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Purification and Characterization of Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Cow Milk

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Abstract: Bacteriocin producing *Lactobacillus plantarum* strain isolated from raw cow's milk samples, showed broad range of antibacterial activity against food borne pathogens. The bacteriocin was purified in two step procedure involving ammonium sulfate precipitation and gel filtration (Sephadex G-100 column). The molecular weight of the purified bacteriocin was found to be 9.5 kDa by SDS-PAGE. It showed high thermal stability (up to 121°C) and was active over wide range of pH (3 to 11) and complete inactivation in the presence of trypsin enzyme. The study revealed the possibility of using bacteriocin as a food preservative and the *L. plantarum* as probiotic.

Key words: Bacteriocin · *Lactobacillus plantarum* · Antimicrobial Activity

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in the food industry as starter culture for fermentation. *Lactobacilli* have been used since decades against infectious diseases [1] and have been extensively studied for their ability to protect against pathogens. These organisms have been widely used as probiotics [2, 3]. Many of these lactic acid bacteria are known to produce antibacterial substances including bacteriocins which can inhibit the growth of several pathogenic bacteria. Bacteriocins from lactic acid bacteria are natural antimicrobial peptides or small proteins with bactericidal or bacteriostatic activity against genetically closely related species [4]. Bacteriocins can be classified broadly as those synthesized by Gram-positive and those by Gram-negative organisms. Among those synthesized by Gram-positive organisms, *Lactobacilli* bacteriocins are of commercial value [5].

Lactobacillus bacteriocins are grouped as class-I bacteriocins (lantibiotics), class-II bacteriocins (heat-stable, non-lantibiotics), class-III (heat-labile proteins with large molecular mass) and class-IV (hydrophobic and heat-stable proteins, associated with lipids or carbohydrates). The lantibiotics in general have wider spectrum of activity than the non-lantibiotics. Nisin produced by *Lactobacillus lactis* is the best-studied and the only lantibiotic approved in more than 60 countries including USA to be used in food industry [6, 7].

Lactobacillus plantarum has been isolated from various habitats and several bacteriocins (antimicrobial peptides) have been described in strains from milk [8, 9], cheese [10], sorghum beer [11], barley beer [12] and fermented cucumber [13].

Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized [14-18]. Because of the increasing demand for more natural and microbiologically safe food products, there is a need for bio preservation methods. Bacteriocins have considerable potential for food preservation, as well as for human therapy as potential supplements or replacements for currently used antibiotics [19, 20]. Therefore, in this paper we reported on the antimicrobial properties of bacteriocin produced from *Lactobacillus plantarum*, isolated from raw cow's milk which has been purified and characterized.

MATERIALS AND METHODS

Isolation of Bacteriocin Producing Bacteria: Raw milk (unpasteurized) samples of cow were collected from the local dairy farms of Hyderabad, India. The samples were collected in a sterile screw cap tubes and serially diluted

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($10^{-1} - 10^{-6}$) in sterile distilled water. The diluted samples were plated (0.1 ml suspension) onto de Man Rogosa Sharpe (MRS) agar [21] plates and incubated at 37°C for 48 h. In a total of 13 different colonies initially observed, screening of bacteriocin producing isolates was done by well-diffusion method [22] against the indicator bacteria *i.e*., *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Enterococcus fecalis*. Only 3 isolates showed antibacterial activity against tested indicators and one of the best strain was selected and subjected to morphological and biochemical characteristics as described by Michael [23]. The strain was sub cultured onto MRS agar slants which were incubated at 37°C for 24 h and preserved in 20% glycerol at -20°C.

Production of Bacteriocin: The isolated strain was propagated in MRS broth (1000 ml) seeded with 10% inoculum (10^8 CFU/ml) of overnight culture and incubated for 48 h at 150 rpm at 37°C. After incubation, the whole broth was centrifuged at 10,000×g for 15 min and the cell-free supernatant was used as crude bacteriocin [20].

Purification of Bacteriocin: The cell-free culture supernatant (crude bacteriocin) was saturated with 70% ammonium sulfate and stored at 4°C to precipitate out the proteins. The pellet was collected after centrifugation at 10,000×g at 4°C for 30 min. The pellet was dissolved in phosphate buffer (0.1M, pH 7.0) and dialyzed against the same buffer at 4°C overnight [20]. The dialyzed protein was applied to a Sephadex G-100 column (1.6 × 36 cm) pre-equilibrated with phosphate buffer (pH 7.0). The flow rate was adjusted to 24 ml/h and fractions (1 ml each) were collected. The fractions showing high bacteriocin activity were pooled and concentrated in a lyophilizer.

Bacteriocin Assay: The antibacterial activity of the bacteriocin isolated from *L. plantarum* was determined using the well diffusion method as described by Ivanova *et al*. [24]. 50 µl of the bacteriocin were placed in 5-mm diameter wells that had been cut in agar plates previously seeded with the indicator bacteria. The plates were incubated at 37°C for 24 h. After incubation, the diameter of zone of growth inhibition was measured. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition [24].

Determination of Protein: Protein concentration of the bacteriocin in supernatant was determined by the method of Lowry *et al*. [25], using bovine serum albumin as the standard.

Molecular Weight Determination: The molecular weight of the purified bacteriocin was determined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [26]. After electrophoresis, the gel was stained with Commassie Brilliant Blue R-250 and destained by washing overnight with mixture of acetic acid-methyl alcohol-water (5:5:1 v/v). The low range molecular weight marker was used as standard marker (Genei, India).

Characterization of Bacteriocin

Effect of pH: To determine effect of pH, 0.5 ml of purified bacteriocin was added into 4.5 ml of nutrient broth at different pH values (3 to 11) and incubated for 30 min at 37°C. Each of the bacteriocin samples treated at different pH values was assayed against indicator bacteria by well diffusion method [27, 28].

Effect of Temperature: Purified bacteriocin (0.5 ml) was added into 4.5 ml of nutrient broth in the test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation and then heated at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100°C) for 10 min. The preparations containing nutrient broth (4.5 ml) and bacteriocin (0.5 ml) in test tubes were plugged with non-absorbent cotton and covered with aluminum foil and kept in an autoclave at 121°C or 15 lbs pressure for 10 min to check its activity at very high autoclaving temperature. The bacteriocin activity of above different heat-treated was measured by well diffusion method [29].

Effect of Proteolytic Enzyme: Effect of proteolytic enzyme trypsin on the activity of purified bacteriocin was studied as described by Paik *et al*. [30], where enzyme control 1 (C1) contained 0.3 ml of phosphate buffer (0.5M, pH 7.0), enzyme control 2 (C2) contained 0.15 ml of bacteriocin and 0.15 ml of phosphate buffer (0.5M, pH 7.0), while for the enzyme reaction (ER) 0.15 ml of trypsin (0.25 mg/ml) and 0.15 ml of purified bacteriocin were used. The effect of the proteolytic enzyme-trypsin on bacteriocin activity was studied by well diffusion method using above three preparations against the indicator bacteria. Reduction in zone size clearly indicated inactivation of bacteriocin (protein) due to the proteolytic enzyme [30].

RESULTS AND DISCUSSION

The bacteriocin producing strain was isolated from cow milk samples and the selected strain was identified as *Lactobacillus plantarum*.

Table 1: Summary of the purification profile for bacteriocin from *L. plantarum*

Purification steps	Volume (ml)	Total activity (AU/ml)	Total protein (mg)	Specific activity (AU/mg)	Purification fold	Recovery (%)
Culture filtrate	100	6.5×10^8	213.2	27.3	1.0	100.0
Ammonium sulfate	20	5×10^7	25.3	155.7	5.3	63.1
Sephadex G-100	10	4×10^7	1.5	1023.1	13.5	21.3

Table 2: Inhibition of various indicator bacteria by bacteriocin produced by *L. plantarum*

Test organisms	Zone of inhibition (mm)
Enterococcus fecalis	17
Listeria monocytogenes	11
Staphylococcus aureus	21
Escherichia coli	18

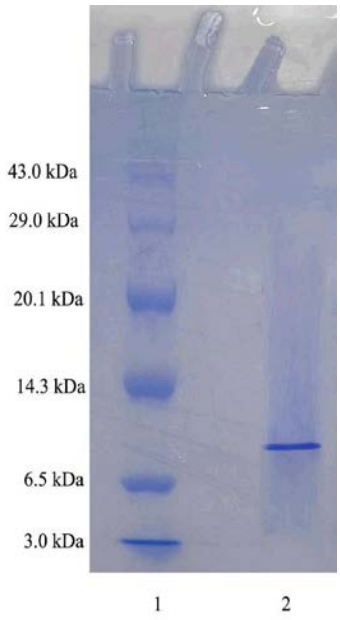

Fig. 1: SDS-PAGE of purified bacteriocin. Lane 1: Molecular weight marker; Lane 2: Purified bacteriocin

Purification of Bacteriocin: The results of the purification procedure were summarized in table 1. After final purification step, the bacteriocin was purified 13.5-fold with a recovery of 21.3%. The purified bacteriocin appeared as a single band in SDS-PAGE with molecular weight approximately 9.5 kDa (Fig. 1). Similarly Todorov *et al*. [12] reported the molecular weight of the bacteriocin from *L. plantarum* ST13BR as 10 kDa. The bacteriocins of lactic acid bacteria belonging to class-I and II have molecular weight (<5 kDa) and (<10 kDa) respectively e.g., *Pediococcus acidolactici* (3.5 kDa), *L. cin* C-TA33a (4.6 kDa) and *L. curvatus* SB13 (10 kDa) [31-33]. The lower molecular mass of bacteriocin of *L. plantarum* (9.5 kDa) suggested that it might belong to class-II bacteriocin group.

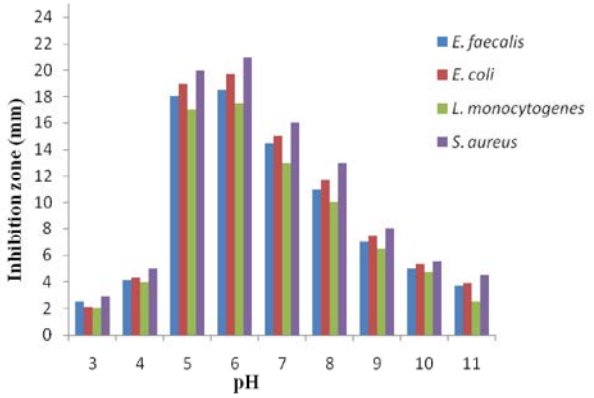

Fig 2: Effect of pH on the activity of the purified bacteriocin

The susceptibilities of various Gram-positive and Gram-negative bacteria to growth inhibition by the bacteriocin of *L. plantarum* were presented in table 2. It shows antibacterial activity against *Staphylococcus aureus*, *Enterococcus fecalis*, *Escherichia coli* and *Listeria monocytogenes*. Among these, highest growth inhibition was recorded against *S. aureus* and minimum activity was observed against *L. monocytogenes*. Similarly, bacteriocin from *L. plantarum* was found to be active against pathogenic bacteria including *Clostridium sporogenes, Enterococcus fecalis, E. coli* and *S. aureus* [11, 34, 35]. These results indicated that the presence of bacteriocin in lactic acid bacteria is responsible for their antimicrobial activity.

Effect of pH: Bacteriocin was active in a wide range of pH, but the maximum activity was observed at pH 5.0 and 6.0 (Fig. 2). Bacteriocin could retain its antimicrobial activity partially when there was a shift to acidic or basic range. Stability of bacteriocin at different pH scale is a limiting factor for recommending its use in food items. Bacteriocins produced by *L. plantarum* and *L. brevis* OGI retained their antimicrobial activity in an acidic pH range of 2.0 to 6.0, while inactivation occurred at pH 8.0 to 12.0 [22].

Effect of Temperature: Figure 3 shows the effect of temperature on bacteriocin activity in terms of inhibition zones. It has been found to be thermostable in nature as

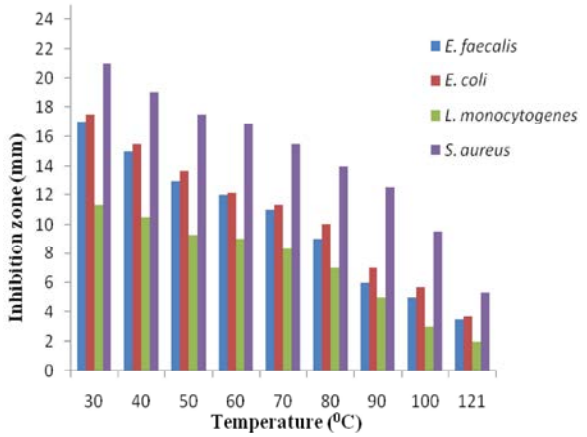

Fig. 3: Effect of temperature on the activity of the purified bacteriocin

it can withstand high temperature up to 121°C, although a partial loss in the activity was observed with a continuous increase in temperature. Thermo stability of bacteriocin at high temperature makes it possible to sterilize the food products even at room temperature, thus avoiding their storage at low temperature. Earlier studies revealed that bacteriocins produced by *L. paracaseii, L. lactis, L. plantarum* and *L. pentosus* remained active after heating till 121°C for 20 min [32].

Effect of Proteolytic Enzyme: The activity of bacteriocin decreased in presence of trypsin enzyme at its tested dose of 0.25 mg/ml. When purified bacteriocin was treated with trypsin in the ratio of 1:1 and welled into Petri dishes containing indicator bacteria, there was either minimum or no inhibition zone formation in the agar plates, indicating zero activity of bacteriocin. This proves that bacteriocin is basically a protein in the nature and therefore it can be broken down by gastric juices, thus making it completely safe for human consumption.

In conclusion, the bacteriocin was purified from *L. plantarum*, isolated from cow milk samples. The strong antagonistic effect of bacteriocin against the food borne pathogens indicated its usefulness in the preservation of different food products enhancing their shelf life. Its high heat stability, wider pH tolerance and sensitivity to trypsin enzyme suggested it as unique bacteriocin. It can be suggested that *L. plantarum* can be industrially exploited for the synthesis of antimicrobial peptides and strain improvement studies can be carried out to enhance bacteriocin production. Extensive toxicological and acceptability tests should be performed before the product is approved for large scale consumption.

REFERENCES

1. Bernet, M.F., D. Brassart, J.R. Meeser and A.L. Servin, 1994. Lactobacillus acidophilus LA1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell-invasion by enterovirulent bacteria. Gut., 35: 483-489.
2. Tannock, G.W., 1999. A fresh look at the intestinal microflora. In: Probiotics. A critical review. Ed., Tannock, G.W., Horizon Scientific Press, Norfolk, UK, pp: 5-14.
3. Sgouras, D., P. Maragkoudakis, K. Petraki, B. Martinez-Gonzalez, E. Eriotou, S. Michopoulos, G. Kalantzopoulos, E. Tsakalidou and A. Mentis, 2004. *In vitro* and *In vivo* inhibition of *Helicobacter pylori* and *Lactobacillus casei* strain Shirota. Appl. Environ. Microbiol., 70: 518-526.
4. Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. Biochem., 70: 337-349.
5. Garneau, S., N.I. Martin and J.C. Vederas, 2002. Two-peptide bacteriocins produced by lactic acid bacteria. Biochem., 84: 577-592.
6. Federal Register, 1998. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Federal Register, 54: 11247-11251.
7. Janes, M.E., R. Nannapaneni and M.G. Johnson, 1999. Identification and characterization of two bacteriocin-producing bacteria isolated from garlic and ginger root. J. Food Prot., 62: 899-904.
8. Rekhif, N., A. Atrih, M. Michel and G. Lefebvre, 1995. Activity of plantaricin SA6, a bacteriocin produced by *Lactobacillus plantarum* SA6 isolated from fermented sausages. J. Appl. Bacteriol., 78: 349-358.
9. Mohan Kumar, A. and N. Murugalatha, 2012. Isolation of *Lactobacillus plantarum* from cow milk and screening for the presence of sugar alcohol producing gene. J. Microbiol. Antimicrobials, 4(1): 16-22.
10. Gonzales, B., P. Arca, B. Mayo and J. Suarez, 1994. Detection, purification and partial characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin. Appl. Environ. Microbiol., 6: 2158-2163.
11. Van Reenen, C.A., L.M.T. Dicks and M.L. Chikindas, 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. J. Appl. Microbiol., 84: 1131-1137.

12. Todorov, S.D., C.A. Van Reenen and L.M.T. Dicks, 2004. Optimization of bacteriocin produced by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer. J. Gen. Appl. Microbiol., 50: 149-157.
13. Atrih, A., N. Rekhif, J.B. Milliere and G. Lefebvre, 1993. Detection and characterization of a bacteriocin produced by *Lactobacillus plantarum* C19. Can. J. Microbiol., 39: 1173-1179.
14. Daeschel, M.A., M.C. Mc Keney and L.C. Mc Donald, 1990. Bactericidal activity of *Lactobacillus plantarum* C-11. Food Microbiol., 7: 91-98.
15. Kato, T., T. Matsuda, E. Ogawa, H. Ogawa, H. Kato, U. Doi and R. Nakamura, 1994. Plantaricin-149, a bacteriocin produced by *Latobacillus plantarum* NRIC 149. J. Ferment. Bioeng., 77: 277-282.
16. Kelly, W.J., R.V. Asmundson and C.M. Huang, 1996. Characterization of plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*. J. Appl. Bacteriol., 81: 657-662.
17. Lash, B.W., T.H. Mysliwiec and H. Gourama, 2005. Detection and partial characterization of a broad-range bacteriocin produced by *Lactobacillus plantarum* ATCC 8014. Food Microbiol., 22: 199-204.
18. Ravi, V., M. Prabhu and D. Subramanyam, 2011. Isolation of bacteriocin producing bacteria from mango pulp and its antimicrobial activity. J. Microbiol. Biotech. Res., 1(2): 54-63.
19. Fricourt, B.V., S.F. Barefoot, R.F. Testin and S.S. Haysaka, 1994. Detection and activity of plantaricin F an antimicrobial substance from *Lactobacillus plantarum* BF001 isolated from processed channel catfish. J. Food Protection, 57: 698-702.
20. Ogunbanwo, S.T., A.I. Sanni and A.A. Onilude, 2003. Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and Lactobacillus brevis OGI. African J. Biotechnol., 2: 219-227.
21. De Man, J.C., M. Rogosa and M.E. Sharpe, 1960. A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol., 23: 130-135.
22. Bromberg, R., I. Moreno, C.L. Zaganini, R.R. Delboni and J.D. Oliveira, 2004. Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. Braz. J. Microbiol., 35: 137-144.
23. Michael, T., 1981. Family Lactobacillaecea. In: Star, P (eds). The prokaryotes. New York, Blackwell Press, USA, pp: 609-619.
24. Ivanova, I., P. Kabadjova, A. Pantev, S. Danova and X. Dousset, 2000. Detection, purification and partial characterization of a novel bacteriocin substance produced by *Lactococcus lactis* subsp. lactis b14 isolated from Boza-Bulgarian traditional cereal beverage. Biocatalysis, 41(6): 47-53.
25. Lowry, O.H., N.J. Rosenbrough, A.I. Farr and R.J. Randall, 1951. Protein measurement with the folin-phenol reagent. J. Biol. Chem., 193: 265-271.
26. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 277: 680-685.
27. Motta, A.S. and A. Brandelli, 2002. Characterization of an antibacterial peptide produced by *Brevibacterium lines*. J. Appl. Microbiol., 92: 63-70.
28. Sharma, N. and N. Gautam, 2007. Use of bacteriocin as potential bio-preservative in milk, cheese and apple juice. Bev. Food World, 34: 44-47.
29. Sharma, N., G. Kapoor and B. Neopaney, 2006. Characterization of a new bacteriocin produced from a novel isolated strain of *Bacillus lentus* NG121. Antonie Van Leeuwenhoek, 89: 337-343.
30. Paik, H.D., S.S. Bae, S.H. Park and J.G. Pan, 1997. Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp. tochigiensis. J. Ind. Microbiol. Biotechnol., 19: 294-298.
31. Jeevaratnam, K., M. Jamuna and A.S. Bawa, 2005. Biological preservation of foods-bacteriocins of lactic acid bacteria. Indian J. Biotechnol., 4: 446-454.
32. Papanthanasopoulos, M.A., F. Krier, A.M.R. Junelles, G. Lefebvre, J.P. Le Caer, A. Von Holy and J.W. Hastings, 1997. Multiple bacteriocin production by Leuconostoc mesenteroides TA33a and other Leuconostoc/Weissella strains. Current Microbiol., 35: 331-335.
33. Sudirman, I., F. Mathieu, M. Michel and G. Lafebvre, 1993. Detection and properties of curvaticin 13, a bacteriocin-like substance produced by *Lactobacillus curvatus* SB13. Curr. Microbiol., 27: 35-40.
34. Aly, E.A., 2007. Characterization of a bacteriocin-like inhibitory substance produced by *Lactobacillus plantarum* isolated from Egyptian home-made yogurt. Science Asia, 33: 313-319.
35. Atta, H.M., B.M. Refaat and A.A. El-Waseif, 2009. Application of biotechnology for production, purification and characterization of peptide antibiotic produced by probiotic *Lactobacillus plantarum* NRRL B-227. Global J. Biotechnol. Biochem., 4(2): 115-125.