

Optimized culture conditions for bacteriocin production by *Pediococcus acidilactici* LAB 5 and its characterization

Vivekananda Mandal^{1*}, Sukanta Kumar Sen² and Narayan Chandra Mandal^{#2}

¹P.G. Department of Botany, Darjeeling Govt. College, Darjeeling 734 101, India

²Microbiology Laboratory, Department of Botany, Visva-Bharati, Santiniketan 731 235, India

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A strain of *Pediococcus acidilactici* LAB 5 was isolated from vacuum-packed fermented meat product, in order to obtain a novel bacteriocin from food-grade organisms. Optimized culture conditions for bacteriocin production in different media (viz., MRS, TGE, TGE + buffer, TGE + Tween 80, and TGE + Tween 80 + buffer) and at different temperatures and pH conditions were reported. TGE + Tween 80 + buffer medium was found to be most effective for bacteriocin production (about 2,400 AU/ml) by this strain, when incubated at 37°C for 24 h. Bacteriocin, partially purified by adsorption-desorption method showed molecular mass of 10.3 kDa and produced prominent inhibition zone in activity gel. It showed significant storage stability both at high as well as in low temperatures for up to 6 months and retained its activity in a number of organic solvents, except in 2-mercaptoethanol. The treatment with amylase and lysozyme did not change its activity, but it lost its activity on proteinase K treatment. Antibacterial efficacy of bacteriocin was proved against some food spoilage and human pathogenic bacteria like *Enterococcus*, *Leuconostoc*, *Listeria*, *Staphylococcus* and *Streptococcus*.

Keywords: Antibacterial, Bacteriocin, Cystibiotics, *Pediococcus acidilactici*

Lactic acid bacteria (LAB) are a group of Gram-positive, catalase-negative bacteria that produce various types of compounds such as bacteriocin, organic acid, diacetyl, and hydrogen peroxide during lactic acid fermentation¹. Among these compounds, bacteriocins are the most effective as they can kill or inhibit bacteria closely related to the producer strain. Bacteriocins are proteinaceous compounds that are inhibitory towards sensitive bacterial strains of their closely related members and are produced by both Gram-positive and Gram-negative bacteria². Their efficacy has also been exploited in food preservation³⁻⁵.

Although most of the bacteriocins have a very narrow range of inhibitory spectrum, nisin^{6,7} from *Lactococcus lactis* subsp. *lactis* and pediocin PA-1⁸ from *Pediococcus acidilactici* PAC 1.0 have a very wide range of inhibitory spectrum. *Pediococcus* is a mesophilic, microaerophilic bacterium that produces acids from glucose, galactose, fructose etc. Pediocin AcH produced by *P. acidilactici* H has extensively been studied^{9,10} for development of a potential food preservative.

In order to isolate a potent bacteriocin producing strain for possible use as food preservative, in the present study, we report optimum conditions for bacteriocin production by our laboratory isolate *P. acidilactici* LAB 5 from vacuum-packed fermented meat product. Its partial characterization with respect to pH, temperature and organic solvents stability, and enzyme sensitivity is also reported.

Materials and Methods

Bacterial strains and media

A strain of *Pediococcus acidilactici* LAB 5, confirmed by morphological, biochemical and 16 Sr DNA studies, was isolated from vacuum-packed fermented meat product (Ahrambagh chicken) and screened for bacteriocin production against two bacteriocin sensitive indicator strains *Enterococcus faecalis* MB1 and *Leuconostoc mesenteroides* Ly¹¹. Identification of the producer strain was done based on the cell morphology and carbohydrate fermentation profile by API CH 50 test and 16 Sr DNA sequencing.

Bacteriocin producer *P. acidilactici* LAB 5 and indicator LAB strains were cultured in *Lactobacillus* MRS medium (Hi Media) and maintained in 10% glycerol-skimmed milk at -4°C. Other non-lactic pathogenic bacteria were maintained as per MTCC guidelines.

*Author for correspondence:

E-mail: mandal_vivek@yahoo.co.in

Ph: 091-354-2254316 (O)

091-9434556340 (R)

Activity detection and assay

Antimicrobial substance (bacteriocin) produced by the strain was detected by deferred method¹¹ and confirmed by spot-on-lawn method¹¹. For deferred method, overnight culture of the producer strain was serially diluted and spreaded on to the surface of MRS agar plates and incubated for 24 h at 28°C to allow the colonies to appear and then overlaid with 5 ml of melted MRS soft agar (0.7%), seeded with overnight (O/N) grown 6×10^7 CFU/ml of *E. faecalis* MB1. The plates were incubated at 28°C for 24 h and observed for clear zone of inhibition. For spot-on-lawn method, pre-poured MRS agar plates were overlaid with 5 ml melted soft agar (0.7%), inoculated with 50 µl of O/N grown 6×10^7 CFU/ml of indicator strain. 5 µl of both boiled and un-boiled cell-free aliquot of the producer strain was spotted on the lawn and incubated at 28°C for 24 h. The amount of bacteriocin production was calculated as arbitrary activity units. One arbitrary activity unit (AU) was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain¹².

Optimization of media, pH and temperature for growth and bacteriocin production

The optimal growth conditions and media requirements was determined by growing the strain in different media like MRS, tryptone glucose yeast extract (TGE), TGE + Tween 80 (0.05%), TGE + buffer (containing sodium citrate, sodium acetate and dipotassium hydrogen phosphate, 0.2% each), TGE + Tween 80 + buffer broth adjusted at different pH (from pH 2.0-12.0) with 2 N HCl and 2 N NaOH and individual sets were incubated at 20°C, 28°C and 37°C. Growth was monitored as optical density (O.D.) at 620 nm and pH change and bacteriocin production were determined at every 3 h interval for 24 h and tested for both boiled and unboiled cell-free culture aliquot following spot-on-lawn method.

Partial purification of bacteriocin

Partial purification of bacteriocin was done following the adsorption-desorption method¹³. For purification, the producer strain was grown in TGE + Tween 80 + buffer medium (pH 6.8) at 37°C for 24 h in a rotary shaker at 100 rpm. The culture was heat-killed and then cooled to room temperature. The pH of culture aliquot was adjusted to 6.0-6.3 and shaken at 100 rpm at room temperature for 2 h to adsorb bacteriocin to the producer cells. Such cells adsorbed with bacteriocin molecules on to their surfaces were

harvested by centrifugation at 6,000 rpm for 15 min and washed with sterile 5 mM Na-P buffer (pH 6.5) and then centrifuged at 6,000 rpm for 15 min. The pellet obtained was resuspended in 100 mM NaCl (pH 1.5-2.0) for desorption. Cell-free supernatant was collected by centrifugation (at 8,000 rpm for 15 min) and dialyzed in 1,000 Da cut-off dialysis bag (Sigma) for 24 h against double-distilled water with frequent changes of water. The dialysate was then lyophilized.

Stability of bacteriocin to different pH, temperatures and organic solvents

To determine the sensitivity to different pH, temperatures and organic solvents, partially purified bacteriocin was used to perform the following experiments. The aliquots were exposed to different temperatures such as 100 and 121°C for 20 min and at -4°C for 6 months and residual activity was measured by using spot-on-lawn method through dilutions. For stability at different pH, the aliquots were adjusted to range of 2.0-12.0 pH using 2 N HCl and 2 N NaOH and incubated for 2 h. The residual activity was measured after neutralizing the aliquots to pH 6.0.

To investigate the stability in different organic solvents like acetone, acetonitrile, benzene, chloroform, dimethyl sulphoxide (DMSO), ethanol, ethyl acetate, isopropanol, methanol and 2-mercaptoethanol, 10% solution of each was prepared and to each 1 ml of 10% organic solvent and 1 ml of purified aliquot was added and mixed vigorously. Thereafter, 10 µl of each mixture was spotted on lawned indicator strain of *E. faecalis* MB1 and *L. mesenteroides* Ly in MRS agar plates for three different incubation periods viz., immediately after mixing and after 1 h and 4 h incubation at room temperature (~28°C). The purified aliquot (control 1) and 10% organic solutions (control 2) were used as positive and negative control, respectively. The residual activity was measured as the diameter of inhibition zone (mm) against the indicator strain.

Enzyme sensitivity of bacteriocin

Partially purified lyophilized bacteriocin (2,000 AU/ml) was mixed with enzymes (Genei) solutions of α -amylase (200 AU/ml), lysozyme (15,000 AU/ml) and proteinase K (200 AU/ml) and incubated for 2 h at 30-37°C and the residual activity was measured in same method as stated earlier.

Determination of MW and activity of bacteriocin in SDS-PAGE

The partially purified bacteriocin was detected through 18% separating gel and 5% stacking gel with

acrylamide and bis-acrylamide ratio 30:1 under denaturing condition. 20 µl of partially purified bacteriocin (2 mg/ml) was mixed in 4: 1 ratio with denaturing sample buffer and loaded in a well. Culture supernatant and the blank medium were loaded in separate wells. Electrophoresis was done at 20 mA for first 30 min and then next 2 h at 30 mA. Thereafter, the gel was removed and cut into two vertical halves. One part of the gel containing marker proteins was stained with Coomassie brilliant blue G-250 and de-stained to visualize the bands and the other part was tested for antimicrobial activity after washing repeatedly for 24 h in sterile double distilled water¹⁴. The gel was then placed on a pre-poured MRS plate and overlaid with 5 ml melted MRS soft agar (0.7%) seeded with 5×10^6 cells of sensitive indicator *E. faecalis* MB1. The plate was then incubated at 28°C for next 24 h and observed for zone of inhibition.

Results and Discussion

The species of *Pediococcus* isolated from vacuum-packed fermented meat product using MRS as selective medium was found to be Gram-positive and catalase-negative. The strain was identified by sugar utilization profile as per API CH-50 biochemical profiles (data not provided) and microscopic observations – paired cocci and non-spore forming and confirmed by partial 16S r DNA sequencing and phylogenetic analysis (Fig. 1). It showed 100% similarity with *P. acidilactici* through neighbour-joining method. So, the isolated producer organism was *P. acidilactici* and we designated the strain as LAB 5 and bacteriocin produced by the strain as pediocin NV 5. Earlier¹⁰, bacteriocin-producing lactic acid bacteria have also been isolated from similar types of fermented food products.

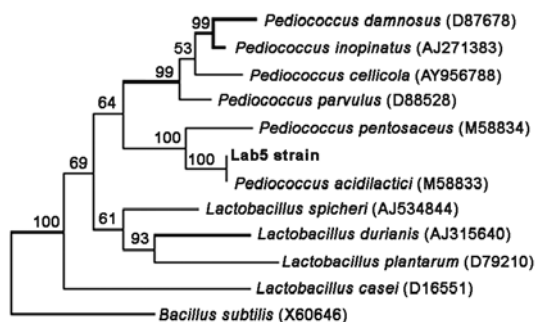


Fig. 1—Phylogenetic tree of producer strain LAB 5 with respect to other lactic acid bacteria as inferred by neighbour-joining method of partial 16S r DNA sequences [Number in parenthesis represents accession number of the strain]

The inhibitory activity of bacteriocin against other non-lactic acid bacteria is shown in Table 1. Some species of *Enterococcus*, *Leuconostoc* and *Listeria* are highly pathogenic to human beings. The isolated *P. acidilactici* LAB 5 and its bacteriocin product (pediocin NV 5) showed strong cidal activity against these species at very low concentration. The bacteriocin also effectively killed *Staphylococcus aureus*, a surface-infecting human pathogen. All these indicated a quite broad antibacterial spectrum of the bacteriocin.

Production and partial purification of bacteriocin

The optimal growth conditions and media requirements for bacteriocin production were found to be in TGE + Tween 80 + buffer (pH 6.8) medium, which supported maximum bacteriocin production (2,400 AU/ ml), when grown at 37°C for 24 h (Fig. 2A-C). Bacteriocin activity was detected at early exponential phase and maximized at stationary phase. Cell growth was also quickly maximized in this medium. Increased bacteriocin production in TGE + Tween 80 + buffer, compared to TGE + Tween 80 showed that TGE + Tween 80 + buffer contained some more buffering components (like sodium acetate, sodium citrate and dipotassium phosphate, each 0.2%) that controlled the ultimate pH after growth to pH 4.4, on contrary to the pH 3.8 in TGE+ Tween 80. As Tween 80 is a non-ionic detergent, it might help in releasing bacteriocin molecules from

Table 1—Antimicrobial spectrum of bacteriocin pediocin NV 5 produced by strain *Pediococcus acidilactici* LAB 5

Bacterial strains	Deferred method	Spot-on-lawn method
<i>Bacillus megaterium</i> 1684 [#]	Nil	Nil
<i>Bacillus subtilis</i>	Nil	Nil
<i>Enterococcus faecalis</i> MB 1*	+	+
<i>Escherichia coli</i>	Nil	Nil
<i>Leuconostoc mesenteroides</i> Ly*	+	+
<i>Listeria innocua</i>	+	+
<i>Listeria monocytogenes</i> 657	+	+
<i>Pentoea ananetis</i> 2307	Nil	Nil
<i>Pseudomonas aeruginosa</i>	Nil	Nil
<i>Pseudomonas flurocence</i>	Nil	Nil
<i>Salmonella typhimurium</i> 98	Nil	Nil
<i>Staphylococcus aureus</i> 96	+	+
<i>Streptococcus</i> sp.	+	+
<i>Xanthomonas campestris</i> 2286	Nil	Nil

+, Indicator strain inhibited by bacteriocin; nil, indicator strain not inhibited by bacteriocin; *, indicator strains were kindly provided by Prof. Bibek Ray, Wyoming University, USA; #, The number at end of strain name is MTCC code no. MTCC, microbial type culture collection, Chandigarh, India

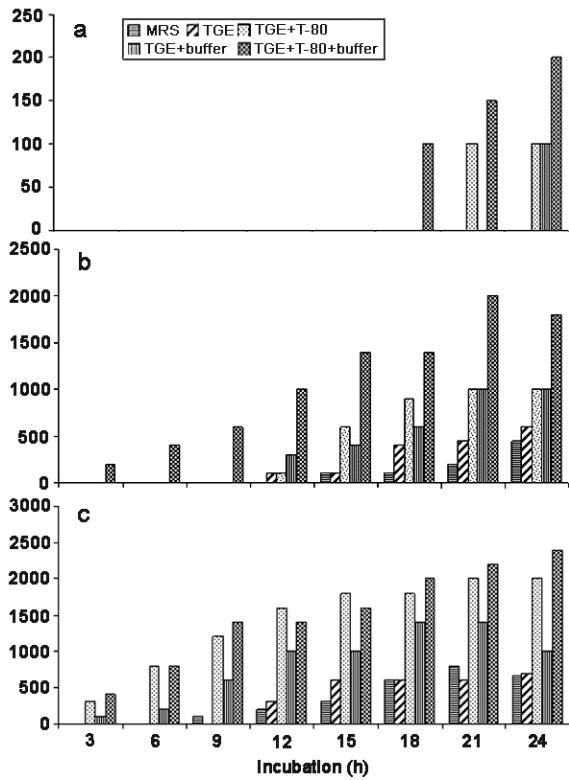


Fig. 2—Production of bacteriocin at different temperatures by *Pediococcus acidilactici* LAB 5 in different media [(A): at 20°C; (B): at 28°C; and (C): at 37°C]

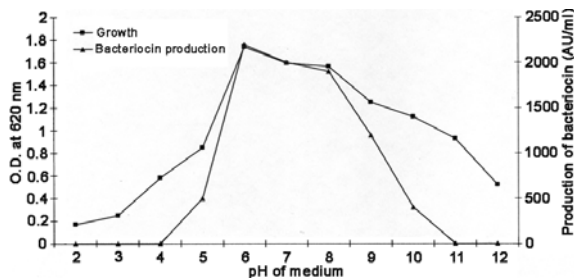


Fig. 3—Effect of pH on growth and production of bacteriocin in TGE + Tween 80 + buffer medium by *P. acidilactici* LAB 5 at 37°C

the producer cell wall into the medium¹⁵. This strain failed to produce bacteriocin significantly in MRS medium. The amount of free bacteriocin in the medium decreased with prolonged incubation period, due to self-proteolytic enzymes, synthesized in greater amount in the late stationary phase¹⁶. The pH suitability for better production of bacteriocin at different pH conditions of TGE + Tween 80 + buffer was in the range of pH 5.0-7.0, with optimum pH at 6.6 ± 0.2 (Fig. 3). The bacteriocin production by this strain showed primary metabolic kinetics, as evidenced by the higher bacteriocin production with increase of cell density in the logarithmic phase of growth curve. The partially purified product showed a significant increase in inhibitory activity, which was equivalent to 20,000 AU/ml, as compared to the initial culture aliquot activity of 2,400 AU/ml (Table 2).

Sensitivity of bacteriocin to pH, temperature, organic solvents and enzymes

The bacteriocin was stable to a wide range of acidic pH (2.0 to 8.0), but not in the high alkaline condition, due to alkali lysis. It was resistant to the high temperature treatments (like 100°C, 121°C for 20 min) and produced the same AU/ml as non-treated one. It retained its antimicrobial potency up to 6 months in cold storage (-4°C). It retained its activity in positive control, and in mixture with 10% organic solvents (acetone, acetonitrile, benzene, chloroform, DMSO, ethanol, ethyl acetate, isopropanol, and methanol) in fresh, 1 h and 4 h incubation, but not with 2-mercaptoethanol. Each of these 10% organic solvents (negative control) failed to produce any inhibition zone against the indicator strains. Retention of antimicrobial activity in organic solvents mixture and not in solvent solution suggested that inhibition was due to bacteriocin molecule and solvents had no effect on its structure. This finding suggested that bacteriocin molecule was the pure protein and not the conjugated lipoprotein. Failure to retain antibacterial activity in 2-mercapto ethanol indicated that bacteriocin

Table 2—Purification and recovery of bacteriocin by adsorption-desorption method (AU/ ml) [5 µl of each component was applied on the indicator overlay to check the activity]

Components at different stages of purification	Activity (inhibition zone produced)	AU/ml	Total AU
Untreated producer culture (Final vol. = 1 L)	Inhibition zone up to dilution 12	$12 \times 200 = 2400$	24×10^5
Heat-killed producer culture (Final vol. = 1 L)	Inhibition zone up to dilution 11	$11 \times 200 = 2200$	22×10^5
Discarded sup after centrifugation (pH 6.0) (Final vol. = 1 L)	Inhibition zone up to dilution 2	$1 \times 200 = 200$	2×10^5
Discarded cell pellets (pH 2.0)	Inhibition zone up to dilution 5	$5 \times 200 = 1000$	10×10^5
Dialyzed bacteriocin solution (Final vol. = 100 ml)	Inhibition zone up to dilution 100	$100 \times 200 = 20000$	2×10^7
Lyophilized bacteriocin solution (Final vol. = 2 ml)	Inhibition zone up to dilution 500	$500 \times 200 = 100000$	10×10^7

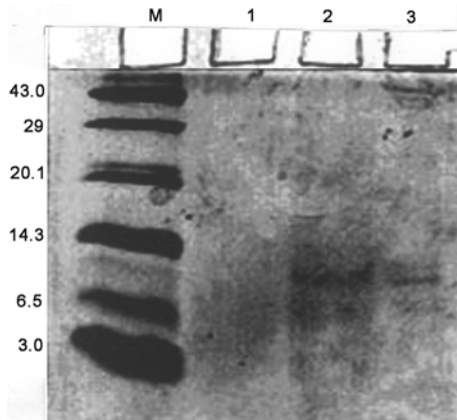


Fig. 4—18% Tricine SDS-PAGE of partially purified bacteriocin Pediocin NV 5 [Lane M, molecular wt. marker; lane 1, blank media; lane 2, culture aliquot of TGE + Tween 80 + buffer; and lane 3, lyophilized bacteriocin pediocin NV 5]

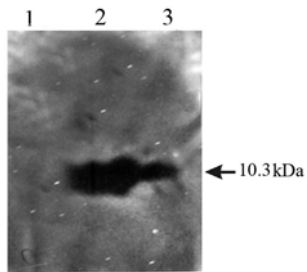


Fig. 5—Activity gel of pediocin NV 5 against *E. faecalis* Ly by overlay method [Lane 1, blank medium; lane 2, culture aliquot; and lane 3, lyophilized bacteriocin pediocin NV 5]

molecules might have cystine disulphide bridge(s), which maintains its native conformation with bacteriocin action¹⁷.

Enzyme assay with proteinase K showed loss of antimicrobial activity, but not with α -amylase and lysozyme, suggesting again that proteinaceous nature of bacteriocin molecule and not conjugated with carbohydrates. Thus, the above studies indicated that pediocin NV 5 was similar with other pediocin molecules like pediocin PA 1.0 or pediocin AcH and belonged to the cystibiotics class, but with different antimicrobial spectrum. The present investigation also indicated the novelty of bacteriocin in being tolerant to different organic solvents.

MW and activity of bacteriocin in SDS-PAGE

The estimated molecular mass of partially purified bacteriocin was found to be 10.3 kDa, as evidenced in Tricine-SDS PAGE (Fig. 4), which was quite high in comparison with bacteriocin like pediocin AcH (4.628 kDa)¹⁸ but much below than pediocin PA 1.0 (16.5 kDa)¹⁹. It produced prominent inhibition zone in

the activity gel against *E. faecalis* MB1 as shown in Fig. 5.

Conclusion

The present study demonstrated the production of pediocin NV 5 by *P. acidilactici* LAB 5 under different cultural conditions. The bacteriocin appeared to be a new candidate in pediocin family. Its antimicrobial potency, pH stability, activity retention in different organic solvents and high temperature stability suggested its wide applicability in acidic pH conditions and in solvent-rich environment and also in pre-processed fermented products. The strong antilisterial activity at 28°C, as well as at -4°C indicated that it could be used to control listerial growth in cold storage meat products.

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