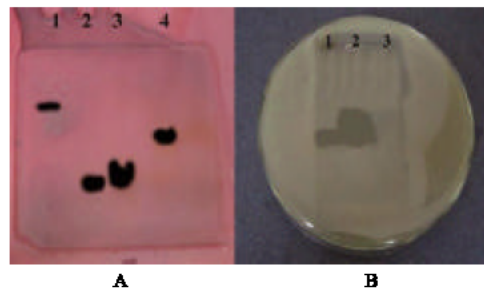


Fig. 1: Polyacrylamide gel (20%) electrophoresis of the bacteriocin. (A) Silver stained polyacrylamide gel (20%), Lane 1: Trypsin marker (13 kD); Lane 2: New bacteriocin; Lane 3: Commercial Nisin; Lane 4: *Lb delbrukii*. (B) Antimicrobial activity of bacteriocins separated by Polyacrylamide gel. After fixing and washing, the gel overlaid with indicator strain *Lactobacillus delbrukii* PTCC1333. Lane 1: New bacteriocin; Lane 2: Commercial Nisin; Lane 3: *L. delbrukii* PTCC1333

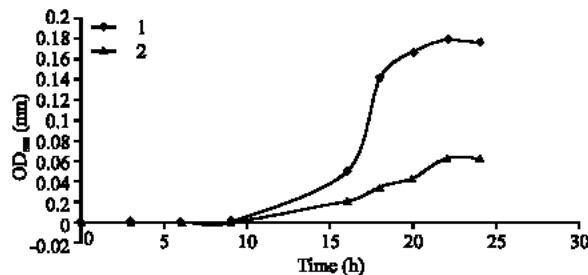


Fig. 2: The effect of bacteriocin *L. lactis* on *Bacillus cereus* in BHI media. (1) Control without bacteriocin, (2) Bacteriocin produced by strain P1

on these characteristics, the isolate was classified as *Lactococcus lactis*. Unlike Nisin which is active in a wide range of pH (pH 2-10), the new bacteriocin is only active at around pH 6. It completely was inactivated by the proteolytic enzymes proteinase k, but not by pepsin, suggesting the bacteriocin produced by P1 strain is proteinaceous in nature. The new bacteriocin showed remarkable heat stability at autoclaving temperature (Table 1). The apparent molecular weight of bacteriocin was determined by SDS-PAGE (Fig. 1). In silver stained SDS-PAGE the bacteriocin migrates at the same

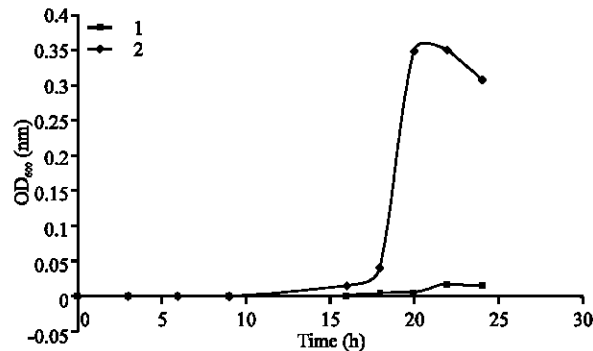


Fig. 3: The effect of bacteriocin *L. lactis* on *Listeria monocytogenes* in BHI media. (1) Control without bacteriocin, (2) Bacteriocin produced by strain P1

position as commercial nisin (MW = 3 kD). When another half of SDS-PAGE after removal of SDS was used for antimicrobial inhibition test clear zones were observed for both nisin and new bacteriocin at the same position. These results indicate that the new bacteriocin has the same molecular weight as nisin. In an attempt to determine whether the bacteriocin produced by strain P1 was nisin or not, PCR analysis using specific primers for nisin gene and 16s rRNA gene primers for control was used. The DNA amplification of *L. lactis* P1 yielded the expected DNA fragment for the control (16s rRNA gene) but no DNA product observed for the nisin primers, (data not shown), although we tried hard by using different PCR conditions.

The antimicrobial spectrum of the bacteriocin against *Bacillus cereus* and *Listeria monocytogenes* was also studied (Fig. 2 and 3). The new bacteriocin produced by strain p1 inhibited the growth of both *Listeria monocytogenes* and *Bacillus cereus* with higher activity towards *Listeria monocytogenes* (Fig. 2 and 3).

Discussion

In this study, a bacteriocin producing *L. lactis* (P1), was identified from traditionally fermented dairy product from Iran. Isolation and identification of a strain of *L. lactis* from cheese, is consistent with previous reports associating the occurrence of the organism with dairy products (Olasupo *et al.*, 1999; Ko and Ahn, 2000; Schillinger and Luck, 1987). The new bacteriocin from strain P1 had similar enzymatic and heat sensitivity pattern to nisin, but it had different stability in acidic pH in comparison to Nisin. Unlike Nisin which is active in a wide range of pH and with highest activity at pH 2-4, the new bacteriocin is only active at narrow pH (pH 6). Also the bacteriocin from strain P1 had similar molecular weight and inhibitory effect on SDS-PAGE to nisin (Ko and Ahn, 2000). For further identification, PCR was carried out with Nisin specific primers although positive result was obtained for the control (primers against 16s rRNA), no result was obtained with specific primers. As with Nisin the growth of strains *L. monocytogenes* and *B. cereus* were inhibited by the new bacteriocin and more inhibition was observed on *Listria monocytogenes* than *Bacillus cereus*. Finally, to optimize the production of bacteriocin the effect of pH and temperature was studied. The optimum conditions for bacteriocin producing bacterium were pH 6 and 37°C. The new bacteriocin probably could be used in liquid food as preservative or as starter culture in fermentation to inhibit the growth of *Listeria monocytogenes* and *Bacillus cereus* to reduce food poisoning for fermented foods.

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