

Biopreservation of apple and pomegranate juice using bacteriocin of *Lactobacillus acidophilus* NCDC 343

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Abstract

Bacteriocins are antimicrobial metabolites produced by lactic acid bacteria, which are being used as additives to prevent microbial spoilage of food items. Bacteriocins in free form are degraded by the enzymes present in the fruit juices. Encapsulation matrix protects the bacteriocin from food components. The present study was designed to elucidate the effectiveness of calcium alginate entrapped bacteriocin of *Lactobacillus acidophilus* in preservation of fruit juices. Calcium alginate gel was chosen as immobilization matrix because it is cheap, abundant and bio safe. Bacteriocin of *Lactobacillus acidophilus* NCDC 343 showed wide spectrum of antimicrobial activity against pathogenic and food spoilage microorganisms. It inhibited the growth of *Staphylococcus aureus*, *Erwinia* sp., *Bacillus subtilis*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroids* and *Listeria monocytogenes*. Immobilized bacteriocin treatment resulted in maximum reduction of bacterial population in apple and pomegranate juice as compared to chemical preservative at 37°C. Calcium alginate entrapped bacteriocin proved to be better and safe preservative as compared to chemical preservatives in fruit juices.

Keywords bacteriocin, biopreservation, fruit juice, immobilization, *Lactobacillus acidophilus* NCDC 343

Introduction

Fruit juices contain several antioxidants, vitamins and minerals required for the growth of human being and serve significant part in fighting against cardiac disorders and disastrous diseases like cancer

[1]. Fruit juices are spoiled due to the growth of acid tolerant bacteria, yeasts and molds that is favored by essential nutrients presents in juices [2]. Microbial spoilage leads to off flavor, off odor, flat sour, ethanol production, formation of CO₂, turbidity and changes the color of the juices [3-4].

Traditionally, the shelf life of fruit juices was enhanced by thermal processing. But, it tends to reduce the product quality and freshness [5]. Therefore, some non-thermal pasteurization methods have been proposed during the last couple of decades, including ultrasound, high pressure homogenization (HPH), high hydrostatic pressure (HHP) and pulsed electric field (PEF) [6]. However, not all these newer methods are employed industrially because of the expenses incurred. Apart from these physical methods, chemical preservatives such as sodium benzoate, potassium sorbate and potassium metabisulphite are used to increase the shelf life of juices [7]. Consumption of these preservatives is linked to the increasing incidence of allergies [8]. Therefore, the use of food preservatives from natural sources has been increased.

Biopreservation is a promising technique for extending shelf life of food products. Bacteriocins are important sources of biopreservation. These are ribosomally synthesized, antimicrobial metabolites produced by lactic acid bacteria [9]. *Lactobacillus* is an important genus of lactic acid bacteria. Many *Lactobacillus* sp. like *Lactobacillus plantarum*, *L. acidophilus*, *L. pentosus*, *L. paracasei* subsp. *paracasei* and *L. delbrueckii* [10-13] have been exploited as bacteriocin producers by many researchers.

Bacteriocin as a biopreservative provides new opportunities to improve the microbial safety

of fruit juices. Bacteriocins like Nisin, Enterocin AS-48 and Bificin C6165 etc. have been used by many workers in free form for the fruit juice preservation [14-16]. But, loss of bacteriocin activity occurs when the bacteriocin interacts with the food components and it is also degraded by the proteolytic enzymes present in food items. Therefore, present study was designed to increase the bacteriocin activity in juices by calcium alginate entrapment.

An attempt has been made to observe the preservative efficacy of immobilized bacteriocin in juices and comparative account of bacteriocin and chemical preservatives was made for enhancing shelf life of juices.

Methodology

Microorganisms and Media

Lactobacillus acidophilus NCDC 343 was employed as bacteriocin producer and grown on de Man Ragosa Sharp (MRS) medium and incubated at 37°C/48h. The indicator strains used were *Staphylococcus aureus* NCDC 109, *Erwinia* sp. MTCC 2760, *Bacillus Subtilis* MTCC 2451, *Pseudomonas fluorescens* MTCC 103, *Escherichia coli* NCDC 135, *Leuconostoc mesenteroides* NCDC 29 and *Listeria monocytogenes* MTCC 657. All the microbial cultures were sub-cultured periodically.

Preparation of crude bacteriocin

Crude bacteriocin was prepared as per the method of Joshi et al. [17]. Overnight grown metabolically active culture of *Lactobacillus acidophilus* NCDC 343 was inoculated in one liter of MRS broth and incubated at 37°C for 24 hours. After completion of incubation time, culture broth was centrifuged at 8000 rpm for 10 min at 4°C (REMI-CPR-30 PLUS). The pellet was discarded and pH of supernatant was neutralized with 0.1N NaOH to exclude the antimicrobial effect of organic acids. The crude bacteriocin was stored at -10°C for further studies.

Protein estimation

Protein concentration of crude bacteriocin preparation was determined by the method of Lowry et al. [18].

Antibacterial efficacy and quantification of bacteriocin

Well diffusion assay described by Fleming et al. [19] was performed to determine antibacterial activity of crude bacteriocin preparation against fruit juice spoiling bacteria (*Staphylococcus aureus*, *Erwinia* Sp., *Bacillus subtilis*, *Pseudomonas fluorescens*, *Leuconostoc mesenteroides*, *Listeria monocytogenes* and *Escherichia coli*). For quantification of bacteriocin, serial 10 fold dilutions of the bacteriocin preparation were used to perform the well diffusion assay. The antimicrobial activity of the bacteriocin is the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units per ml (AU/ml).

Preparation and Characterization of calcium alginate beads

Beads were prepared using the method described by Hermes et al. [20]. Sodium alginate solutions at concentration of 2.5%, 3.0%, 3.5%, 4.0% and 75 mM and 100 mM calcium chloride solutions were prepared. Capsules were formed by drop wise addition of sodium alginate solution into a gently stirred CaCl₂. After membrane formation, the capsules were removed, washed with distilled water and transferred into hardening solution (100 mM CaCl₂) for further studies.

Physical property of beads such as weight of beads on wet basis and dry basis was noted. Diffusion properties of beads were also checked [21].

Determination of release of bacteriocin from beads

As bacteriocins are ribosomally synthesized protein molecules, so amount of release of bacteriocin from calcium alginate beads was studied by determining the protein content. Fifteen beads weighing 1.2 g were added to 3 ml of saline and kept on incubator shaker at 37°C/150 rpm. After the interval of 6 hours, 2 ml sample was withdrawn and 2.5 ml of saline was added periodically for 8 days. Then, samples were subjected for protein estimation.

Comparison of chemical preservatives and immobilized bacteriocin for preservation of fruit juices

Efficiency of Potassium metabisulphite (KMS) and immobilized bacteriocin was determined for the preservation of commercially available fruit juice (Pomegranate and Apple). Ten ml of juice sample was taken and KMS at concentration of 0.7 mg/ml

was added, which was permitted by FPO to be used safely as preservative in fruit juices. In another 10 ml of juice sample, bacteriocin immobilized in calcium alginate beads (weight of beads- 1gm) was added. A control was also run simultaneously.

Three sets of above mentioned combinations were prepared and kept at 37°C /150 rpm. Samples were withdrawn periodically for 8 days each after the interval of 2 days. Bacterial colony count (log CFU/ml) in juice sample was then determined by pour plate technique.

Statistical Analysis

All experiments were performed in triplicate, and the data was presented as mean $\pm$ standard deviation (SD). Data was subjected to statistical analysis by one way ANOVA using Daniel's XL Toolbox. Statistical significance was considered to exist at $p < 0.05$.

Results and Discussion

Antibacterial efficacy and quantification of crude bacteriocin

Activity spectra of bacteriocin of *Lactobacillus acidophilus* were determined against fruit juice spoiling bacteria. *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Erwinia* sp., *Leuconostoc mesenteroides*, *Listeria monocytogenes* and *Bacillus subtilis* were found to be sensitive to bacteriocin. However, no inhibitory activity was observed against *Escherichia coli* (Table 1). Maximum inhibitory activity was observed against *Staphylococcus aureus*, so it was selected as an indicator strain for further studies. Three times 10 fold dilution of bacteriocin has zone of inhibition of 4.3 mm Therefore, the crude

Table 1. Antibacterial activity of crude bacteriocin preparation of *Lactobacillus acidophilus* NCDC 343 against fruit juice spoiling bacteria

S. No	Fruit juice spoiling bacteria	Zone of inhibition (mm)
1.	<i>Staphylococcus aureus</i>	22 $\pm$ 0.4
2.	<i>Pseudomonas fluorescens</i>	19 $\pm$ 0.57
3.	<i>Erwinia species</i>	16 $\pm$ 0.65
4.	<i>Bacillus subtilis</i>	14 $\pm$ 0.9
5.	<i>Leuconostoc mesenteroides</i>	20 $\pm$ 0.8
6.	<i>Listeria monocytogenes</i>	15 $\pm$ 0.76
7.	<i>Escherichia coli</i>	Nil

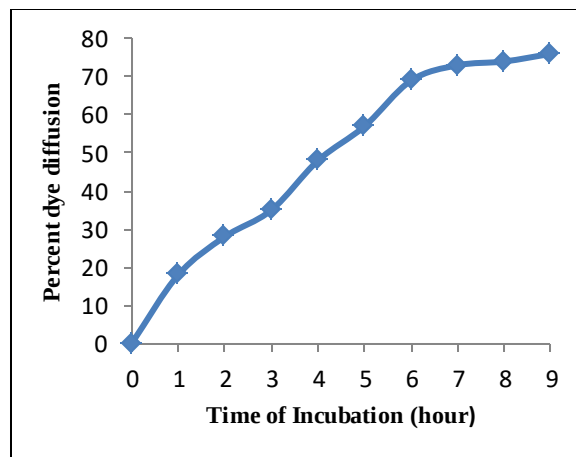


Figure 1. Dye diffusion from calcium alginate beads

bacteriocin prepared in this study contained 1000 AU/ml of bacteriocin. AU/ml is defined as the reciprocal of highest dilution which shows minimum zone of inhibition of 2 mm [22].

Protein content

As bacteriocin are proteinaceous in nature, protein content of crude bacteriocin preparation was determined and it was found to be 7500 μ g/ml i.e. 7.5g/l.

Characterization of calcium alginate beads

Beads prepared from 3.5% sodium alginate were found to be most stable. When the concentration of sodium alginate was lower than 3.5 %, round beads were not obtained. Weight of bead on wet basis i.e. 0.042 g was more than on dry basis (i.e. 0.028 g). Percent dye diffusion was calculated to study the diffusion property of beads. As shown in Figure 1, absorbance increased with an increase in time period from 0 to 7 hours and after 7th hour of incubation, it was maximum i.e. 0.073. Percent dye diffusion was 73% and thereafter, sustainable release of dye occurred.

Release of bacteriocin from calcium alginate beads

After entrapment of bacteriocin in calcium alginate beads, release of bacteriocin from the beads was checked for 8 days at 37°C/150rpm. Maximum protein was released after 174 hours i.e. after 7th day and sustained release of protein content was found up to 8 days. Moreover, 7.5 mg/ml of protein was immobilized and 4.8 mg/ml was released after 8 days (Figure 2), i.e. 64% of the total immobilized

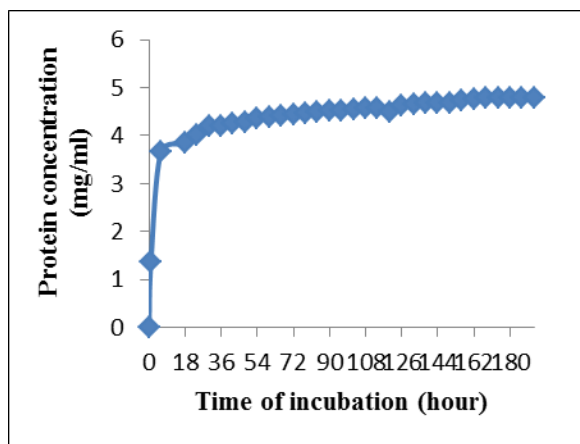


Figure 2. Amount of bacteriocin released from calcium alginate beads

bacteriocin was released efficiently after 8 days of incubation.

Comparative analysis of effectiveness of chemical preservatives and immobilized bacteriocin in preservation of fruit juices

The effectiveness of immobilized bacteriocin and chemical preservative (KMS) was studied for enhancing the shelf life of pomegranate and apple juice for 8 days at 37°C/150 rpm. Bacteriocin treatment resulted in decreasing the bacterial colony count more efficiently as compared to chemical preservatives. Table 2 and 3 exhibits the record on comparative efficiency of Potassium metabisulphite (KMS) and immobilized bacteriocin to enhance

Table 2 Comparative analysis of preservative efficiency of immobilized bacteriocin of *Lactobacillus acidophilus* NCDC 343 and chemical preservative (potassium metabisulphite) in pomegranate juice

		Number of cells (log CFU/ml)					
S. No	Combinations	Time of incubation (Days)					
		0	2	4	6	8	
1.	Pomegranate Juice (control)	3.33 ± 0.04	3.37 ± 0.04	3.53 ± 0.04	3.64 ± 0.02	3.81 ± 0.02	
2.	Pomegranate Juice + preservative (KMS)	3.28 ± 0.04	3.14 ± 0.04	3.49 ± 0.03	3.62 ± 0.02	3.71 ± 0.03	
3.	Pomegranate Juice + immobilized bacteriocin	3.09 ± 0.05	2.54 ± 0.01	2.63 ± 0.01	2.77 ± 0.01	2.84 ± 0.05	

(Results are expressed as Mean ± S.E.M and differences are considered significant at $p < 0.05$)

Table 3 Comparative analysis of preservative efficiency of immobilized bacteriocin of *Lactobacillus acidophilus* NCDC 343 and chemical preservative (KMS) in apple juice

		Number of cells (log CFU/ml)					
S. No.	Combination	Time of Incubation (Days)					Mean
		0	2	4	6	8	
1.	Apple Juice (Control)	3.30 ± 0.05	3.49 ± 0.02	3.56 ± 0.04	3.79 ± 0.03	3.97 ± 0.05	3.691
2.	Apple Juice + Preservative (KMS)	3.29 ± 0.02	3.08 ± 0.05	3.21 ± 0.06	3.26 ± 0.01	3.94 ± 0.03	3.457
3.	Apple Juice + immobilized bacteriocin	3.12 ± 0.05	2.84 ± 0.01	2.88 ± 0.01	2.91 ± 0.01	3.71 ± 0.04	3.064

(Results are expressed as Mean ± S.E.M and differences are considered significant at $p < 0.05$)

shelf life of pomegranate and apple juice respectively.

In pomegranate juice, bacteriocin treatment resulted in decreasing bacterial colony count after 2 days and noted to be 2.54, 3.14 and 3.37 for immobilized bacteriocin, for KMS and control, respectively. On 4th day, log CFU/ml for immobilized bacteriocin, KMS and control were 2.63, 3.49 and 3.53, respectively. The log CFU/ml was lowest in juice sample treated with immobilized bacteriocin on 6th day. However, after 8 days of incubation, increase in log CFU/ml for all the three treatments i.e. for immobilized bacteriocin, KMS and control was observed. The bacterial colony count (log CFU/ml) was recorded to be 2.84 in pomegranate juice sample containing immobilized bacteriocin while it was 3.71 for juice sample containing chemical preservative (KMS) after 8 days of incubation (Table 2, Fig. 3). Addition of calcium alginate entrapped bacteriocin resulted in reducing the bacterial colony count by 25.4% whereas KMS reduces it by only 2.6% after 8 days as compared to control.

For apple juice, there was a decline in the log CFU/ml after storage for 2 days and was noted to be 2.84 for immobilized bacteriocin, 3.08 for KMS while it reached 3.49 for control. On 4th day log CFU/ml for immobilized bacteriocin, KMS and control were 2.88, 3.21 and 3.56, respectively (Table 3, Fig. 4). The log CFU/ml was lowest for immobilized bacteriocin on 6th day. After 8 days of treatment, immobilized bacteriocin resulted in

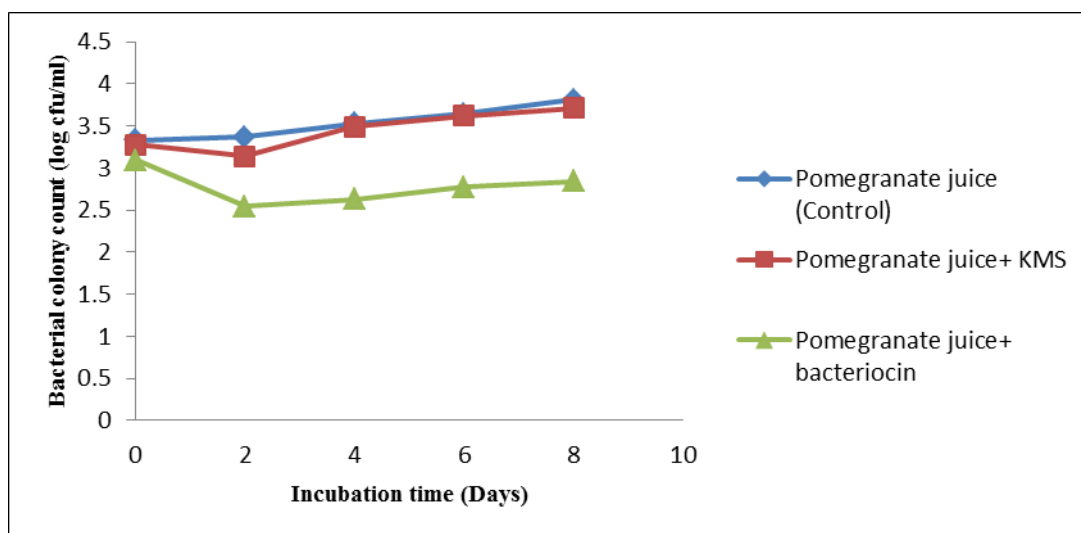


Figure 3 Comparative analysis of immobilized bacteriocin and chemical preservative in enhancement of shelf life of pomegranate juice

reducing bacterial colony count by 17% and KMS by 6.3% only.

From the above results, it is clear that among both the preservatives that were used to enhance the shelf life of pomegranate and apple juice, bacteriocin proved to be better preservative as compared to chemical preservatives. Bacteriocins from LAB have been generally recognized as safe substances and these are also non-toxic on eukaryotic cells. Their use as a preservative in fruit juices can reduce the application of chemical preservatives [23]. Therefore, bacteriocins may be used as an alternative tool to satisfy the increasing consumer demand for safe, fresh-tasting, ready-to-eat and minimally-processed foods.

Bacteriocins in free form have been used as preservative in fruit juices by many researchers. Yamazaki et al. [24] observed that commercially available bacteriocin, nisin has inhibitory effect against *Alicyclobacillus acidoterrestris* in fruit juices. Purified bacteriocin from *Lactococcus lactis* AP2 has better potential in enhancement of the shelf life of orange and mixed fruit juice as compared to sodium benzoate [25]. Enterocin AS-48 is also considered as a good candidate for the preservation of fruit juices. Burgos et al. [15] observed that HIPEF treatment in combination with enterocin AS-48 results in the inactivation of *Listeria monocytogenes*, *Bacillus licheniformis* and *Staphylococcus aureus* in juices.

Direct application of bacteriocins in food system may result in a decrease or loss of

antimicrobial activity due to the interaction with food components [26]. As Cleveland et al. [27] reported Nisin inactivation by some meat components, such as phospholipids and glutathione S-transferase. Encapsulation technology has been shown to protect bacteriocins from interfering food components, potentially enhancing their efficacy and stability. Pei et al. [16] proved that encapsulation of bificin C6165 in calcium alginate beads has better preservative effect in fruit juices as compared to free bificin after 8 days.

In the present study, calcium alginate entrapped bacteriocin has been proved as an efficient biopreservative as compared to the chemical preservatives that are used routinely in fruit juices preservation. Moreover, bacteriocins are not known to cause allergies. As these are produced by lactic acid bacteria, they are probiotic in nature also and help in restoring the gut microflora [23].

Conclusion

Purified bacteriocin of *Lactobacillus acidophilus* NCDC 343 has antagonistic activity against food spoilage organisms. Bacteriocin immobilized in calcium alginate beads showed high potential in the reduction of microbial population in fruit juices and is more effective than chemical preservatives. Bacteriocin treatment resulted in reducing the bacterial load by 14% and 25.4% in apple and pomegranate juice, respectively. Hence, it may be recommended to use the bacteriocins in fruit juice preservation as they are safe and nontoxic.

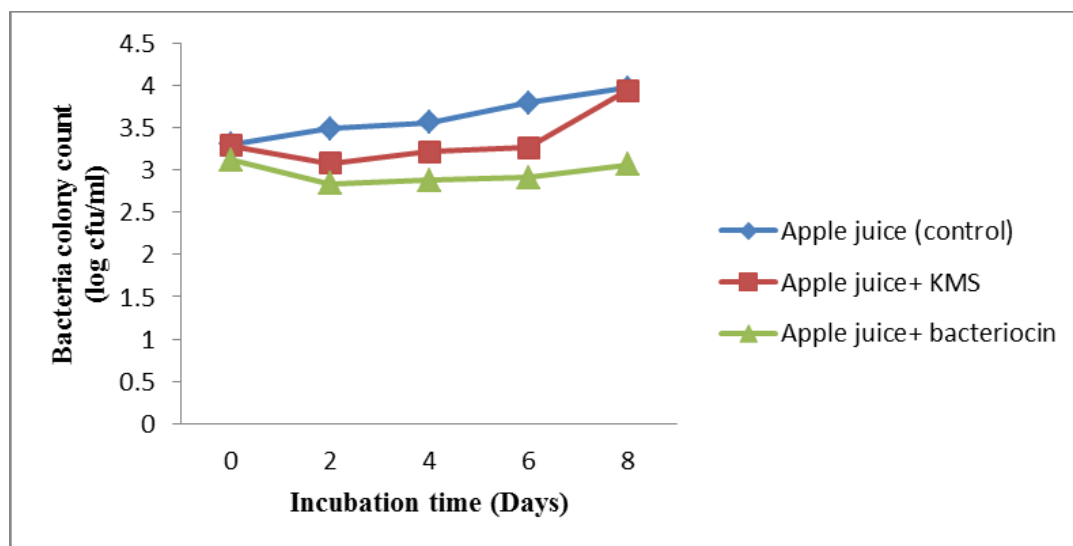


Figure 4 Comparative analyses of immobilized bacteriocin and chemical preservative in enhancement of shelf life of apple juice

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