Purification and Characterization of Bacteriocin Produced by *Lactobacillus brevis* UN Isolated from Dhulliachar: a Traditional Food Product of North East India

Neha Gautam · Nivedita Sharma · O. P. Ahlawat

Received: 9 December 2012 / Accepted: 20 September 2013 / Published online: 1 October 2013
© Association of Microbiologists of India 2013

Abstract A bacteriocin producing strain *Lactobacillus brevis* UN isolated from Dulliachar—a salted pickle and identified by biochemical and molecular methods. *L. brevis* UN was found to produce bacteriocin with broad spectrum activity against spoilage causing/food borne pathogens viz. *L. monocytogenes*, *C. perfringens*, *S. aureus*, *L. mesenteroides*, *L. plantarum* and *B. cereus*. Bacteriocin production was optimized through classical one variable at a time method. The isolate showed maximum bacteriocin production at early stationary phase, pH 4.0, temperature 35 °C and with an inoculum size of 1.5 OD @ 10 %. Bacteriocin produced by *L. brevis* UN was purified to homogeneity by single step gel exclusion chromatography and was most active at pH 6.0 and 7.0, stable up to 100 °C and was proteinaceous in nature. The results of NMR revealed the presence of proline, glutamic acid, aspartic acid, leucine, isoleucine and serine in its peptide structure. PCR amplification analysis determined that bacteriocin encoded gene in *L. brevis* UN was plasmid bound.

Keywords Lactic acid bacteria · Bacteriocins · Nuclear magnetic resonance · *Listeria monocytogenes*

To harmonize consumer demands with the necessary safety standards, modern technologies implemented in food processing and microbiological food-safety standards have diminished and are being replaced by combinations of innovative technologies that include use of natural antimicrobial metabolites [1]. Among natural antimicrobials use of bacteriocins, may be an efficient way of extending food safety without altering the nutritional quality of food products. Apart from this the increasing consumption of precooked food has led to food borne illness worldwide. The bacterial pathogens that account for many of these cases include *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum*, *Campylobacter jejuni* and *Escherichia coli* 0157:H7. Until now, approaches to seek suppression of these pathogens have relied upon the search for more efficient chemical preservatives or on the application of more drastic physical treatments. Nevertheless, these types of solutions have many drawbacks viz., the proven toxicity of commonest chemical preservatives, the alteration of the organoleptic and nutritional properties of foods, and especially recent consumer trends in purchasing and consumption, with demands for safe but minimally processed products without additives.

Hence, the last two decades have seen in intensive investigation on natural antimicrobial products synthesized by food grade lactic acid bacteria that can be used as food preservatives in place of chemical preservatives. The use of generally regarded as safe lactic acid bacteria and their antimicrobial compounds especially bacteriocins is a promising ongoing development in food preservation as these have strong antagonistic effect against most of the food pathogens. Bacteriocins are ribosomally synthesized extracellularly released bioactive peptides or peptide complexes that vary in spectrum of activity, mode of action, molecular weight, genetic organization and considered to be

N. Gautam (✉) · N. Sharma
Microbiology Research Laboratory, Department of Basic Science, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan 173 230, HP, India
e-mail: neha_mbg@yahoo.com

O. P. Ahlawat
Directorate of Mushroom Research, Chambaghat, Solan 173 212, HP, India

safe biopreservatives since they are assumed to be degraded by proteases into gastrointestinal tract [2]. As different types of bacteria produce different kind of bacteriocins, therefore, there is a pressing need to explore the nascent field of natural food biopreservation by isolating different bacteria from new sources capable of producing novel bacteriocins and to characterize them to be added to food as these have a positive effect on food preservation and safety.

Materials and Methods

Isolation of Lactic Acid Bacteria

Lactic acid bacterial strain was isolated from Dhulliachar (a traditional salted pickle of North East India, prepared by seeds of *Cucurbita pepo* and *Sesamum indicum*). Isolation was carried out on MRS agar plates by standard spread plate method under anaerobic condition at 37 °C for 72 h. Anaerobic conditions were maintained under anaerobic gas jars by using gas pack system (Hi-media, Make). The bacterial colonies obtained on MRS agar were purified by streaking twice on MRS agar. The pure cultures were preserved at −20 °C on MRS medium containing 40 % glycerol (v/v) in deep freezer.

Screening of Isolates on the Basis of Biochemical Characteristics

The isolates with characteristics LAB profile viz. catalase negative, oxidase negative and non motile were selected to check their bacteriocin producing potential.

Screening of Isolates on the Basis of Bacteriocin Producing Potential

The bit disk method and well diffusion method was used to screen out bacteriocin producer [3, 4].

Molecular Identification of Isolate Exhibiting Bacteriocin Activity [5]

Potent screened bacteriocin producer isolate UN was subjected to 16S r RNA gene sequencing. Genomic DNA of UN was isolated by using standard protocol of DNA prep kit (Bangalore Genei, India Pvt. Ltd. Make).

PCR Amplification of 16S rRNA Region

The PCR analysis was carried out with following concentration of reagents i.e. Taq buffer (10×)—5.0 µl; dNTP

2 mM—2.5 µl; primer (F)—1.0 µl; primer (R)—1.0 µl; Taq polymerase—0.2 µl; glycerol—0.5 µl; water—12.8 µl; DNA—1 µl; MgCl₂—1 µl. The procedure consisted of 35 cycles of 92 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. The universal primers of expected product size i.e. (1,500 bp) used for amplification were BITS-1-(5'-AGAGTTTGATCCTGG) and BITS-4-(5'-TACCTTGTTACGACTT). The amplified PCR product were clean up using PCR clean up kit (Real Genomics Hi yieldTM Make). Eluted PCR product of UN was sequenced by commercial available services of (Genei, Bangalore, India). The sequences obtained were analyzed using the NCBI nucleotide BLASTN bioinformatics tool.

Identification

On the basis of 16S r RNA gene technique UN identified as *Lactobacillus brevis*. The sequences so obtained were submitted in National Centre for Biotechnology Information (NCBI) to get an accession number. *L. brevis* UN registered under the accession no. JX046150.

Optimization of Process Parameters for Bacteriocin Production by Using Classical One Variable at a Time Method

Maximum bacteriocin production by *L. brevis* UN was optimized by growing it at different time interval, temperature, pH and inoculum size.

Extraction and Purification of Bacteriocin

Lactobacillus brevis UN having OD 1.5 was inoculated in MRS broth (pH 4.0) @ 10 %. Inoculated flasks were kept at 35 ± 2 °C at 120 rpm for 34 h.

Lyophilization of Bacteriocin

Thirty-four-hour-old culture of *L. brevis* UN was centrifuged at 20,000 rpm for 20 min at 4 °C which was lyophilized in a lyophilizer (Allied Frost, Make) for 5 h.

Gel Filtration Chromatography

Sephadex G-75 (5 g) was suspended in 500 ml of distilled water for 48 h. It was packed into the glass column having dimensions of (31 × 2.5 cm) avoiding entrapment of any air bubble in the gel bed. It was then equilibrated with the three

bed volumes of 0.5 M phosphate buffer (pH 7.0). Lyophilized protein sample (2 ml) was loaded on the Sephadex G-75 column. It was then eluted with three bed volumes of 0.5 M phosphate buffer (pH 7.0) and 3 ml fractions were collected. A flow rate of 3 ml in 7 min was maintained. The protein content of collected fractions were measured at 280 nm and fractions showing maximum absorbance were analyzed for bacteriocin activity. The most active fractions were pooled and stored at 4 °C. Purity of sample was checked out by 12 % SDS polyacrylamide slab gel electrophoresis.

Characterization of Purified Bacteriocin of *L. brevis* UN

Effect of pH, temperature and proteolytic enzymes on activity of purified bacteriocin was determined by following the methods as described by El-Shafie et al. and Sharma and Gautam [6, 15].

Identification of Genes Encoding the Bacteriocin Production

Genomic DNA of *L. brevis* UN was isolated as described previously while plasmid DNA of *L. brevis* UN was isolated by alkaline lysis method [7]. The PCR analysis of chromosomal DNA and plasmid DNA of *L. brevis* UN was carried out with a volume of 25 µl mixture in a thermocycler (Eppendorf, Make).

PCR Mixture

Taq buffer (10×)—5.0 µl; dNTP 2 mM—2.5 µl; primer (F)—1.0 µl; primer (R)—1.0 µl; Taq polymerase—0.2 µl; glycerol—0.5 µl; water—12.8 µl; chromosomal/plasmid DNA—1 µl; MgCl₂—1 µl. The primer used for amplification was (5'-CTGCCATACTTGCGTTCC-3') (5'-CCTCTGCC ATACTTGCGTTC-3'). The procedure for amplification consisted of 35 cycles of 92 °C for 1 min, 46.6 °C for 1 min, 72 for 1 min. PCR product so obtained after amplification with specific primers at standardized amplification temperature was clean up using PCR clean up kit (Real Genomics Hi yield™ Make). Eluted PCR product was sequenced by commercial available service of Xcelaris, Ahmadabad, India. The sequences so obtained were analyzed using the NCBI nucleotide BLASTN bioinformatic tool.

Detection of Chemical Structure of Purified Bacteriocin Through NMR

NMR of purified bacteriocin of *L. brevis* UN was recorded on a ECX-300MHZ spectrometer (JEOL, JAPAN Make).

Result and Discussion

In total seven different lactic acid bacteria were isolated from Dhulliachar—a powdered mixture of seeds of *C. pepo* and *S. indicum* which is consumed by people of North East India as a condiment of food. 'Dhulliachar' is entirely different from other fermented vegetable products of North East India viz., Marcha and Hamei which are used as starter cultures for preparation of various indigenous alcoholic beverages [8]. Out of all isolated strains one isolate i.e. UN was found to have characteristic LAB profile viz. catalase negative, oxidase negative and non motile therefore, it was selected to check its bacteriocin producing potential. Bacteriocin producing potential of selected isolate UN was checked in well diffusion method against test indicators after ruling out the effect of hydrogen peroxide and organic acids in culture supernatants [9]. The culture supernatant of isolate UN was found active against spoilage causing deadly food borne pathogens viz. *L. monocytogenes*, *S. aureus*, *C. perfringens*, *L. mesenteroides*, *L. plantarum* and *B. cereus*. Bacteriocin producing isolate UN was identified as *L. brevis* by 16S rRNA gene technique. Sequences so obtained were submitted to NCBI database and matched with already existing sequences. Sequence similarity search for bacteriocin producer strain UN showed 99 % homology with the available nucleotide sequence of *L. brevis* with accession number NR_044704.1. The 16S rRNA sequence of *L. brevis* UN is registered in genbank under the accession number JX04615. Maximum bacteriocin production was optimized through classical one variable at a time process which was observed at 34th h i.e., early stationary phase, at pH 4.0, temperature 35 °C with an inoculum size of 1.5 OD @ 10 %. In case of *L. brevis* UN percent increase in bacteriocin production after optimization through classical one variable at a time process was 40, 20 and 70 % against *L. monocytogenes*, *S. aureus* and *C. perfringens* respectively. Purification was done by single step gel exclusion chromatography on Sephadex G-75. Fraction no 11-15 were pooled together based upon highest antagonistic activity. The purity rate and molecular weight of the *L. brevis* UN was confirmed through SDS PAGE and was found to be 14 kDa.

After purification there was an increase in activity units of bacteriocin of *L. brevis* UN. Activity units increased from 2×10^3 to 8×10^3 . The specific activity was raised to 45,977 in the purified bacteriocin with a final recovery of 37 %. As the bacteriocin titers had increased after purification there was percent increase in antagonistic potential against test indicators. The zone size increased by 50 % against *L. monocytogenes*, 16.6 % against *S. aureus* while 33.3 % against *C. perfringens* after purification. To confirm the bacteriocin potential of purified single band of *L.*

brevis UN, it was cut from the gel and its antimicrobial activity was checked against *L. monocytogenes*. Pure bacteriocin of *L. brevis* UN was characterized to assess its capability to work in different environmental conditions. Bacteriocin of *L. brevis* UN was maximum active at pH 7.0 against all the three test indicators. Though, bacteriocin of *L. brevis* UN was found partially active against *S. aureus* on acidic side up to pH 2.0 and on alkaline side up to pH 12.0 whereas it lost its activity against *L. monocytogenes* and *C. perfringens* on acidic side as well as on basic side. The stability of this bacteriocin at neutral pH made it suitable for their use in neutral food products where use of nisin (a commercial preservative) is limited as it is only active at pH 5.0 and 5.5. Till date many bacteriocins have been reported which have different behavior at different pH. Some of them are active at neutral pH, some at acidic and some are at alkaline pH [10].

As far as temperature stability is concerned the bacteriocin of *L. brevis* UN was most active at 40 and 50 °C when treated for 10 min. In present study, it was found that bacteriocin of *L. brevis* UN retained its partial activity against *C. perfringens* even after heating to 100 °C. This moderate thermostable character of bacteriocin make it desirable for preservation of heat processed food products. It has been well reported in literature that few bacteriocins are extremely heat stable and can withstand high heat of 100 °C and even autoclaving temperature of 121 °C. While some bacteriocins are extremely heat sensitive and start losing activity from 50 °C onwards because a modest increase in temperature results in unfolding and loss of secondary and tertiary structure of protein that may cause denaturation of protein following its destabilization to almost complete loss of native confirmation [11].

Bacteriocin of *L. brevis* UN was found completely inactive in presence of proteolytic enzymes viz. trypsin and proteinase k @ 0.25 mg, reflecting its proteinaceous nature and renders it safe for human consumption. Bacteriocinogenic strain *L. brevis* UN was analyzed in PCR reactions for the presence of known bacteriocin gene. Plasmid DNA was isolated by using alkaline lysis method [12]. The isolated plasmid DNA was used in PCR to amplify bacteriocin encoded gene using selected primers. Simultaneously, chromosomal DNA was also run as a control in PCR. The primers were selected by taking into account nucleotide sequences of bacteriocin encoded genes of *L. brevis* available after searching in the GeneBank databases i.e. EMBL and NCBI. The selected sets of primers were synthesized from Sigma–Genosys. In total 4 set of primers were used to amplify bacteriocin producing gene. The amplification was achieved with one set of primer, i.e. (5'-CTGCCATACTTGCGTTCC-3') (5'-CCTCTGCCA-TACTTGCGTTCC-3') of expected amplicon size 453 bp using a gradient PCR. Annealing temperature was varied in

between 45 and 55 °C to see the effect of temperature on the specific amplification. The annealing temperature 46.6 °C was best for the amplification of bacteriocin encoded gene of *L. brevis* UN as a clear expected amplification product of ~450 bp was obtained. Nil amplification was observed in control confirming that gene that account for bacteriocin production is plasmid mediated rather than chromosomal bound. Other three sets of primers that were used in PCR amplification were failed to amplify with bacteriocin mediated genes as nil amplification or a nonspecific amplification was observed. PCR product was purified and submitted for sequencing to Xcelaris, Ahmadabad, India. The sequence of amplified PCR product showed 93 % homology with *Lactococcus lactis* NC 0136561 and *L. lactis* NC 002662.1—a nisin producing isolate and *E. coli* NC 017906.1—a colicin producer. It is further to be added to the fact that bacteriocin produced by LAB may share consensus sequences [13]. Our results are in accordance to one of the study in which authors reported the presence of pediocin encoded gene in *L. plantarum* LB-BI [14].

¹H-NMR was carried out to find out different amino acids that are forming molecular structure of bacteriocin of *L. brevis* UN. ¹H-NMR experiments were recorded on JOEL, JAPAN ECX = 300 MHz spectrometer at IM-TECH, Chandigarh. The NMR spectrum so obtained was compared with standard ¹H-NMR spectrum of 20 different amino acids. After comparing the peaks it was observed that out of 20 amino acids only six amino acids viz. proline, glutamic acid, arginine, leucine, isoleucine and serine were involved to form peptide structure of purified bacteriocin of *L. brevis* UN as the obtained chemical shift/ppm of purified bacteriocin of *L. brevis* UN matched with the reported chemical shift of these amino acid. A brief explanation related to chemical shift of different amino acids have been presented in Table 1.

Similar study has been reported in literature where authors determined the sequence of amino acid residues for purified *L. lactis* by ¹H-NMR spectrum and found that bacteriocin exhibited aspartic acid, serine, glycine,

Table 1 ¹H-NMR spectral data, delta (ppm) of various amino acids being detected in the purified bacteriocin of *L. brevis* UN

Name of amino acid	H α (ppm)	H β (ppm)	H γ (ppm)	Amide proton (ppm)
Proline	4.48	4.21	2.26–2.51	3.38–3.46
Glutamic acid	3.7	2.51	2.19	5.0
Arginine	3.74–3.78	1.86–1.94	1.59–1.77	3.21–3.26
Leucine	3.88	3.36	1.73	2.0
Isoleucine	–	–	3.53–3.58	–
Serine	4.47	3.5–3.8	1.5	3.3–3.8

histidine, proline, valine, methionine leucine and lysine amino acids.

Conclusion

It may be concluded from above study that bacteriocin of *L. brevis* UN isolated from Dhulliachar has bright prospects to be used as a food biopreservative as it poses a combination of desirable characteristics viz., bacteriocin is secreted from food grade bacteria already existing in edible food sample, thus rendering it completely safe for consumption, strong antagonism against a broad range of serious and challenging food borne pathogens/spoilage causing microorganisms, action of bacteriocin at higher temperature and wider pH range, imparting it stability; degradation of bacteriocin in the presence of proteolytic enzyme making it completely safe for human consumption. Molecular determinants for bacteriocin production in *L. brevis* UN showed that gene for bacteriocin production was plasmid bound. ¹H-NMR revealed the unique combinations of different amino acids in biochemical structure of purified bacteriocin which has been reported for the first time in present study. All the above mentioned attributes advocate strongly that bacteriocin of *L. brevis* UN isolated from Dhulliachar has potential to be used as effective food biopreservative in place of chemical preservatives thus keeping intact the nutritive properties of processed food as well as safer for consumption.

References

- Çakmakci S, ÇetİN B, Turgut T, Gürses M, ErdoĖan A (2012) Probiotic properties, sensory qualities and storage stability of probiotic banana yogurt. *Turk J Vet Animal Sci* 36:231–237
- Balgir PP, Bhatia P, Kaur B (2010) Sequence analysis and homology based modeling to assess structure-function relationship of pediocin CP2 of *Pediococcus acidilactici* MTCC 5101. *Indian J Biotechnol* 9:431–434
- Mandal V, Sen SK (2011) Isolation and characterization of pediocin NV 5 producing *Pediococcus acidilactici* LAB 5 from vacuum-packed fermented meat product. *Indian J Microbiol* 51:22–29
- Deraz SF, El-Fawal GF, Abd-Ellatif SA, Khalil AA (2011) Autohydrolysed *Tilapia nilotica* fish viscera as peptone source in bacteriocin production. *Indian J Microbiol* 51:171–175
- Özdemir GB, Biyik HH (2012) Isolation and characterization of a bacteriocin-like substance produced by *Geobacillus toebii* strain HBB-247. *Indian J Microbiol* 52:104–108
- El-Shafie HA, Ibrahim N, Abd EI-Sabour HA, Mostafa YA (2008) Purification and characterization of bacteriocin produced by isolated strain of *Lactococcus lactis*. *J Appl Sci Res* 4:1315–1321
- Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7:1513–1523
- Tamang JP, Dewan S, Tamang B, Rai A, Schillinger U, Holzapfel WH (2007) Lactic acid bacteria in Hamei and Marcha of North East India. *Indian J Microbiol* 47:119–125
- Yang E, Fan L, Jiang Y, Doucette C, Fillmore S (2012) Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. *AMB Express* 2:48
- Khalil R, Elbahloul Y, Fatima DF, Omar S (2009) Isolation and partial characterization of a bacteriocin produced by a newly isolated *Bacillus megaterium* 19 strain. *Pak J Nutr* 8:242–250
- Baldwin RL, Frieden C, Rose GD (2010) Dry molten globule intermediates and the mechanism of protein unfolding. *Proteins* 78:2725–2737
- Sambrook J, Russell DW (1989) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Faye T, Holo H, Langsrud T, Nes IF, Brede DA (2011) The unconventional antimicrobial peptides of the classical propionibacteria. *Appl Microbiol Biotechnol* 89:549–554
- Xie Y, An H, Hao Y, Qin Q, Huang Y, Luo Y, Zhang L (2010) Characterization of an anti-listeria bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from Komiss, a traditionally fermented dairy product from China. *Food Contam* 22: 1027–1031
- Sharma N, Gautam N (2008) Antibacterial activity and characterization of bacteriocin of *Bacillus mycoides* isolated from whey. *Indian J Biotechnol* 7:117–121