Inhibition spectrum, purification and characterization of bacteriocin from *Leuconostoc* NT-1

The fermentation of foods is an ancient method of producing preserved, shelf-stable, microbiologically safe foods, which also possess additional desirable organoleptic characteristics. The bacteria that play a crucial role in the production of these fermented foods are called lactic acid bacteria (LAB). These LAB produce bacteriocins which exhibit a narrow antimicrobial spectrum and they are modified or unmodified ribosomally synthesized peptides. They damage the target cell membrane and inhibit cell wall synthesis[1]. There are four types of bacteriocins produced by *Leuconostoc* spp. – mesenteriocin 5 by *Leuconostoc mesenteroides*-5 (ref. 2), leucocin A by *Leuconostoc geladium*[3], leuconocin-S by *Leuconostoc paramesenteroides* OX[4] and carnocin produced by *Leuconostoc carnosum* LA 44A (ref. 5).

The present study was carried out to assess antibacterial spectrum of the isolate, partial purification, characterization and effect of some physical and chemical factors on the activity of bacteriocin.

The bacteria were isolated from Indian curd (dahi). Biochemical tests for identification of the isolate were performed according to the procedure given by Collins *et al*.[6] and identification of the isolate was done according to the *Bergey's Manual of Systematic Bacteriology*[7].

Bactericin was purified by 21–60% ammonium sulphate precipitation method and fractions were dialysed against disitilled water[8,9] for 24 h. The active fractions were further fractionated by column diethylaminoethyl (DEAE)-cellulose (anion-exchanger). The column was eluted with 0.1, 0.2, 0.3, 0.4 and 0.5 M sodium chloride (NaCl) in the same buffer at a flow rate of 20 ml/h and fractions of 2 ml volume were collected. Each fraction was checked for the presence of protein by measuring absorbance at 280 nm and the fractions were pooled and concentrated to 2–3 ml by dialysis against sucrose gradient[8,10].

Sephadex G-100 was packed into the column (1 × 15 cm) and equilibrated with 0.1 M phosphate buffer (pH 7.0). One ml of concentrated partially purified bacteriocin protein was applied to the gel column and the column was eluted with the same buffer at the flow rate of 24 ml/h. Next 2 ml of fractions was eluted after every 5 min. Each fraction was checked for the presence of protein by measuring absorbance at 280 nm on a UV spectrophotometer.

Purity of protein fraction after gel filtration and molecular weight determination was estimated by 12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). PAGE was performed as described by Laemmli[11].

The effect of temperature on activity of bacteriocin was determined[10] by heating at different temperatures – 60°C, 70°C, 80°C, 90°C, 100°C and 105°C. Purified bacteriocin (400 µl) was adjusted to pH 2.0, 3.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and incubated at 37°C for 4 h. The residual activity was assayed after neutralizing the pH to 7.0 by addition of NaOH or HCl. Purified bacteriocin was assessed for its sensitivity to two enzymes, viz. trypsin and pepsin[12]. A 10 ml aliquot of partially purified bacteriocin was placed in a sterile petri dish and exposed to UV light for 0, 2, 3, 5, 6. After each time interval, bacteriocin activity was analysed by agar well diffusion method[12]. Non-ionic surfactants Triton X-100, Tween-80, Tween-20 and anionic SDS were tested for their effect on bacteriocin activity[12].

The organism was a Gram-positive coccus and found in pairs but with rare short chains when grown in liquid medium. Cells were oval in shape, but not spherical. There was no tetrad formation. Optimum temperature was 25–30°C. Milk was not coagulated when incubated at 27°C for 24–48 h. The organism could produce acid from glucose, lactose, maltose and mannitol, but not from arabinose, sucrose and glycerol. When grown on sucrose-containing medium, the isolate did not produce dextran. The organism was micro-aerophilic and heterofermentative. The organism grew in medium containing 3.0% NaCl, but not in that with 6.5%.

The inhibitory action of the bacteriocin was studied against nine pathogenic and three spoilage-causing bacteria. Of the nine pathogens tested, six were inhibited by culture filtrates of *Leuconostoc* NT-1. The pathogens that were inhibited are *Staphylococcus aureus* MTCC 96 (zone of inhition 19 mm), *Salmonella typhi* (14 mm), *Enterococcus faecalis* MTCC439 (14 mm), *Klebsiella pneumoniae* (12 mm), *Bacillus cereus* (12 mm) and a methicillin resistance *Staphylococcus aureus* (11 mm). No inhibitory activity was observed against *Moraxella* MTCC 445, *Acinetobactor baumanii* MTCC 1425 and *Listeria monocytogenes* MTCC 657. Of the three spoilage bacteria tested, only two, viz. *Serratia marcescens* (13 mm) and *Clostridium* sp. (15 mm) were inhibited, whereas there was no activity against *Micrococcus luteus* MTCC 106.

The bacteriocin from the culture filtrate of *Leuconostoc* NT-1 was precipitated by addition of 21–60% $(NH_4)_2SO_4$. This fraction contained about 25% of total protein (Table 1).

Ammonium sulphate-precipitated proteins were further fractionated using DEAE-cellulose column. Bacteriocin activity was eluted in peaks I–III which were pooled. This pooled preparation contained 10.20% of the protein present in the sample (Figure 1).

The protein preparation obtained after ion-exchange chromatography was further fractionated by gel filtration through Sephadex G 100 column (Figure 2).

Bacteriocin activity was detected in the fraction under peak A. Active fraction contained 4.9% of the total proteins.

The purity of the bacteriocin was checked by SDS–PAGE with 12.5% gel; the protein standards (Genei) of molecular weight 3500–43,000 Da were also run simultaneously. The purified protein formed a single band at the dye front corresponding to about a molecular weight of 3500 Da.

A partially purified preparation of bacteriocin in phosphate buffer (pH 7.0) was heated at 60–100°C for 1 h (60 min) and at 105°C for 10 min. The bacteriocin was thermostable for heating at 70°C for one hour. There was slight reduction in the bacteriocin activity at 80°C as a zone diameter of 21 mm was obtained.

It can be observed that a part of bacteriocin activity was lost if it was incubated at extreme pH values, but activity was not affected by pH values around

Table 1. Purification of bacteriocin by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration

Fraction (ml)	Volume (ml)	Protein concentration (mg ml^{-1})	Total protein (mg)	Activity	Diameter (mm)	Precipitation of protein (%)
Homogenate	100	5.10	510	+	17	–
Ammonium sulphate precipitation 21–60%	14	9.10	127.40	+	25	25.0
DEAE-cellulose	2	6.5	13.0	+	19	10.20
Sephadex G-100	1	6.2	6.2	+	20	4.9
TCA precipitation	5	7.44	37.2	+	74	–

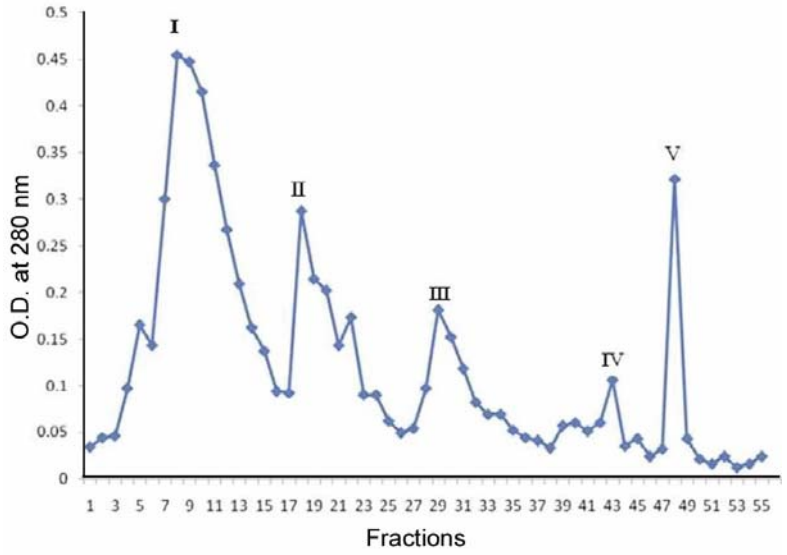

Figure 1. Ion exchange.

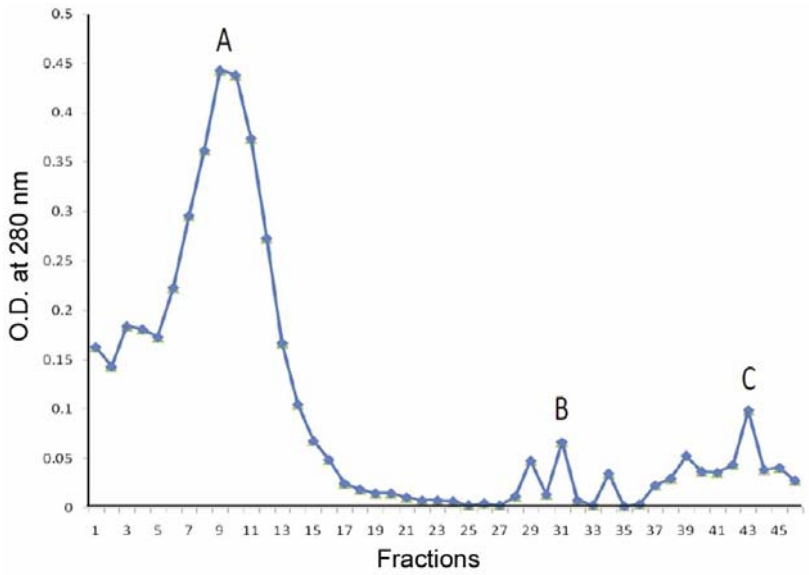

Figure 2. Gel filtration.

neutrality. The activity of bacteriocin was stable when incubated at pH 6.0 and 7.0 for one hour. There was slight reduction in activity at pH values of 5.0 and 8.0. The activity was greatly affected at low pH of 2.0 and 3.0. Reduction in activity was also observed at alkaline pH of 9.0 and 10.0, but adverse effect was not as pronounced as at extreme acid pH of 3.0 and 2.0.

The bacteriocin was stable for UV exposure up to 2 min, but activity was slightly affected for 3 min.

It was observed that Tween-80 adverseley affect the activity of bacteriocin,

whereas Triton X-100 and SDS also exerted appreciable adverse effects on bacteriocin. However, bacteriocin activity was enhanced in the case of Tween-20. The enzymes trypsin and pepsin subsequently assayed for activity, no reduction in bacteriocin activity was recorded.

Bacteriocin produced by *Leuconostoc* NT-1 could inhibit several pathogenic and spoilage-causing bacteria. The inhibitory activity against some potent pathogens such as *S. aureus*, including a MRSA, *S. typhi* and *K. pneumoniae* is an interesting feature of the bacteriocin. Lewas *et al.*[4] have reported bacteriocin from *Leuconostoc paramesenteroides* OX. It showed inhibitory action against *L. monocytogenes*, *S. aureus*, *Lactobacillus sake*, *Aeromonas hydrophila*, *Yersenia enterocolitica* and a few strain of *Clostridium botulinum*. Bacteriocin was purified by ammonium sulphate precipitation (21–60% saturation level) and concentration of protein was 127.40 mg. However Novotny *et al.*[13] could precipitate a bacteriocin from culture filtrate of *Bacillus thermoleovorans* by adding $(NH_4)_2SO_4$ to saturation level of 65%. By ion-exchange (DEAE-cellulose) 13 mg of protein was purified. However Gonzalez and Kunka[14] used DEAE-Sephadex for partial purification of bacteriocin Pediocin PA-1, produced by *Pediococcus acidilactci*. Using gel filtration (Cephadex G 100) 6.2 mg of total protein was purified. However Varadaraj *et al.*[15] used Sephadex G 50 column for purification of a bacteriocin from *Lactobacillus acidophilus*. They could achieve 15-fold purification of bacteriocin. The eluted peak was further analysed by SDS–PAGE and it appeared as a single band, thus indicating that the protein was purified to homogeneity. The molecular mass of the protein was found to be around 3.5 kDa.

The effect of UV rays was studied; after 3 min of exposure the activity was slightly reduced. Ogunbanwo *et al.*[12] studied the effect of surfactants like Tween-20, Tween-80, Triton X-100 and SDS. These were found to increase bactericidal activity of both the bacteriocins. There was no effect of two enzymes, viz. trypsin and pepsin on the activity when bacteriocin preparations were incubated with enzymes for 1 h. However, Hastings *et al.*[16] found that the bacteriocin was sensitive to all of trypsin and pepsin enzymes.

With respect to medical applications, antimicrobial production by *Leuconostoc* NT-1 might play a role during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut health. Also the potential of some of the bacteriocins in antibacterial creams for control of skin infections, burns and facial acne, cosmetics and beauty products, cream, powder and soap and probiotic, for oral hygiene like mouth wash, toothpaste and chewing gum is being studied.

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ANAND PRAKASH MAURYA[1,*]
R. L. THAKUR[2]

[1]*Department of Microbiology,*
Assam University,
Silchar 788 011, India
[2]*Department of Microbiology,*
Sardar Bhagwan Singh Post Graduate Institute,
Balawala,
Dehradun 248 161, India
**For correspondence.*
e-mail: anandmaurya02@yahoo.in