Biopreservation of Fresh Orange Juice Using Antilisterial Bacteriocins101 and Antilisterial Bacteriocin103 Purified from Leuconostoc mesenteroides

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Abstract

Recently, as the consumption of fresh fruit juice has been increasing the demand for natural and potent biopreservative has also been increased. *Listeria monocytogenes* is one of the most important and serious intracellular foodborne pathogen that threatens immunocompromised individuals. The main objective of this study is to investigate the biopreservative potential of Antilisterial Bacteriocin101 and Antilisterial Bacteriocin103 on comparison with a chemical preservative. Preservation of fresh orange juice was carried out in two sets i.e., unpasteurized (no heat pretreatment) and pasteurized (pretreated at 72°C for 2 min). *Listeria monocytogenes* MTCC 657 at an initial concentration of 6.75 log CFU/ml was inoculated into both unpasteurized and pasteurized fresh orange juice then chemical preservative (12 ppm), Antilisterial Bacteriocin101 (40 ppm) and Antilisterial Bacteriocin103 (40 ppm) were added and stored for 12 days at 4°C and checked for the viability of the indicator strain at 24 h regular intervals. Antilisterial Bacteriocins showed potential reduction of viable counts upto 4th day in unpasteurized fresh orange juice and upto 6th day in case of pasteurized fresh orange juice. This study has revealed that both the Antilisterial Bacteriocins show high potential in reduction of microbial population and is more effective than chemical preservative even at low concentration.

Keywords: Antilisteria; Bacteriocins; *Listeria monocytogenes*; Biopreservation; Pasteurization

Introduction

The increasing demand for fresh-tasting, healthy, nutritious and ready-to-eat foods has stimulated the expansion of minimally processed fruit and vegetable markets worldwide [1-3]. Processing of the products resulting from natural fruits and vegetables have been observed to increase certain reactions leading to susceptibility to microbes [4]. From a consumer perspective, increasing scientific evidence for consumption of fresh fruits for prevention of biological problems, demand for low-calorie diet and increasing microbiological and pesticide content in processed food has increased the consumption of ready-to-eat vegetables and fruits [5]. This health based option for customers has been short lived due many inappropriate or manipulative storage conditions that again lead to microbiological spoilage and diseases [6].

*Listeria monocytogenes*, a major contaminant of food products, is a Gram positive, facultative, intracellular bacterium that multiplies mainly on dairy, milk and meat products including industrial food products that are distributed or procured under refrigerated conditions [7,8]. *L. monocytogenes*, is considered a serious food-borne pathogen in immune-compromised individuals such as cancer subjects, HIV patients, elderly and pregnant women. Ingestion of *L. monocytogenes* can cause listeriosis in human and animals [9,10] which lead to gastroenteritis, septicaemia, perinatal infections, stillbirth, abortion, meningitis and meningoencephalitis in immunocompromised individuals [11]. Listeriosis affects a wide variety of mammals including monogastric, ruminants (mostly sheep) and human with mortality rate of 20-30% [12].

Traditionally, the shelf-life stability of juices has been achieved by thermal processing. Low temperature long time (LTLT) and high temperature short time (HTST) treatments are the most commonly used techniques for juice pasteurization. However, thermal pasteurization tends to reduce the product quality and freshness. Apart from thermal pasteurization, some chemical preservatives are also widely used for the extension of the shelf-life of fruit juices and beverages. Two of the most commonly used preservatives are potassium sorbate and sodium benzoate. However, consumer demand for natural origin, safe and environmental friendly food preservatives has been increasing since 1990s. Apart from thermal pasteurization, some chemical preservatives are also widely used for the extension of the shelf-life of fruit juices and beverages. Two of the most commonly used preservatives are potassium sorbate and sodium benzoate [13]. Nitrate and nitrite as sodium or potassium salts has also been used as food additives to improve the microbiological safety of food and to extend their safe shelf life [14].

Use of microorganisms in food fermentation is one of the oldest methods for producing and preserving food [15]. Lactic acid bacteria (LAB) have an important role in preserving foods and preventing poisoning [16]. This inhibition is due to the production of various compounds as organic acids, diacetyl, hydrogen peroxide and bacteriocins [17]. Bacteriocins as biopreservatives against food spoilage especially led by microbiological contamination are generally regarded as safe (GRAS) and include proteins with varying molecular weight, biochemical properties and mode of activity [18].

The keen interest towards bacteriocin of lactic acid bacteria worldwide is due to their essential role in majority of food fermentation, flavour development and preservation of food products along with proving safer for health. In the present study, antilisterial bacteriocin isolated from lactic acid bacteria has been targeted for its biopreservative potential.

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Materials and Methods

Maintenance of *Listeria monocytogenes* MTCC 657

In the present study *Listeria monocytogenes* was used as indicator microorganism [19,20]. The indicator microorganism *Listeria monocytogenes* MTCC 657 was obtained from IMTECH, Chandigarh and revived using Brain Heart Infusion broth (Himedia, India) at 30°C for 2 days. *Listeria monocytogenes* MTCC 657 was subcultured and maintained in Tryptone Glucose Extract agar (Casein enzymic hydrolysatse - 5 g/l, Yeast extract-3 g/l, Glucose -1 g/l, Agar - 15 g/l and Final pH at 25°C-7.0 ± 0.2).

Isolation of lactic acid bacteria

Toddy was selected as a source for isolating Bacteriocin-producing Lactic Acid Bacteria. Toddy is one of the traditional, social and local drinks extracted from either a coconut tree or a palm tree flower and called as ‘kallu’. Toddy samples were collected from Kerala and Tamil Nadu, serially diluted and spread plated on Man Rogosa Sharpe (MRS) agar (Himedia, India; Final pH at 25°C, 6.5 ± 0.2). The plates were incubated overnight at 37°C and further screening of the isolates was done by inoculating the colonies into MRS medium.

Screening of the isolates for antilisterial bacteriocin production

The individual colonies that showed growth in MRS agar (*Leuconostoc mesenteroides* BPB GRD 101 and *Leuconostoc mesenteroides* BPB GRD 103) were selected and screened for the production of bacteriocin by spot-on-lawn method. 4 ml of TGE soft agar (0.7%) containing 30 µl of overnight culture of the indicator strain *Listeria monocytogenes* MTCC 657 was overlaid on petriplates pre poured with TGE hard agar (1.8%). Each of the individual colony isolates were spotted on the lawn and the plates were incubated for 24 h at 30°C.

Production and purification of antilisterial bacteriocins using amberlite XAD-16

A single colony of the isolated *Leuconostoc mesenteroides* BPB GRD 101 and *Leuconostoc mesenteroides* BPB GRD 103 strains were inoculated into 10 ml of production media (Tryptone-2%, Glucose-1%, Yeast extract- 2%, MnSO4 - 0.005%, MgSO4 - 0.005%, Tween 80-0.2%, pH-6.8) and incubated overnight at 30°C without shaking. This 10 ml culture was used as the inoculum for 250 ml of production media. The culture was grown at 30°C without shaking for 16-18 hours and centrifuged at 10,000 rpm for 10 minutes. The supernatant was heated at 100°C for 10 minutes to inactivate proteases before further purification. The heat inactivated cell-free supernatant was used for further purification steps.

Five grams of Amberlite XAD-16 was soaked in 50% isopropanol and stored at 4°C. Traces of isopropanol was removed by washing repeatedly with deionized distilled water before use. Five grams of Amberlite XAD-16 was added to 250 ml of heat inactivated cell-free supernatant and incubated with shaking at room temperature for four hours. The mixture was transferred to a chromatographic column and the matrix was washed with 20 ml of deionized distilled water and 20 ml of 40% (v/v) ethanol. The ALB101 and ALB103 were eluted with 20 ml of 70% (v/v) isopropanol followed by washing with 20 ml of absolute isopropanol. The eluted fraction was evaporated to half of its volume and the pH was adjusted to 3.7 with 5N NaOH. This fraction termed as Amberlite fraction was determined for Antilisterial Bacteriocin activity by agar well diffusion method. The protein concentration of this fraction was estimated by measuring the absorbance at 280 nm.

Antilisterial bacteriocin101 and antilisterial bacteriocin103 activity assay

The activity of Antilisterial Bacteriocin101 (ALB101) and Antilisterial Bacteriocin103 (ALB103) was assayed based on the critical dilution of antagonistic activity caused by the Antilisterial Bacteriocin producing strain. The crude ALB101 and ALB103 samples were prepared and assayed against the indicator organism *Listeria monocytogenes* MTCC 657 by agar well diffusion method. Wells were punched on TGE agar (1.8%) seeded with the indicator strain (overnight culture of *Listeria monocytogenes* MTCC 657). Dilutions were aliquoted into the appropriately labelled wells and incubated at 4°C for 4 h and incubated at 30°C overnight. The Antilisterial Bacteriocin activity was defined as the reciprocal of the highest two fold dilution showing complete inhibition of the indicator lawn and was expressed in activity units (AU) per ml of culture media. The temperature based activity of the isolated bacteriocins were undertaken by subjecting them to varied temperatures and the activity was assayed.

Biopreservative potential of antilisterial bacteriocin101 and antilisterial bacteriocin103 in unpasteurized and pasteurized fresh orange juice at (4°C)

The effect of Antilisterial Bacteriocins (ALBs) as biopreservative was checked on fresh orange juice. The juice samples were procured from local juice corner (Coimbatore). The experiment was carried in two sets of four containers (A, B, C and D) each containing 10 ml of juice samples inoculated with indicator strain i.e. *L. monocytogenes* MTCC 657 (6.75 log CFU/ml). First bottle (A) labelled as ‘Control’ (without any preservative), second and third bottle (B and C with purified ALBs) labelled as ‘Test’ and fourth bottle (C) having sodium nitrite (chemical preservative). Purified ALBs and sodium nitrite were added (40 ppm and 12 ppm) respectively into each pathogen treated food sample to observe biopreservative effect of different preservatives against test indicator and the assay was performed in triplicates. The storage studies for stability were done for 12 days at 24 h interval and log CFU/ml was noted. The procured juice samples were also taken in sterilized glass containers and pasteurized by keeping it in hot water at 72°C for 2 min and subjected to the above mentioned analysis.

Results and Discussion

Isolation of lactic acid bacteria

Lactic acid bacteria isolated from toddy on MRS agar plate were subjected to morphological, biochemical identification and assayed for its bacteriocin activity. Two strains designated as BPB GRD 101 and BPB GRD 103 were subjected to 16S rRNA sequencing and phylogenetic analysis. Based on the microbiological, physiological tests and 16S rRNA phylogeny, the strains BPB GRD 101 and BPB GRD 103 showed 99% similarity with *Leuconostoc mesenteroides*. The sequences BPB GRD 101 and BPB GRD 103 defined as *Leuconostoc mesenteroides* GRD BPB 101 and *Leuconostoc mesenteroides* GRD BPB 103 was deposited in NCBI under the GenBank accession number JX174179 and JX174180.

Screening of antilisterial bacteriocin activity

The Spot-On-Lawn antimicrobial activity was the conventional method of monitoring bacteriocin production which is fast and reliable. Various authors had used Spot-On-Lawn method...
for screening of bacteriocin activity [17,18,21]. The Antilisterial Bacteriocins (ALB101 and ALB103) were tested for antimicrobial activity against L. monocytogenes MTCC 657 by using the spot-on-lawn method with modifications. ALB101 and ALB103 showed a largest clear zone of inhibition against L. monocytogenes MTCC 657 (Figure 1). The Antilisterial Bacteriocins producing LAB strains were further characterized for bacteriocin activity. The crude and partially purified Antilisterial Bacteriocins were tested for their antimicrobial activity against L. monocytogenes MTCC 657 by two fold dilution method and well diffusion assay (WDA). The crude and partially purified ALB101 and ALB103 showed the antilisterial activity against L. monocytogenes MTCC 657. Activity was measured as the reciprocal of the highest dilution showing antimicrobial activity. When the bacteriocin was subjected to various temperatures ranging from -80°C to -121°C, the stability of the bacteriocin was observed as the activity remained stable.

Production and purification of antilisterial bacteriocins

Antilisterial bacteriocin production was carried out using the TGE + Tween 80 (pH 6.8) medium, which supported maximum bacteriocin production. Bacteriocin activity was detected at early exponential phase and maximized at stationary phase. Tween 80 is a non-ionic detergent, it might help in releasing bacteriocin molecules from the producer cell wall into the medium [22]. Numerous purification strategies have been reported for bacteriocins all with varying degrees of success, which may be attributable to the extremely heterogeneous nature of bacteriocin [23]. The purification methods commonly employed include ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic chromatography coupled with cation-exchange column chromatography, reverse-phase high-performance liquid chromatography (HPLC), Amberlite XAD- 2, Sephadex G-25 gel filtration, ultrafiltration and gel permeation chromatography and ethanol precipitation. Each purification method has its own drawbacks, which may include issues with low yield and purity, cost, and the requirement for a skilled operator [24]. Among the methods tested, the Amberlite XAD-16 method seemed the most appropriate in partially purifying the sample, especially since it is used in the previously reported purification scheme of the NKR-5-3 enterocins [25]. Among the methods tested, the Amberlite XAD-16 method seemed the most appropriate in partially purifying the sample, especially since it is used in the previously reported purification scheme of the NKR-5-3 enterocins.

Biopreservative potential of antilisterial bacteriocin101 and antilisterial bacteriocin103 on total viable counts of L. monocytogenes mtcc 657 in unpasteurized fresh orange juice

Microbial counting was carried out using pour plate method [26].

Figure 1: Spot on lawn method using top agar method for confirmation of bacteriocin activity against L. monocytogenes MTCC 657.

Figure 2: The zone of inhibition by ALB101 and ALB103 against Listeria monocytogenes MTCC 657 when subjected to various temperatures. (A) ALB101 at -20°C; (B) ALB103 at -20°C; (C) ALB101 at -80°C; (D) ALB103 at -80°C; (E) ALB101 at 4°C; (F) ALB103 at 4°C; (G) ALB101 at 121°C; (H) ALB103 at 121°C.

Figure 3: The viability of Listeria monocytogenes MTCC 657 inoculated onto unpasteurized orange juice during 12 days storage at 4°C.

which is a standard method for viable counting. Bacteriocins used in this study, when applied to fresh orange juice had inhibited the multiplication of L. monocytogenes MTCC 657 when compared to the control and showed maximum reduction on bacterial population (Figures 2 and 3). The results further revealed that microbial count drastically decreased in both the fresh and pasteurized sample.

The initial density of L. monocytogenes MTCC 657 on introduction to unpasteurized fresh orange juice was 6.75 log CFU ml⁻¹ on day 0. On day 12, L. monocytogenes MTCC 657 was increased to 7.14 log CFU ml⁻¹ in control. Addition of ALB101, decreased the viable count of microbial population slowly from 6.75 to 6.56 log CFU ml⁻¹ till 4th day after which an increase in proliferation was observed. For ALB103, the reduction potential was observed as from 6.75 to 6.49 log CFU ml⁻¹ during 4th day of storage which followed by proliferation of viable counts. Reduction of viable count was observed from 6.75 to 6.7 log CFU ml⁻¹ at 2nd day on addition of sodium nitrite (Figure 2).

Biopreservative potential of antilisterial bacteriocin101 and antilisterial bacteriocin103 on total viable counts of L. monocytogenes mtcc 657 in pasteurized fresh orange juice

The initial density of L. monocytogenes MTCC 657 on introduction to pasteurized fresh orange juice was 6.75 log CFU ml⁻¹ on day 0.
ALB101 showed an obvious gradual decrease in viable extent from 6.75 log CFU ml⁻¹ as 6.54 log CFU ml⁻¹ and 6.66 log CFU ml⁻¹ for 4th and 5th days of storage, respectively, followed by new viable cells. Potential cells from three to six days as 6.58, 6.51, 6.61 and 6.69 log CFU ml⁻¹. In case of chemical preservative (Sodium nitrate), reduction of microbial inhibition of ALB103 was obvious in reduction of viable cells from three to six days as 6.58, 6.51, 6.61 and 6.69 log CFU ml⁻¹. These findings are consistent with the work of several other investigators who studied the antimicrobial activity of Nisin. The increased concentration of bacteriocins isolated from CA44 showed strong antagonism against indicator microorganism. The biopreservative potential of ALB101 and ALB103 along with chemical preservative (Sodium nitrite) was carried out using fresh orange juice (unpasteurized and pasteurized) and the results showed that low concentrations (40 ppm) of antilisterial Bacteriocins were potentially reduced the viability of *L. monocytogenes* MTCC 657 in unpasteurized as well as pasteurized orange juice than chemical preservative. However, ALB103 was more potent than ALB101. So it would be recommended to use these Antilisterial Bacteriocins as biopreservatives during the food preservation process.

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### Conclusion

LAB are known for the safety and shelf life extension of the food products and also serve as alternatives to chemical preservatives/additives in food preservation. Bacteriocin-producing strains of LAB are of great interest as they are generally recognised as safe (GRAS) organisms and their antimicrobial products as biopreservatives. To meet the demand for very potent natural biopreservative, bacteriocin producing lactic acid bacteria was isolated from toddy. Isolated bacteriocins were thermostable and active against the foodborne pathogen *L. monocytogenes* MTCC 657. As a conclusion of spot-on-lawn and well diffusion assay, the Antilisterial Bacteriocins (ALB101 and ALB103) isolated from *Leuconostoc mesenteroides* stains showed strong antagonism against indicator microorganism. The biopreservative potential of ALB101 and ALB103 along with chemical preservative (Sodium nitrite) was carried out using fresh orange juice (unpasteurized and pasteurized) and the results showed that low concentrations (40 ppm) of antilisterial Bacteriocins were potentially reduced the viability of *L. monocytogenes* MTCC 657 in unpasteurized as well as pasteurized orange juice than chemical preservative. However, ALB103 was more potent than ALB101. So it would be recommended to use these Antilisterial Bacteriocins as biopreservatives during the food preservation process.

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**Figure 4:** The viability of *Listeria monocytogenes* MTCC 657 inoculated onto pasteurized orange juice during 12 days storage at 4°C.
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