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Purification and characterization of bacteriocin produced by strain of *Lactobacillus brevis* MTCC 7539

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Bacteriocin, an antimicrobial agent having potential for food biopreservation was purified from *Lactobacillus brevis* (a safe food-grade bacteria isolated from *Vari Kandal*, a traditional fermented food of Himachal Pradesh by adopting a novel repeated washing method. Its purity was confirmed by SDS-PAGE and Native-PAGE. The relative molecular mass of bacteriocin was 93.74 kD, while specific activity and recovery were 35.52 folds and 17.13%, respectively. It showed high thermal stability and was active over wide range of pH and exhibited sensitivity to trypsin.

Keywords: Bacteriocin, *Lactibacillus brevis*

Food safety is an important issue of international concern[1]. Prepacked food items available in food market contain variety of chemical preservatives[2], which may alter chemical constituents, nutritional and organoleptic qualities of food, thus may have serious adverse effects on health[3]. Thus, biopreservation of food has emerged as an attractive and safe approach. Among biopreservatives, bacteriocins have received increasing attention due to their unique properties of inhibiting food-borne pathogens and spoilage causing microorganisms. Bacteriocins produced by lactic acid bacteria have been evaluated for the preservation of milk, meat and vegetables due to their capacity to inhibit the growth of narrow range of pathogenic, spoilage causing bacteria[4].

In the present study, a bacteriocin produced from a food-grade bacteria *Lactobacillus brevis,* isolated from *Vari Kandal*, a traditional fermented food of Himachal Pradesh has been purified and characterized.

Materials and Methods

Isolation of bacteriocin producing strain

Bacteriocin-producing lactic acid bacteria were isolated from *Vari Kandal*, a traditional fermented locally available food item of Himachal Pradesh prepared by fermenting *Phaseolus radiates*. For isolation of bacteriocin, fermented material was soaked in 10 ml of distilled water at 27°C for 24 h and after crushing in sterilized pestle mortar was serially diluted. Isolation of bacteria was carried out by pour plate method on MRS agar at 37°C for 24 h[5]. In total, 128 different colonies were initially observed, following the dilution series method (10^{-2}-10^{-8}). Screening of bacteriocin producing isolates was done by Bit/disc method and well-diffusion method against the indicators[6] i.e., *Listeria monocytogenes*, *Staphylococcus aureus*, *Leuconostoc mesenteroides*, *E. coli* and *Enterococcus faecalis*. Only 7 isolates showed antibacterial activity against tested indicators, and one of the best strain was selected for further study based on its antagonistic activity against the serious food pathogens like *L. monocytogenes* and *S. aureus*. It was identified as *L .brevis* MTCC 7539 at Institute of Microbial Technology (IMTECH), Chandigarh, India.

Purification of bacteriocin

The culture broth was seeded with culture of *L. brevis* at 10% of inoculum having 10^8 CFU/ml (2.0 OD) and incubated for 72 h at 150 rpm at 37°C. Partial purification of the sample was done by salt saturation method[7]. The culture of *L. brevis* was saturated with 70% ammonium sulphate and stored at 4°C to precipitate out the proteins. After 24 h, centrifugation was done at 20,000 *g* at 4°C for 30 min and pellet was stored at –42°C[6]. The precipitates collected after centrifugation were insoluble in the tried buffer/solvents such as phosphate buffer (0.5 M, pH 7.0), Tris.HCl, methanol and Tween-80, while impurities present in the sample were found soluble in water. Due to insolubility and unstability of bacteriocin in different solvents, it was separated out from rest of the impurities (which were found soluble in water) by washing method. The detailed procedure followed was as follows:

The partially purified bacteriocin (1000 µl) was taken in eppendorf tube and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet containing bacteriocin was washed with water and centrifuged again at 10,000 rpm for 10 min

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at 4°C. The washing process, followed by centrifugation was repeated six-times consecutively and during washing, loss of activity of bacteriocin was checked at every step by well-diffusion method against sensitive indicators. No inhibition zone was formed against the indicators with the collected washing, thus indicating retention of activity only in the pellet. After final washing, sodium dodecyl sulphate (SDS) (0.6% w/v) was added in phosphate buffer, pH 7.0 to facilitate the solubility of purified bacteriocin in it. Activity of bacteriocin was confirmed in the sample by well-diffusion assay showing inhibition of 16 mm and 18 mm against *L. monocytogenes* and *S. aureus*, respectively.

SDS-PAGE and native-PAGE were run to check the purity of sample and also to determine molecular mass of the sample[8]. The activity units (AU) of crude (culture supernatant), partially purified and purified bacteriocins were estimated by critical dilution method[9]. These were calculated as reciprocal of the highest dilution showing definite inhibition of the indicator lawn and expressed as arbitrary units i.e., AU/ml[10,11]. The highest dilutions leading to smallest detectable zone of inhibition were 10^{-2}, 10^{-6} and 10^{-12} in culture supernatant, partially purified and purified bacteriocin respectively. Therefore, AU/ml were calculated as follows:

For culture supernatant: 0.3 µl = 200; 1 ml = 200/0.3; 1 ml = 200/3 × 1000 × 10 = 6.6×10^5 AU/ml; For partially purified bacteriocin: 0.3 µl = 600; 1 ml = 600/0.3; 1 ml = 600/3 × 1000 × 10 = 20×10^5 AU/ml; For purified bacteriocin: 0.3 µl =1200; 1ml = 1200/0.3; 1 ml = 600/3 × 1000 × 10 = 4×10^6 AU/ml.

Characterization of purified bacteriocin

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 (50, 60, 90, 100°C) each for 10 and 20 min, respectively. The preparation containing nutrient broth and bacteriocin (4.5 ml + 0.5 ml) in test tubes, plugged with cotton and covered with aluminum foil was kept in autoclave and treated at 121°C or 15 lbs pressure for 10 min and 20 min, respectively to check its activity at very high autoclaving temperature. Well-diffusion method was performed with above heat-treated bacteriocin to detect the inhibition zone[9].

To determine effect of pH, purified bacteriocin (0.5 ml) was added to 4.5 ml nutrient broth of different pH of (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) and incubated for 30 min at 37°C. Each pH treated bacteriocin was assayed by well diffusion method[12,13].

Effect of proteolytic enzyme trypsin on activity of purified bacteriocin was studied as described previously[14]. Three types of preparations were taken i.e., enzyme reaction (E_R): 0.15 ml of phosphate buffer (0.5 M, pH 7.0) + 0.15 ml of bacteriocin + 0.15 ml of trypsin (0.25 mg/ml); enzyme control (EC_1): 0.3 ml of phosphate buffer (0.5M, pH 7.0); and enzyme control II (EC_2): 0.15 ml phosphate buffer (0.5 M, pH 7.0) + 0.15 ml bacteriocin. The effect of proteolytic enzyme-trypsin on bacteriocin activity was studied by well-diffusion method using above three preparations against the indicators. Reduction in zone size clearly indicated inactivation of bacteriocin (protein) due to the proteolytic enzyme[14].

Results and Discussion

Isolation and screening of bacteriocin producing strain

The culture supernatant of the selected strain *L.brevis* MTCC 7539 showed antibacterial activity against serious food spoilage causing micro-organisms. Initially, *L. brevis* formed zone of inhibition of 10 mm against *S. aureus* and 2 mm against *L. monocytogenes* and activity units for crude supernatant were found to be 6.6×10^5 AU/ml. Due to hyperbacteriocin production and high activity units of *L. brevis*, it was further evaluated for production, purification and characterization.

Purification of bacteriocin

Partial purification of cell-free supernatant was done by 70% ammonium sulphate precipitation. The precipitates, so formed showed complete insolubility in water, phosphate buffer (pH 7.0) and Tris HCl (pH 7.0) but were soluble in methanol and lost their activity completely after 3 days. Thus, crude bacteriocin differed from other bacteriocin preparations reported so far, which are readily soluble in water and other buffers (phsosphate buffer, Tris.HCl, sodium acetate, etc.). After washing out the impurities, bacteriocin was found soluble in SDS (0.6% w/v) containing phosphate buffer (0.5 M, pH 7.0). The activity of bacteriocin was also retained completely in presence of SDS. The activity units were also increased to 4×10^6 AU/ml in the purified sample (Table 1). SDS, a negatively-charged detergent unfolds the proteins and binds stoichiometrically to them giving each protein approximately the same charge to mass ratio[15].

Table 1—Purification of bacteriocin produced by *L. brevis*

	Volume (ml)	Activity unit (AU/ml)	Total activity	Protein (mg/ml)	Specific activity (10^4)	Purification fold	Recovery (%)
Crude (Culture supernatant)	1000	6.6×10^5	6.6×10^8	8.627	7.6	1.00	100.00
Purified bacteriocin	10	4×10^6	4×10^7	1.478	270	35.52	17.13

[Protein concentration was determined by Lowry's method. Purification fold represents increase in the specific activity. Recovery % is remaining protein concentration as % of the initial protein concentration]

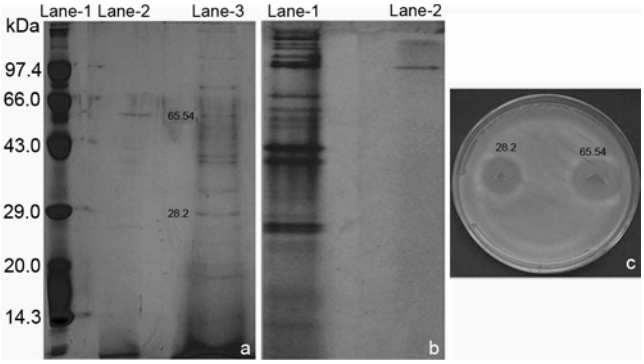

Fig. 1—(a): SDS-PAGE of purified (lane 2) and partial purified bacteriocin (lane 3), and markers (lane - 1); and (b): Native PAGE of partially purified (lane 1), and purified bacteriocin (lane - 2); and (c): Inhibition spectrum of purified bacteriocin against *L. monocytogenes* after SDS-PAGE

Though reports of insolubility of bacteriocin in buffers such as phosphate buffer, Tris HCl etc. are lacking, but similar problem of solubilizing of helviticin, a bacteriocin from *L. helviticus* in a number of solvents was also encountered which showed dissolution in SDS-aided sodium acetate buffer only[16].

The purified bacteriocin of *L. brevis* showed high molecular mass of 93.74 kD (Fig. 1a), whereas the bacteriocins of lactic acid bacteria belonging to class I and II had shown fairly low molecular mass e.g., *Pediococcus acidolactici* (3.5 kD), *L. curvatus* SB 13 (10 kD), *L. cin* C-TA33a (4.6 kD) and *L. cin* B-TA33a (3.5 kD), respectively[17-19]. So far highest molecular mass of 37 kD and 52 kD were reported for bacteriocin of *L. helviticus* 481 and *L. brevis* SD27, respectively[16,20]. The higher molecular mass of bacteriocin of *L. brevis* suggested that it might belong to class IV of bacteriocins, which included large thermostable protein complexes containing other essential moieties, in addition to protein. The class III and IV of bacteriocins were reported to have higher molecular mass, but class III contains heat-labile proteins while class IV belongs to thermostable protein[16].

Native-PAGE was run to find out the status of denatured protein subunits formed in SDS-PAGE. It revealed only the presence of a single band (Fig. 1b) confirming that two bands (65.54 + 28.2 kD = 93.74 kD) formed in SDS-PAGE were the subunits of same purified bacteriocin. These subunits were formed as a result of denaturation of native protein due to the action of SDS and heat treatment in SDS-PAGE[21,22]. The results were further supported by similar inhibition activity spectrum exhibited by both the bands of bacteriocin against their sensitive indicators after SDS-PAGE (Fig. 1c).

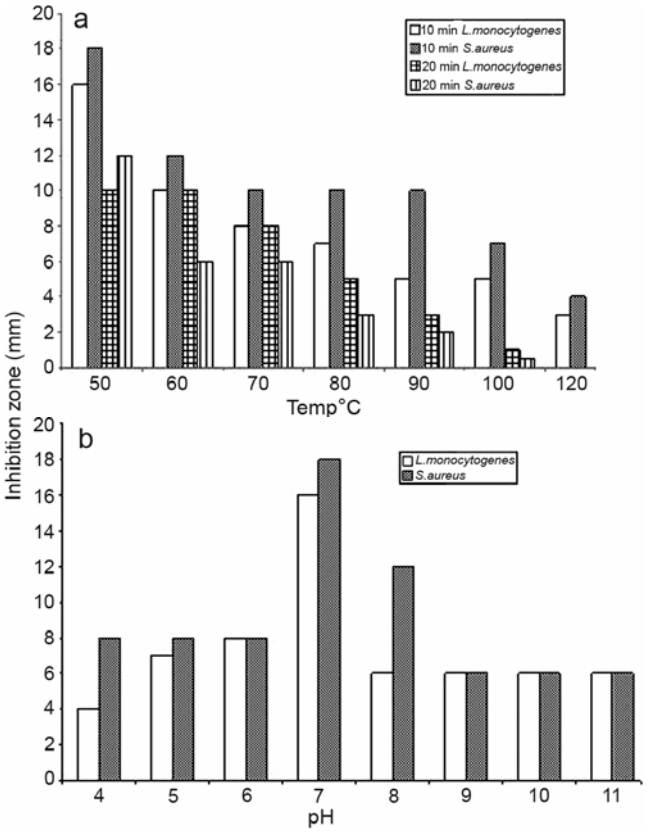

Fig. 2—Effect of temperature (a) and pH (b) on activity of purified bacteriocin

Characterization of purified bacteriocin

Effect of temperature, pH and proteolytic enzyme

Figure 2a shows the effect of temperature on bacteriocin activity in terms of inhibition zones, heat-

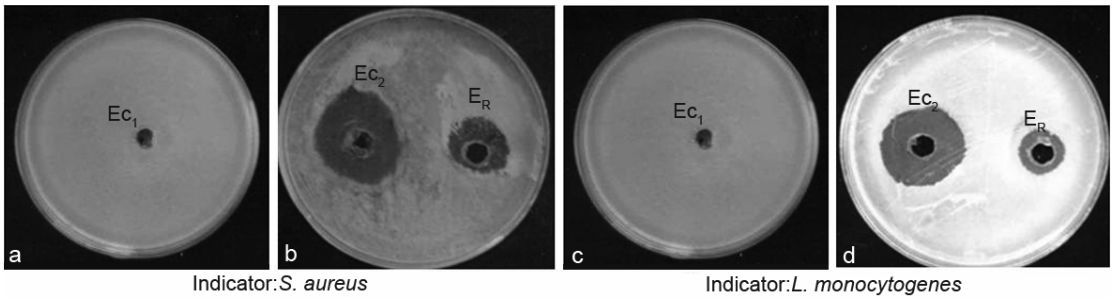

Fig. 3—Effect of trypsin on activity of bacteriocin against *S. aureus* (a,b) and *L. monocytogenes* (c,d)

treated in range of 50 to 121°C for 10 and 20 min. Bacteriocin retained its activity upto 121°C for 10 min and till 100°C for 20 min, though partial loss of activity was noticed with continuous increase in temperature. Earlier studies revealed bacteriocins produced by *L. paracaseii*, *L. pentosus*, *L. plantarum* and *L. lactis*i sub. sp. *lactis* remained active after heating till 121°C for 20 min[19].

Bacteriocin of *L. brevis* was active in a wide range of pH (3-10), but the maximum activity was observed at neutral pH (Fig. 2b). Thus the bacteriocin of *L. brevis* showed an advantage over commercial bacteriocin–nisin for its application as a food biopreservative, as it can remain stable in all types of foods acidic, alkaline or neutral in nature. Bacteriocins produced by *L. plantatum* and *L. brevis* OG1 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[6].

The activity of bacteriocin decreased in presence of trypsin 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 lawns of sensitive indicators in enzyme reaction (E_R) minimal zones of clearance i.e. 7 mm (*L. monocytogenes*) and 10 mm (*S. aureus*) were formed as compared to enzyme control (EC_2), where zones of 16 and 18 mm were recorded against them. No zone formed in EC_1 indicated that phosphate buffer (0.5 M, pH 7.0) did not have any effect on the growth of indicators (Fig. 3a-d). A significant decrease in size of inhibition zones in presence of trypsin suggested the breakdown of bacteriocin, thus confirming its proteinaceous nature. Lactococin R 9/2, lactococcin R10/1 and enterocin R 18 were inactivated by proteinase k and α chymotrypsin[23].

In conclusion, the bacteriocin was purified from *L. brevis*, isolated from the fermented food *Vari Kandal* of Himachal Pradesh. Its insolubility in water/various buffers, solubility in SDS, high heat stability, wider pH tolerance, sensitivity to trypsin suggested it as an unique bacteriocin. The strong antagonistic effect of bacteriocin against the food borne pathogens indicated its usefulness in the preservation of different food items enhancing their shelf life.

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References

1 Park S H, Itoh K E & Fujisaiua T (2003) *Curr Microbiol* 46, 385-388
2 Silva J, Cavalho A S, Teiveira P & Gibbs P A (2002) *Lett Appl Microbiol* 34, 77-81
3 Messi P, Guerrieri E & Bondi M (2003) *FEMS Microbiol Lett* 220, 121-125
4 Sharma N & Gautam N (2007) *Bev Food World* 34, 44-47
5 Chen Y S, Yanagida F & HSU J S (2006) *J Appl Microbiol* 101, 125-130
6 Bromberg R, Moreno I, Zaganini C L, Delboni R R & Oliveira J de (2004) *Braz J Microbiol* 35 (1-2) 1-13
7 Sharma N & Kapoor G (2004) In: *Intellectual Property Rights in Horticulture* (Jindal K K & Baba R, eds), pp. 175-179, S Publishers, Dehradun
8 Ren W, Zhang S, Song D , Zhou K & Yang G (2006) *Indian J Biochem Biophys*, 43, 88-93
9 Sharma N, Kapoor G & Neopaney B (2006) *Antonie van Leeuwenhoek, http/www.springerlink*
10 Barefoot S F& Klaenhammer T R (1983) *Appl Environ Microbiol*. 45, 1808-1815
11 Ogunbanwo S T, Sanni A I & Onilude A A (2003) *African J Biotechnol* 2, 219-227
12 Motta A S and Brandelli A (2002) *J Appl Microbiol* 92, 63-70
13 Sharma N & Gautam N (2007) *Indian J Biotechnol* 7, 117-121
14 Paik H D, Bae S S, Park S H & Pan J G (1997) *Indian J Microbiol Biotechnol* 19, 294-298
15 Rawn J D (1989) *Biochemistry*, 2nd edn, pp. 51-73, Neil Patterson Pub, North Carolina

16 Joerger M C & Klaenhammer T R (1986) *J Bacteriol* 167, 439-446
17 Jeevaratnam K, Jamuna M & Bawa A S (2005) *Indian J Biotechnol* 4, 446-454
18 Sudirman I, Mathieu F, Michel M & Lafebvre G (1993) *Curr Microbiol* 27, 35-40
19 Papanthanasopoulos M A, Krier F, Junelles A M R, Lefebue G, Lecaer J P, Von Holy A & Hastings J W (1997) *Curr Microbiol* 35, 331-335
20 Drider D, Fimland G, Hechard Y, Mcmullen L M & Prevost H (2006) *Microbiol Mol Biol Rev* 70, 564-582
21 Burgress R R *et al* (2000) *Meth Enzymol* 328, 141-157
22 Einarson M B, Orlinick J R (2002) *Protein-Protein Interactions* (Golemis E, ed), pp. 37-57, Cold Spring Harbor Laboratory Press, New York
23 Elotmani F, Juneelles A M R, Assobhei O & Milliere J B (2002) *Curr Microbiol* 44, 10-17