

Bacteriocin mediated bio preservation of fortified sugarcane juice and detection of spoilage microbes by MALDI-TOF

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Abstract

Quality storage of ready-to-serve (RTS) sugarcane juice is still debatable. The present study aimed to screen the effect of bacteriocin, and nisin @ 10ppm for the preservation of citrus lime fortified sugarcane juice at a storage temperature of 2-8°C to provide an RTS beverage. Raw juice was extracted, filtered and fortified with 10% citrus lime extract, pasteurised added with 10 ppm of nisin. The results of the shelf life studies revealed that the total viable count of bacteria, mould and yeast on the 14th day of storage was 2.76 cfu/ ml, 2.95 cfu/ ml, 2.10×10^3 cfu/ml respectively. The total viable count of bacteria was found to be below the detectable limit; the count of yeast exceeded the permissible limit and caused browning of cane juice, and cracking of bottles thereafter. The rapid identification of different bacteria and yeasts was performed using MALDI-TOF MS. Overall analysis results showed matching hints of bacterial pathogens such as *Acinetobacter calcoaceticus*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Escherichia coli*, *Salmonella choleraesuis*, *Serratia marcescens*, *Veillonella atypica*. Though many bacterial species matching patterns were obtained, Bacteria like *Staphylococcus epidermidis* and yeasts like *Candida krusei* and *C. tropicalis* were found to have high consistency and high confidence score values of 2.00 – 3.00. Few other significant common pathogenic fungi like *Cryptococcus neoformans* and *Trichophyton tonsurans* were identified with score values of 0.00 - 1.69. While the physicochemical properties, pH, total soluble solids (TSS), total sugars, and reducing sugars were determined, we found the stored juice to be deteriorating significantly from day 1 to day 14 respectively. Therefore, the deteriorating effect on the quality of the juice could be due to microbial growth even after nisin treatment. Hence, from the present study, it is evidenced that sugarcane juice even under refrigerated conditions harbours the contaminant bacteria and yeasts. The study suggests that the bottled cane juice could be preserved using bacteriocin like nisin at 2-8°C for 5 days.

Keywords: Ready to serve, Sugarcane juice, bacteriocin, nisin, biopreservation, MALDI-TOF.

Introduction

Sugarcane juice is usually served fresh and sugarcane juice could not be served as ready to serve (RTS) beverage in the packed container without chemical preservatives. As with other fruit juices, it is highly impossible to store sugarcane juice for a longer duration due to faster spoilage by the inhabitant bacteria, mould and yeast of the cane juice. The biochemical properties of sugarcane juice act as a substrate enriched with all the nutrients for the fastidious proliferation of multiple numbers of microorganisms which ultimately degrade sucrose into lactic and acetic acid, alcohols and polysaccharides in the sugarcane juice [1].

Sugar cane juice darkens quickly after extraction due to the rapid oxidation of some of the flavonoids, polyphenol and organic acids making it unfavourable for commercialization by making it into an unappealing dark brown colour. It has been a challenging venture to preserve sugarcane juice in bottles and serve a fresh one without adding any preservatives. When preserved, normally Potassium metabisulfite which is yeast and mould inhibitor is being used widely for the preservation of sugarcane juice. It is highly essential and indispensable for quality assurance of the finished products in the market. Nowadays, milk is marketed only after pasteurisation so as to kill harmful pathogens such as *Coxiella* and *Mycobacterium* that are inherently associated with fresh milk. Likewise, certain bacteria, yeasts and fungi are conditional inhabitants in soil or on the surface of the plant, are potentially pathogenic and cause dreadful diseases when ingested without any processing.

Preservation of fresh and raw sugarcane juice is a challenging and an ever ending problem in the beverage industry. Meeting the demands for nutritious and safe foods has resulted in increased interest in non-thermal preservation techniques. Antimicrobial compounds derived from microbial, plant and animal sources that are added either directly or indirectly to fruit juices effectively control and inhibit spoilage microorganisms [2]. There are certain other changes in the physicochemical and biochemical properties of the juice due to the effect of fermenting microbes like *Leuconostoc mesenteroides* and *L. dextranum*. These organisms convert the sucrose molecule into polysaccharides, such as dextran [3]. In certain cases, contamination occurs by Gram-negative bacteria namely, *Gluconacetobacter sucrofermentans*, *G. xylinum*, *G. obediens* that convert sucrose into cellulose biopolymers and gluconic acids [4]. Activation of the invertase enzyme occurs soon after the cutting of sugarcane during harvest and the non-reducing sugars in the cut end begins to reduce into glucose and fructose and the percentage of reducing sugars indicates cane deterioration. Titratable acidity of the preserved juice gradually increases with decreasing pH due to microbial contamination [5]. Therefore, effective antimicrobial preservation would meet the need of the ready to serve (RTS) cane juice with effective physicochemical property.

Nisin, an antimicrobial peptide-based Type A (I) antibiotic substance is secreted by certain Gram-positive bacteria such as *Lactococcus* and *Streptococcus* species as a part of their metabolic activity. These antibiotic substances are ribosomally synthesised peptides that kill and inhibit the growth of wide species of pathogenic bacteria. In 1988, it was approved by the Food and Drug Administration (USA) and designated as a Generally Regarded As Safe (GRAS) preservative. Nisin meets the Qualified Presumption of Safety (QPS) concept and is very easily digested by the gastrointestinal tract of human subjects [6]. The use of bacteriocins as natural food preservatives especially in meats, fish products,

juices, canned foods dairy foods and beverages, fulfills consumer need for high safety and quality foods without the use of carcinogenic chemicals. DuPont, Danisco have commercialized nisin under the trade name Nisaplin® and Pediocin PA1 in the name of Microgard™, Alta 2431 by Quest International, Netherlands. The consumer trend in search of newer organic products of natural origin urges the use of nisin to check the bactericidal capability against Gram-positive bacteria [7].

With the knowledge of use of bacteriocin, it is indispensable to assess the effect of Nisin on the microbial growth associated with preserved sugarcane juice that is served as RTS in packed containers. Added to this, microbial diversity profiling of sugarcane juice enables the development of specific bio-preservatives to eliminate particular organisms to reduce sucrose deterioration of juices. Therefore, this study is aimed at analysing the effect of nisin as a natural preservative and characterising the microbial status of the sugarcane juice and their physicochemical properties at 2-8°C.

MATERIALS AND METHODS

Production of fortified sugarcane juice

Fortified sugarcane juice was standardized and developed using citrus lime extract that acts as both a fortifying agent and also a natural preservative. Sugarcanes were obtained from Sugarcane Research Station, Cuddalore, Tamil Nadu. About 10-11 month cane of variety CoC 24 which had organoleptically high acceptance with an overall acceptability score of 9.7% were collected and the outer ring of the cane was peeled off using a clean knife, washed and crushed using 15 HP pre-sterilised clean cane crusher under aseptic condition.

Assessment of nisin antibacterial effect with different concentration

The concentration of nisin @ 0, 5, 10, 20, 25 ppm were optimised for addition as a bio preservative to arrest the growth of bacteria in fortified sugarcane juice. This was carried out by serial dilution agar plate technique. We used about 10 ml of freshly extracted sugarcane juice sample was diluted with 90 ml of 0.1% sterile peptone water (1 g peptone, 1000 ml sterile distilled water) and plated on nutrient agar at pH 5.5 for enumeration of bacteria. Uninoculated plates of nutrient agar were used as a control. Total plate count was expressed in CFU/ml Concentration of 10 ppm of nisin was standardized to exhibit antibacterial effect and used for further study.

Production of sugarcane juice

Amongst all, then the raw juice was filtered, added with 10 ppm of bio preservative nisin (pre-standardised against LAB test cultures) and fortified with 10 per cent citrus lime extract (membrane filtered) to increase the overall acceptability of the juice for consumption. The fortified sugarcane juice was filled up in pre-sterilised PET bottles and stored at 2-8°C.

Isolation of microorganisms from sugarcane juice

The microorganisms such as bacteria, moulds and yeasts responsible for the rapid deterioration of the sugarcane juice were isolated by the aerobic plate count method. About 35 numbers of different colonies with distinguished morphology were isolated randomly, screened and purified separately in slants.

Preparation of culture samples for MALDI-TOF MS identification

The culture sample was prepared as described by Schulthess et al [8] The cultures grown at 24 hours of incubation were isolated from the bottled sugarcane juice and were prepared for MALDI-TOF MS by direct colony transfer - formic acid method. The culture was smeared using a toothpick on a polished steel MSP 96 target plate and 1 µL of 70% formic acid was added to the culture smear and air-dried. Then, 1 µL of a saturated -cyano-4-hydroxycinnamic acid (HCCA) matrix solution in 50% acetonitrile 2.5% trifluoroacetic acids was overlaid into it and air-dried again at room temperature. The target plate was placed into the plating chamber of the mass spectrometer and closed for performing the analysis.

Rapid Identification of microbial cultures using MALDI-TOF MS

The MALDI-TOF mass spectra measurements of samples loaded in the target plate were performed using a Bruker MALDI TOF Biotyper 4.1.70 (PYTH) at Agricultural College and Research Institute, (TNAU), Trichy. As per the instructions of the manufacturer, calibration was performed using the test standard. Analyses of all the isolates were run in triplicates. The identification results were expressed by BioTyper log (scores): 2.00 - 3.00 indicates high-confidence identification, 1.70 - 1.99 denotes Low-confidence identification and 0.00 - 1.69 signifies No Organism Identification Possible.

Physicochemical properties and microbial quality of sugarcane juice

The various physicochemical properties such as pH, titratable acidity, total soluble solids (TSS), Total sugars, viscosity and microbial counts such as total bacterial count, yeast count and fungal count were determined [9 -11].

RESULTS AND DISCUSSION

The study aimed at analysing the effect of nisin on microbial contamination of sugarcane juice whether it could be a suitable bio preservative for providing as RTS drink. After 14 days of preservation of sugarcane juice, bottle swelling and sedimentation at the bottom of the bottles even at 10°C was observed. This could be due to microbial contamination that occurs at different stages of juice processing such as contamination of sugarcane, roller crusher, collecting vessel, ice, hands of the personnel and filter cloth. About 35 distinct colonies were observed and were chosen for further characterisation using MALDI-TOF. The results revealed matching hints of bacterial pathogens such as *Acinetobacter calcoaceticus*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Escherichia coli*, *Salmonella choleraesuis*, *Serratia marcescens*, *Veillonella atypica*. MALDI analysis revealed that bacteria like *Staphylococcus epidermidis* and yeasts including *Candida krusei* and *C. tropicalis* with high consistency and high confidence scores as given in Table 1. The low-temperature storage would prevent the growth of the organism. Richa Karmakar *et al* have shown that the growth of yeast and fungi in juice stored at 30°C increased significantly after 6 days ($p < 0.05$), but bacteria were found to decrease at the later stage of storage [12]. In another research experiment, *P. membranifaciens* was found to be resistant to heat, moderate amount of salt, SO₂, sorbic, benzoic and acetic acid. *Byssoschlamys fulva*, *B. nivea*, *Neosartorya fischeri*, and *Talaromyces* are the mould that could survive commercial heat pasteurization treatment,

usually applied to fruits and fruit products because of the presence of heat resistant ascospores [13-15]. But in the present experiment different yeast species were exploited.

Nyman *et al* reported that sorbic and benzoic acids are commonly used preservatives in soft drinks. The association of benzoic acid with ascorbic acids generates an accumulation of benzene in soft drinks [16]. Another study reported that compounds like flavanones, polymethoxylated flavones, coumarin and tetrazene in lemon peel extract acts as an antimicrobial agent against *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Micrococcus aureus* [17]. Abhilasha and Pal showed that ozone and nisin treatment of sugarcane juice helped to retain the original colour of the juice by inhibiting the phenoloxidase activity. But ozone-treated samples were not highly accepted for sensory perception. Though the overall acceptability score of sugarcane juice were significantly highest in the ohmic heated sample, nisin treated juice samples was also highly accepted [18]. Nisin acts as an effective agent against a wide range of food spoilage and pathogenic bacteria and also works actively across a range of pH values and could able to resistant high-temperature treatments of processed foods [19]. Conversely, it is not effective against mould and yeasts. Nisin is a heat-stable peptide that inhibits the growth of most Gram-positive bacteria (20-22).

Willard *et al* had reported that milled sugarcane revealed a diverse population of polysaccharide producing bacteria such as *Acinetobacter* sp., *Psychrobacter* sp., *Enhydrobacter aerosaccus*, *Enterobacteriaceae* sp., *Porphorymonas* sp., *Weissella* sp., *Leuconostoc* sp., *Streptococcus* sp, *Bacillus* sp., *Microbacterium ginsengisoli*, *Micrococcus luteus* and *Propionibacterium acnes*. The bacterial genera that are responsible for spoilage of cane juice include *Lactobacillus*, *Leuconostoc*, *Enterobacter*, *Micrococcus*, *Flavobacterium*, *Lactobacillus*, and the spoilage yeasts are *Candida*, *Saccharomyces*, *Pichia* and *Torulopsis*, moulds such as *Aspergillus*, *Cladosporium*, *Monilla* and *Penicillium* cause juice deterioration in less than 24 hours [23]. Also, various phenols in the juice upon rapid oxidation change the appealing colour of the juice and make it unacceptable in storage even under refrigerated conditions [24].

In the present study, we observed that the pH was decreasing as and when the days of preservation are prolonged. While, the Titratable acidity, reducing sugars and viscosity were found to be increased, and total soluble solids, sugars were depleting. Results are depicted in Table 2. This could be attributed due to the presence of gas-producing bacteria and yeasts.

Previous studies of Olaniran *et al* suggested that lime effectively controlled the rate at which increase occurred during storage by double-fold when compared with ascorbic and citric acid used in the study. There was a significant decrease in the pH of the fresh juice from 4.50 to 4.03 in addition to 4% lime. The addition of lime as preservative did not negatively affect the acceptance of the juice thus recommended. In the present research also similar results were obtained [25].

Another study which tested the quality of beverage from sugarcane variety CoP 92226 showed a satisfactory storage stability of 90 days at refrigeration as well as room temperature could be prepared from pasteurized juice after the addition of 40 mg citric acid per 100 ml and 150 ppm of potassium metabisulphite. The citric acid was able to lower the pH of

sugarcane juice to 4.9 which gave a preservative action and inhibited the growth of microorganisms during storage [14]. Tournas *et al* reported that *Zygosaccharomyces bailii*, *Candida krusei*, *Saccharomyces bisporus*, *Schizosaccharomyces pombe* and *Pichia membranifaciens* are resistant to preservatives and tolerate chronic intracellular pH drops with the help of phosphofructokinase enzyme [15].

Study on low temperature storage (10°C) of cane juice by Mishra *et al* had reported that a combination of gamma radiation (5 kGy) with permitted preservatives such as citric acid (0.3%), sodium benzoate (0.015%), potassium sorbate (0.025%), and sucrose (10%) at 10°C could inhibit juice spoilage microorganisms and could able to preserve raw sugarcane juice for more than a month [24]. Studies on the stability of sugarcane juice blended with anola juice at refrigerated and room temperature good quality beverage from sugarcane juice with satisfactory storage stability of 50 days at refrigeration and 20 days at room temperature could be achieved from heat-treated sugarcane juice beverage at 75°C for 10 min after addition of 5% anola juice in sugarcane juice [26].

Preserving the beetroot juice (40% blended) with 20% each of carrot and tomato juice and incorporating bacterial cellulose with 20 ppm of nisin at low pH ensured the microbiological stability of the product for 90 days at room temperature [27]. Another study showed that thymol and nisin can be used as a potential bio preservative component in organic preservation of sugarcane juice which was effective against the control of four species of *Shigella*, such as *S. boydii*, *S. dysenteriae*, *S. flexneri* and *S. sonnei* [28].

About 5% culture-free solution of bacteriocin bio preservative was highly effective in the control of *S. aureus*, *E. coli* and *P. aeruginosa* up to 48 hrs in sugarcane juice preservation [29]. Moshaghi *et al* reported that lysozyme was less effective against *E.coli* in food preservation and nisin was less effective in controlling *Listeria monocytogenes*. Nisin and lysozyme in combination were effective in the control of Gram-positive bacteria, especially *S. aureus* [30]. A similar study by Mwambete and Mpenda (2019) had reported that the sugarcane juice sold in street vending outlets of Tanzania recorded microbial contaminants above the acceptable limits of 10 to 100 folds. Bacterial counts ranged from 1.44×10^5 to 6.0×10^5 cfu/ml and fungal counts from 1.36×10^5 to 2.64×10^5 cfu/ml. About 25 bacteria, 15 yeasts and 8 mould species were isolated from the sugarcane juice. *Escherichia coli*, *Candida albicans* and *Aspergillus flavus* were the most frequently isolated [31]. Sumonsiri showed that nisin at the concentration of 50 and 75 ppm significantly arrested the aerobic microbial counts in micro-filtered coconut water when compared to the control sample without affecting turbidity, colour, and sensory parameters after 7 days of preservation in refrigerated storage [32]. Use of nisin has effectively been a good preservative for them. Still, contrasting reports on use of nisin being reported in controlling bacterial growth with respect to various foods and beverages.

Thus, the present study clearly reveals that use of nisin could curtail the cost involved in the preservation and processing of sugarcane juice without compromising the nutrient parameters of the final product. Also, the study brings the bottling cane juice harbours bacteria and few pathogenic yeasts and thus being controlled with the use of nisin.

Conclusion

Juices are subjected to spoilage by rapid multiplication of acid tolerant and osmophilic microorganisms but immediate consumption of the juicy product is not possible in all situations and preservation by addition of sugar or chemical preservatives, thermal pasteurisation, hurdle Technology, high pressure processing, irradiation technique are used for long time storage of juices. But natural preservatives from microbial origin, especially nisin in sugarcane juice preservation, are very less utilised due to the narrow anti-microbial spectrum of bacteriocins. Nisin proved to control certain Gram Positive Bacteria such as lactic acid bacteria and *Leuconostoc*. These two are basically reported to spoil the sugar cane juice and are most responsible for staling of canes after the harvest as reported in earlier studies. The dominating yeast species present in acidic fruit juices had been reported as *Candida parapsilosis*, *C.stellata*, *Torulaspora delbrueckii* and *Zygosaccharomyces rouxii*. In the present study also similar types of yeasts were reported which remained uncontrolled after 10 days of nisin based cold chain preservation also. But would rather be proven to inhibit the turbidity, pellicle formation, sediment deposition or clumping by organic acid or aldehyde fermenting off flavour production till 10 days of storage. The present investigation reveals that combination of citrus lime extract with nisin reduces the risk of proliferation of wild fermenting bacteria and yeast.

The study showed very few pathogenic microorganisms after 14 days of preservation. So, preservation with nisin at lower temperature for a short period of 5 days at cold storage of 2-8°C could be suggested for RTS bottled cane juice.

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Table 1: Microorganism grown in cultures from stored sugarcane juice identified by MALDI-TOF mass spectrometry

S.No	Identified spoilage organisms	Confidence of Identification	Consistency	Average Score value
	<i>Staphylococcus epidermidis</i>	+++	A	2.015
	<i>Staphylococcus epidermidis</i>	+	B	1.88
	<i>Candida krusei</i>	+++	A	2.09
	<i>Candida krusei</i>	+	A	1.80
	<i>Candida tropicalis</i>	+++	A	1.9
	<i>Candida krusei</i>	+	B	1.67*

A - High consistency; B – low consistency; C – no consistency.

+++ - High Confidence; + - Low confidence.

*No confidence on average score value

Table 2: Physicochemical properties of bottled fortified sugarcane juice on Nisin preservative at 2-8°C

Preservation duration (days)	pH*	Titrateable acidity*(%)	TSS*(°Brix)	Total sugars*(%)	Reducing sugars*(%)	Viscosity* (cps)
0	4.7	0.65	20.20	19.10	0.97	2.77
2	4.6	0.68	20.11	19.05	0.94	2.71
4	4.4	0.72	19.70	18.82	1.77	2.94
6	4.3	0.88	19.15	18.66	1.89	2.99
8	4.3	0.90	18.74	17.98	2.04	3.12
10	4.2	0.99	17.44	17.55	2.35	3.24
12	3.8	1.22	14.77	13.51	3.57	3.88
14	3.5	1.98	12.45	12.89	4.00	3.96

*Mean of three repeated observations on each day.

Table title and legends:

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