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Characterization of a new bacteriocin produced from a novel isolated strain of *Bacillus lentus* NG121

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Abstract

The new bacteriocin is produced from *Bacillus lentus* NG121 isolated from *Khameera* – a traditional fermented food from Himachal Pradesh, India which has been reported for the first time in the literature to produce bacteriocin and exhibited very high activity units of 20×10^5 AU (Arbitrary Units)/ml. This bacteriocin was partially purified and was further characterized to assess its preservation characteristics. It showed strong antimicrobial activity against the most challenging and serious test indicators like *Listeria monocytogens* and *Staphylococcus aureus*. There was a drastic decrease up to 70% in viable cells of the indicators within the first 10 h of adding partially purified bacteriocin thus proving its bactericidal action. It could withstand the high heat of 100 °C for 10 min of heating time without losing any activity. A wide range of pH tolerance i.e. from 5.0–10.0 was expressed by this bacteriocin. It was found completely sensitive to proteolytic enzyme trypsin. The unique combination of all the above mentioned characteristics makes the bacteriocin of newly isolated *Bacillus lentus* NG121, a food grade bacteria, highly desirable for preservation of different food items in the food industry.

Introduction

We live in a sea of bacteria. Though generally it is thought that these single cell organisms are the purveyors of the diseases, but largely these have proven to be friends rather than foes. The list of such examples is endless and biopreservation is one of the finest examples of them.

Biopreservation means the application of biopreservatives at low concentration having GRAS (generally regarded as a safe) status and not imparting any deleterious effects to the food in which it is used (Bizani and Brandelli 2002). Though bacteria have been impregnating the food supply thus serving as a preservating agent for millennia, these natural non-toxic food biopreservatives functioned in obscurity until about a decade back. Recently, scientists have developed a concept of targeting food pathogens/spoilage causing microorganisms with the help of these prokaryotes and thus providing longevity and safety to the food.

Another attractive reason for researchers to work in this field is to find a safer and healthier way to protect the food and to provide longer shelf life in place of harmful chemical preservatives.

There is a popular trend to prevent spoilage with many different types of chemical preservatives and various other shielders that lead to alteration in chemical constituents, nutritional and organoleptic properties of the food and health hazards. The increasing awareness among the consumers of natural healthy food products have necessitated the need to exploit other methods of preservation i.e. biopreservation.

Among food biopreservatives bacteriocins – biologically derived low molecular weight proteinaceous compounds, easily degraded during digestion in human beings (Orgunbanwo et al. 2003) –– can be the best alternative to the predominant method of chemical preservation. Different types of bacteria produce different types of bacteriocins, therefore there is a pressing need to explore the nascent field of biopreservation and isolate more and more bacteria from new sources capable of producing novel bacteriocins and characterize them to be added to food. In the present study the new bacteriocin produced from *Bacillus lentus* NG121 isolated from an unexplored traditional fermented food – *Khameera* of Himachal Pradesh a Himalayan state of India –– has been characterized for various parameters to test its efficacy to be added into food as a potential biopreservative.

Materials and methods

Materials used

Bacillus lentus NG121: *Bacillus lentus* NG121 reported for the first time in the literature to produce bacteriocin was isolated from Khameera, a traditional fermented food of India by dilution series method ($10^2 \times 10^8$) on nutrient agar plates and incubated at 37 °C for 72 h. (Sharma and Kapoor 2004). The identification of the isolated pure culture as *B. lentus* was confirmed at Indian Institute of Microbial Technology, Chandigarh, India.

Nutrient Agar: Beef extract (3.0 g), Peptone (5.0 g), NaCl (8.0 g), Agar (10 g)/1000 ml, pH 6.8
Nutrient Broth: Beef extract (3.0 g), Peptone (5.0 g), NaCl (8.0 g)/1000 ml, pH 6.8
Tris–HCl Buffer: 0.1 M, pH 7.0
Phosphate Buffer: 0.1 M, pH 7.0
Enzyme: Trypsin (Sigma Chemicals)

Partial purification of bacteriocin of Bacillus lentus NG121

Inoculum preparation

10 ml culture of *B. lentus* NG121 (10^{-6} dilution) was added into 90 ml of nutrient broth and was incubated at 37 °C for 72 h at 150 rpm in order to obtain bacterial culture of 2.0 OD.

Ammonium sulfate precipitation

For partial purification, the ammonium sulfate precipitation method was chosen (Orgunbanwo et al. 2003). The bacterial culture (2.0 O.D.) was saturated with different concentrations of ammonium sulfate i.e. 20, 40, 50, 70 and 80% subsequently with constant stirring. When the precipitation occurred at 80% saturation level, the preparation was kept at room temperature (30 °C) for 12 h. Centrifugation of the supernatant was carried out at 20,000*g* at 4 °C for 1 h. The pellet so obtained was dissolved in 100 ml of Tris–HCl Buffer (0.1 M, pH 7.0). The cell free extract of *B. lentus* was dialyzed. After 24 h, the dialyzed bacteriocin suspension was carefully removed from the dialysis bags and was centrifuged at 10,000*g* for 10 min at 4 °C.

Calculation of activity units (Arbitrary Units – AU/ml) of partially purified bacteriocin

Serial two-fold dilution method

The activity units of partially purified bacteriocin of *B. lentus* were calculated by the serial two-fold dilution method of Barefoot and Klaenhammer (1983). Partially purified bacteriocin was diluted in saline water with a range of 10^{-2}, 10^{-4}...10^{-10}. Each dilution was used to estimate AU by the well diffusion method (given below) and dilution corresponding to the smallest detectable zone was marked for further calculation.

Well diffusion method

In nutrient agar plates, lawn of indicators were prepared by swabbing the plates with cotton using indicator (1.0 OD). The wells of 7×3 mm size were cut with a sharp borer in these plates and 0.3 ml of partially purified bacteriocin was added in each well. The plates were incubated for 24 h at 37 °C and the results were noted in terms of zones of inhibitions formed around the wells.

Mode of action of partially purified bacteriocin

To determine the mode of action, bacteriocin of *B. lentus* was mixed with its test indicator in the ratio of 1:1. The preparation was kept for incubation at 37 °C for different time intervals of 1, 2...9, 10 h, respectively. Controls (indicator without bacteriocin) were run in parallel. After every time interval 0.1 ml of the preparation was mounted on the nutrient agar plates by spread plate method. After incubation of Petri plates at 37 °C for 24 h, the results were obtained by counting the number of colonies in the form of c.f.u./ml of bacteriocin treated and bacteriocin untreated indicators on the plates.

Characterization of partially purified bacteriocin

Effect of temperature on activity of partially purified bacteriocin

0.5 ml of bacteriocin was added to 4.5 ml of nutrient broth in a test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation and then treated at different temperature of 40, 50, 60 °C … 90 and 100 °C each for 10 min. The well diffusion method was performed with the above heat treated bacteriocin to detect the inhibition zones.

Effect of pH on activity of partially purified bacteriocin

A 0.5 ml aliquot of each bacteriocin was added to 4.5 ml nutrient broth and this preparation in each test tube were adjusted at different pH of 3.0, 4.0, 5.0, … 10.0, 11.0 and incubated for 30 min at 37 °C. Each pH treated bacteriocin was assayed using well diffusion method.

Effect of proteolytic enzyme – trypsin on activity of partially purified bacteriocin

Lawns of test indicators were prepared in nutrient agar Petri plates and the effect of proteolytic enzyme on activity of partially purified bacteriocin was studied by following the method of Paik et al. 1997.

Enzyme activity

Enzyme control I (E_{c1}) : 0.3 ml phosphate buffer
Enzyme control II (E_{c2}) : 0.15 ml bacteriocin of each isolate + 0.15 ml phosphate buffer
Enzyme reaction (E_R) : 0.5 mg of enzyme – Trypsin (Sigma Chemicals) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.0 and then added to bacteriocin of *B. lentus* in the ratio of 1:1. The enzyme reaction and enzyme control I and II were assayed by the well diffusion method on the indicator plates.

Results and discussion

Partial purification of bacteriocin produced from B. lentus

Bacteriocin secreted from *B. lentus* was partially purified by the ammonium sulfate saturation method. After precipitation bacteriocin of *B. lentus* had produced 20×10^5 AU/ml. The appreciably high Activity Units of bacteriocin of *B. lentus* rendered it very effective for preservation purposes. The partially purified cell free bacteriocin of *B. lentus* showed the same inhibitory spectrum as that of bacteriocin originally secreted by the cells of *B. lentus* i.e. it was antagonistic to *L. monocytogens* and *S. aureus*. The zone size increased in partially purified bacteriocin of *B. lentus* against its corresponding sensitive test strains which indicated higher potency of bacteriocin after its partial purification. Table 1 shows the increase in the size of inhibition zones after partial purification against the indicators. An increase of 46.7 and 33.3% was recorded against *S. aureus* and *L. monocytogens*, respectively. This proves that the bacteriocin of *B. lentus* retained its original antagonistic properties along with the increase in the titre of bacteriocin after partial purification.

The same studies on retaining the full potency of bacteriocin activity after its partial purification were also recorded. Mantovani et al. (2002) revealed that crude extracts and partially purified cultures of *Streptococcus bovis* HC 5 inhibited the same organisms i.e. *S. bovis* 33317 and *S. bovis* 15351. Larsen et al. (1993) reported the antimicrobial activity of *Lactobacillus bavaricus* to be maximum for *L. sake* LMG 9468 after its partial purification and as well as of its bacterial cell culture.

Mode of action of partially purified bacteriocin

Figure 1 reveals the results in the form of c.f.u./ml when sensitive strains were treated with their respective bacteriocin. When sensitive cells of *L. monocytogens* were treated with bacteriocin of

Table 1. Per cent increase in the inhibition zone size (mm) of partially purified bacteriocin from *B. lentus* NG121.

Bacterial isolates	Bac[a] + *S. aureus* (mm)	BP[b] + *S. aureus* (mm)	% increase in zone size [c]	Bac[a] + *L. monocytogenes* (mm)	BP[b] + *L. monocytogenes* (mm)	% increase in zone size[c]
B. lentus	15.0	22.0	46.7	18.0	24.0	33.3

Each value is a mean of three replicates:
[a]Bac – Bacteriocin of cell culture
[b]BP – Partially Purified bacteriocin
[c]$\frac{\text{Zone size (mm) of partially purified bacteriocin–Zone size (mm) of crude bacteriocin}}{\text{Zone size (mm) of crude bacteriocin}} \times 100$

B. lentus, it was observed that with increasing time there was a decrease in the number of cells of bacteriocin treated test indicator as compared to the control (indicators without bacteriocin). There was a continuous decrease in the number of bacteriocin treated *L. monocytogenes* i.e. 40×10^6 c.f.u./ml at 1 h, 30×10^6 c.f.u./ml at 5 h and 14×10^6 c.f.u./ml at 10 h while c.f.u./ml in control remained the same throughout the time period.

Similar was the case with *S. aureus* where c.f.u./ml reduced from 54×10^6 c.f.u./ml at 1 h to 44×10^6 c.f.u./ml at 5 h and further to 28×10^6 c.f.u./ml at 10 h, when this indicator was treated with bacteriocin as compared to the control. These results indicate the bactericidal mode of action of bacteriocin of *B. lentus*.

Bavaricin A of *L. bavaricus* also showed antibacterial effect for *L. sake* LMG946 having bacteriocin activity of 100 AU/ml. For all the concentrations of bavaricin used c.f.u./ml of the sensitive strains decreased with increase in time. *L. sake* LMG 9468 and 100×10^6 c.f.u./ml before 100 AU/ml of bavaricin A (Larsen et al. 1993). Bactericidal mode of action of bacteriocin of *Bacillus thuringiensis* has also been shown by Paik et al. 1997 for indicator *B. thuringiensis* subsp. *thompsoni* where the viability of the cells reduced from 5.5 log c.f.u./ml before 50 min to 25 log c.f.u./ml with 200 AU/ml of bacteriocin.

Characterization of partially purified bacteriocin

Effect of temperature on activity of partially purified bacteriocin

When bacteriocin of *B. lentus* was heat treated in the temperature range 40–100 °C for 10 min and for 20 min, it was observed that the effect of temperature for 10 min on bacteriocin activity was

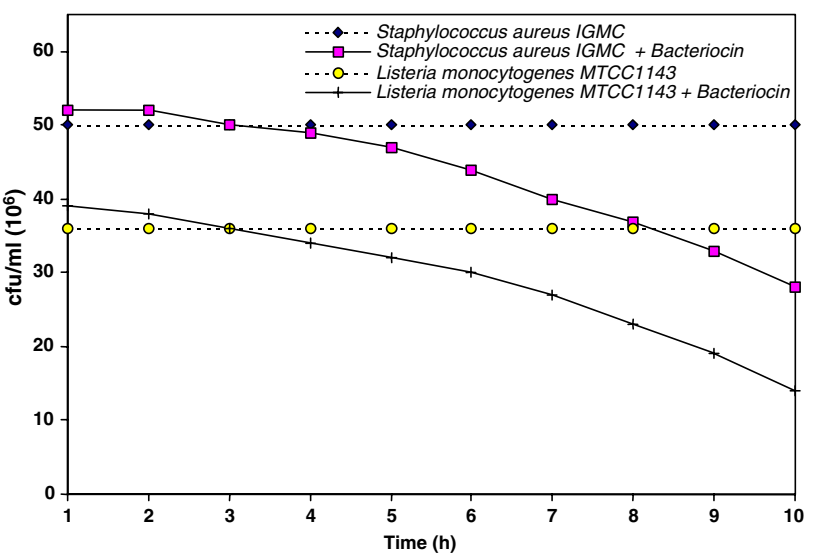

Figure 1. Bactericidal effect of partially purified bacteriocin of *B. lentus* NG121 against indicators.

very slight and it retained activity to 100 °C. There was a little difference in the zone of inhibitions formed after interaction of indicators with heat treated bacteriocin. The zones of diameter 24 and 22 mm were formed against *L. monocytogens* and *S. aureus* after treatment of bacteriocin at 40 and 50 °C. The diameter of the zone was 21 mm for *L. monocytogens* and 17 mm for *S. aureus* at 70 °C. The zones were 20, 18 and 12 mm for *L. monocytogens* and 14, 14 and 11 mm in case of *S. aureus* at 80, 90 and 100 °C, respectively (Figure 2).

The bacteriocin isolated in the present study had shown thermstability thus it was placed under class II of bacteriocins which were found to be heat stable in nature (Klaenhammer 1993). Though the retention of activity of bacteriocin of *B. lentus* till 100 °C for 10 min was a remarkable property to be used for biopreservation of food.

Naclerio et al. (1993) isolated Cerein from *B. cereus* (400 AU/ml) which was partially stable to heat treatment. The activity was maintained during treatment up to 75 °C (50 AU/ml) and disappeared only after 15 min incubation at 90 °C.

Effect of pH on activity of partially purified bacteriocin

Bacteriocin of *B. lentus* showed the maximum activity at pH 7.0 for *L. monocytogens* and *S. aureus*. Bacteriocin retained its activity when the pH was changed from 5.0 to 10.0 though there was a complete loss of activity when the pH was lowered to 3.0, 4.0 and was raised beyond 10.0 (Figure 3). The activity of bacteriocin in the broad pH range further recommends its use in acidic and alkaline foods for biopreservation. Similar studies were reported by Yildrim and Johnson (1998) for bacteriocin of *L. lactis* active in pH range 2.0–9.0 and Bizani and Brandelli (2002) for bacteriocin of *Bacillus* sp. showing activity in pH ranging from 5.0 to 8.0.

Effect of proteolytic enzyme – trypsin on activity of partially purified bacteriocin

When bacteriocin of *B. lentus* was treated with trypsin in the ratio of 1:1 (ER) and then welled into the lawns of indicator strains, no inhibition zone was formed. When phosphate buffer alone (EC_1) was put into the well no zone was formed. Bacteriocin treated with phosphate buffer (EC_2) resulted in zone formation of 22 and 24 mm for *S. aureus* and *L. monocytogens*, respectively. Bacteriocin showed zero activity when trypsin was added into the well along with phosphate buffer thus there was no inhibition zone formed around the wells (ER) (Plate 1). This shows that enzyme trypsin had completely inactivated the bacteriocin of *B. lentus*. The sensitivity of bacteriocin of *B. lentus* to proteolytic enzyme trypsin revealed its

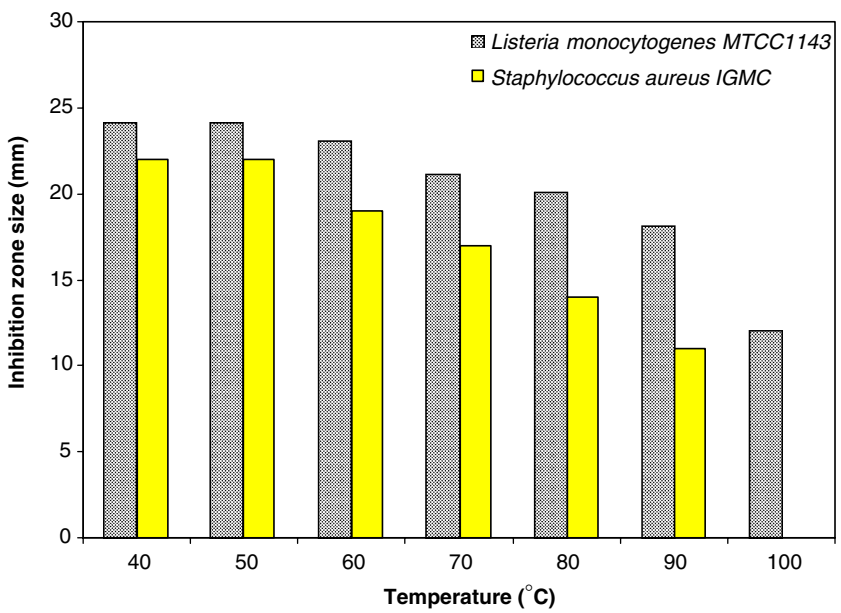

Figure 2. Effect of temperature on activity of partially purified bacteriocin of *B. lentus* NG121.

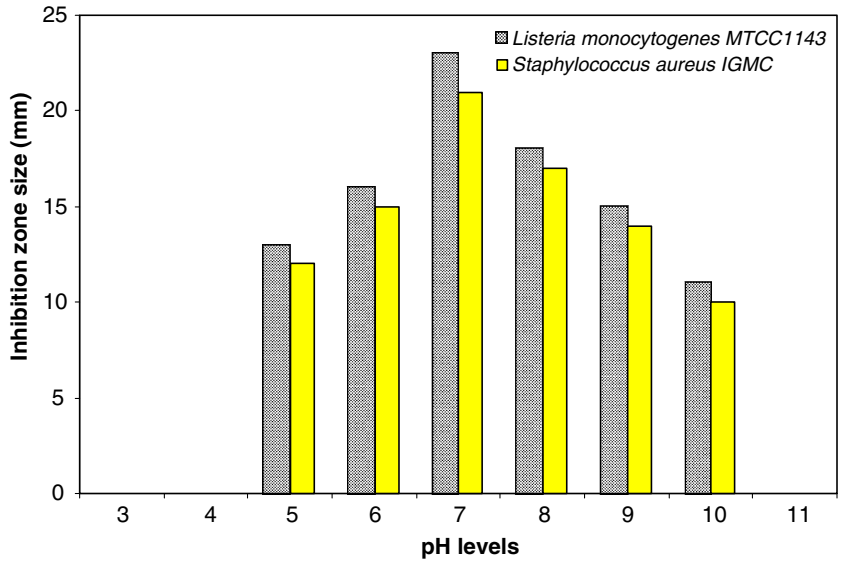

Figure 3. Effect of pH on activity of partially purified bacteriocin of *B. lentus*.

proteinaceous nature and further it supported its use as food biopreservative because of its capability to be easily degraded in the digestive system of human beings.

Conclusion

Bacillus lentus NG121 isolated from Khameera, the least explored fermented traditional wheat product of Himachal Pradesh, a hilly state of India, has been reported for the first time in the literature to produce bacteriocin. The bacteriocin produced from *B. lentus* showed strong antimicrobial activity against most challenging and serious food pathogens like *L. monocytogens* and *S. aureus*.

The bacteriocin of *B. lentus* was partially purified by the salt (ammonium sulfate) saturation method. The partially purified bacteriocin exhibited very high activity of 20×10^5 AU/ml. The viability of the pathogens decreased drastically up to 70% within the first 10 h of adding bacteriocin. The characterization of bacteriocin showed high thermostability i.e. up to 100 °C for 10 min. It expressed a wide range pH tolerance i.e. from 5.0–10.0. It was found to be completely sensitive to proteolytic enzyme – trypsin.

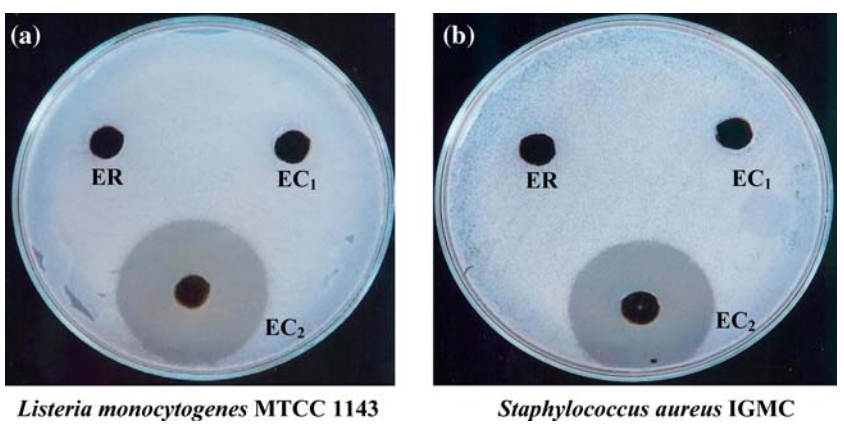

Plate 1. Effect of trypsin on activity of partially purified bacteriocin of *B. lentus* NG121 against indicators.

The unique combination of all the above mentioned properties rendered new bacteriocin of a novel isolated strain of *B. lentus* NG121, a food grade bacteria, as an attractive food biopreservative and thus highly desirable for preservation of food items in the food/food processing industry.

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