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Natural preservation of orange juice to enhance its shelf stability and microbial safety using purified bacteriocin of *Brevibacillus borstelensis*AG1

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ABSTRACT

In present study, Brevicin produced from *Brevibacillus borstelensis* AG1 isolated from Marcha - a natural herbal cake of North East India was used to enhance the shelf life and microbial safety of orange juice. Food borne pathogens viz. *Listeria monocytogenes* MTCC 839, *Bacillus subtilis* CRI and *Clostridium perfringens* MTCC 1739were inoculated at the amount of 8.16, 8.13 and 8.18 log CFU/ml, respectively in orange juice to study the preservative effect of bacteriocin against them as compared to chemical preservative – sodium benzoate and commercial biopreservative i.e. nisin. Viable cells were counted periodically and a consistent reduction in number of viable cells of each tested pathogen was observed. Brevicin when tested was found antagonistic to most challengeable and serious food borne pathogens to control in processed fruit/vegetables products. Brevicin was found active over a wide pH range i.e. 3.0 to 11.0 and thermostable upto 100^{o}C. It showed better preservative potential by reducing the pathogenic load of indicators in orange juiceas compared to control having maximum spoilage proving its potential as a natural preservative to enhance microbial safety and shelf life of different food items.

Keywords: Biopreservation, bacteriocin, application, *Brevibacillus borstelensis* AG1, orange juice

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INTRODUCTION

Nowadays there is an increasing trend to consume ready-to-eat fruit and vegetable products, with minimal processing and preservatives. There are various physical and chemical methods to preserve the food, but the use of chemicals has clinically been proven harmful to our system. For this reason, the food technologists deviating their research towards the screening of natural, eco-friendly safe and broad spectrum preservatives[1]. Biological activity of microorganism for production of a range of harmless metabolites that can suppress the growth and survival of an undesirable microbiota in foods may emerge as an attractive solution for food preservation[2]. One of the most important contributions of these microorganisms is the extended shelf life of the fermented products. Growth of spoilage and pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter derived inhibitors such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins[3]. The *Bacillus* sp./LAB have the ability to produce antimicrobial compounds called bacteriocins. In recent years, bacteriocin had grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of food enhanced in shelf life or safety[4,5]. Bacteriocins are antimicrobial peptides or proteins produced by strains of diverse bacterial species with various mechanisms of action and exhibit bactericidal activity against species closely related to producer strain[6,7,8]. Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved. In recent years, considerable effort has been made to develop food applications for many different bacteriocins using bacteriocinogenic strains[9,8]. Different bacteriocin producing strains of *Bacillus* species as well as Lactic acid bacteria have been isolated for this purpose but the keen interest towards bacteriocin of lactic acid bacteria worldwide is due to their essential role in majority of food fermentation, flavor development and preservation of food products along with proving safer for health[10]. Strains from the *Bacillus* genus produce a diverse array of antimicrobial compounds, with several different basic chemical structures[11,12]. Most studies regarding food applications have focused on LAB bacteriocins, mainly nisin and a few others[13]. Although nisin is the only bacteriocin currently licensed as a commercial biopreservative, its applications are restricted due to its very low activity at a neutral or an alkaline pH. The antimicrobial activity of these natural substances against food borne pathogens, as well as spoilage bacteria, has raised considerable interest for their application in food preservation Therefore, the search for new bacteriocins with improved physico-chemical properties (stability in a wide range

of pH and temperature) and also a broad antimicrobial spectrum is of great interest for their application in foods.

MATERIALS AND METHOD

Bacterial isolate and bacteriocin production

Brevibacillus borstelensis AG1 -a bacteriocin producing strain isolated from Marcha- a traditional starter culture for the production of various indigenous sweet-sour alcoholic beverages of North East Himalayan States of India was used to estimate the antagonistic potential of bactericoin produced by it against spoilage causing and serious food borne pathogens i.e. *Listeria monocytogenes* MTCC839, *Bacillus subtilis* CRI and *Clostridium perfringens* MTCC1739. The antagonistic spectrum was checked by bit disc and well diffusion assay[14,15]. Bacteriocin produced was purified to homogeneity by ammonium sulfate precipitation (50% salt saturation) followed by gel exclusion chromatography. Purity of bacteriocin was checked by SDS-PAGE[16]. The activity of culture supernatant, partially purified and purified bacteriocin was calculated by serial two fold dilution method[14]. Two hundred µl of sample was poured into the wells cut on the lawns of indicators in nutrient agar plates. The plates were then incubated at 35^{o}C for 24 h and the zones of inhibition formed around the wells were measured. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units per ml (AU ml^{-1}). The activity of purified bacteriocin was observed for 4 weeks against test pathogen viz. *L. monocytogenes*MTCC839 to assess its efficacy and stability.

Application of bacteriocin as a biopreservative to increase shelf life of orange juice

The orange juice procured from local market in sterile conditions was used to evaluate biopreservative potential of bacteriocin. Clean sterile 50 ml test tubes were taken and orange juice sample (40 ml) was put in each set of sterile test tubes. Orange juice was pasteurized by keeping in hot water at 72^{o}C for 2 min. The juice was then brought to room temperature. Total Soluble Solids (TSS) and pH of the orange juice were checked. The test strains viz. *Listeria monocytogenes* MTCC839, *Bacillus subtilis* CRI and *Clostridium perfringens* MTCC1739were used for inoculating different sets of orange juice sample at the amount 8.13, 8.16 and 8.18 log CFU/ml, respectively (Table 1) and the activity of partially purified bacteriocin, purified bacteriocin, nisin and chemical preservative was studied against them at room temperature (15-18^{o}C). These tubes were properly plugged, sealed with adhesive tape and hot wax. Biopreservatives i.e. partially purified bacteriocin, purified bacteriocin and nisin were added at the rate of 2000 parts per million

(ppm) while chemical preservative i.e. sodium benzoate was added at the rate of 600 ppm in pathogen treated orange juice for comparative study of different preservatives against test indicators while control was kept as such i.e. without addition of any preservative. The storage studies for stability were done at an interval of 0, 2, 5, 7 and 10 days at room temperature (15-18^{o}C) and the change in log CFU/ml was noted down.

Table 1: Application of purified bacteriocin as a biopreservative

	Tomato paste		
Set A	$O+I_L$+PBac	$O+I_B$+PBac	$O+I_C$+PBac
Set B	$O+I_L$+Bac	$O+I_B$+Bac	$O+I_C$+Bac
Set C	$O+I_L$+nisin	$O+I_B$+nisin	$O+I_C$+nisin
Set D	$O+I_L$+sodium benzoate	$O+I_L$+sodium benzoate	$O+I_C$+sodium benzoate
Set E	$O+I_L$	$O+I_B$	$T+I_C$

Where, i) $O+I_L$ orange juice inoculated with *L. monocytogenes* MTCC 839

ii) $O+ I_B$ orange juice inoculated with *B. subtilis* CRI

iii) $O+I_C$ orange juice inoculated with *Clostridium perfringens* MTCC 1739

iv) $O +I_I$ is control without preservative.

Statistical Analysis

The experimental data were analyzed by using factorial completely randomized design (CRD) at 5 % level of significance. Factorial CRD was used for comparative study of biopreservative with nisin and sodium benzoate against the test indicators.

RESULTS AND DISCUSSION

In the present investigation biopreservative effect of bacteriocin produced by *Brevibacillus borstelensis* AG1to enhance the shelf life of orange juice with other commercial preservatives i.e. nisin and Sodium benzoate was compared. The bacteriocin producing isolate was found to be gram positive, catalase positive, rod shaped having circular and creamish colonies on nutrient agar medium and has been identified using 16S rRNA gene technique as *B. borstelensis* JX129162[16]. The culture supernatant of *B. borstelensis* AG1 showed wider zones of inhibition of 16 mm, 14 mm and 13 mm against *Listeria monocytogenes* MTCC839, *Bacillus subtilis* CRI and *Clostridium perfringens* MTCC1739, respectively indicating high titer of bacteriocin production (Figure 1). After purification, it crude bacteriocin exhibited high potential to inhibit the test indicators. Percent increase in inhibition zone sizes due to partially purified and complete purified bacteriocin against indicators viz. *L. monocytogenes* MTCC839, *B. subtilis* CRI and *C. perfringens* MTCC1739was found to be 20 to 80 %, 20 to 40 % and 37.5 to 62.5 % respectively(Table 2). Bacteriocin produced from *B. borstelensis* AG1 was purified by salt saturation technique (ammonium sulphate

precipitation) followed by gel exclusion chromatography. The precipitation was attained at 50 % level of saturation. After precipitation, bacteriocin of *B. borstelensis* AG1 had produced 30,00,000 AU/ml. The precipitates dissolved in buffer were subjected to gel exclusion chromatography followed by molecular weight determination using SDS-PAGE which was found to be 12 KDa[16]. The activity units of purified bacteriocin were found to be 40,00,000 AU/ml. It was found active over a wider ph range i.e. 3.0-11.0 and was thermostable up to 100^{o}C. Purified bacteriocin was stable when checked against indicator bacteria viz. *L. monocytogenes* for 4 weeks (28 days) enablingit to be used in food products to keep them safe for longer period (Figure2). Orange juice used in the present study has been found to have TSS and pH 8^{o}B and 4.3, respectively. As the bacteriocin was stable at low pH, it was tested to be used as biopreservative in acidic foods having low pH viz. orange juice with pH 4.3. After treating test indicators i.e. *L. monocytogenes* MTCC839, *B. subtilis* CRI and *C. perfringens* MTCC1739inoculated orange juice with bacteriocin, it was found that with increasing time there was a decrease in the number of viable cells as compared to control. In case of orange juice inoculated with *L. monocytogenes* MTCC 839, initially log CFU/ml was 8.13 for partially purified bacteriocin, purified bacteriocin, nisin, chemical preservative and for control having no preservative (Figure 3). A decrease of 1.06, 1.10, 0.97 and 1.06 log CFU/ml was observed on second day for partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. While in control, there was an increase of 1.06 log CFU/ml in case of control. On fifth day increase by 1.11, 1.12, 1.12 and 1.10 log CFU/ml in samples with partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate was observed while in control an increase of 1.11 log CFU/ml was observed. At day 7, an increase of 0.1, 2.12, 1.11, 1.10 and 1.07 log CFU/ml was observed for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. At day 10 the increase in microbial count by 2.13, 2.17, 3.14, 3.13 and 3.06 log CFU/ml for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. The CD value was found to be 0.05. The T value was 0.0020 and D value was 0.0018 and the TxD value was 0.0044. Among all the preservatives used, purified bacteriocin was found statically at par with sodium benzoate having mean log CFU/ml 9.20 and 9.19, respectively followed by partially purified bacteriocin and nisin with mean log CFU/ml 9.47 and 9.39, respectively. Maximum spoilage was observed in control having highest mean i.e. 10.69. Similar preservation studies for orange juice inoculated with *B. subtilis* CRI(8.16 log CFU/ml and 1.0 OD) were also carried for 0, 2, 5, 7 and 10 days. Initially log CFU/ml was 8.16 for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate. The means for control, partially purified bacteriocin, purified bacteriocin, nisin and

sodium benzoate were found to be 10.13, 9.24, 9.21, 9.31 and 9.22, respectively (Figure4). A decrease in log CFU/ml was observed on day 2 (by 1.09, 1.12, 0.97 and 1.06 log CFU/ml) for partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively while in case of control it was increased by 1.07 log CFU/ml. An increase of 1.06, 1.14, 1.13, 1.12 and 1.10 log CFU/ml was observed on day 5 for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. After day 5, there was a continuous increase in log CFU/ml by 0.14, 2.08, 2.10, 2.09 and 2.07 for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. The increase in microbial count on day 10 was reported very high (2.11, 2.16, 2.14, 2.13 and 2.08 log CFU/ml) for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate. The CD value was found to be 0.05. The T value was 0.0014 and D value was 0.0013 and the TxD value was 0.0030. Among applied preservatives, purified bacteriocin was were found to have preservative potential statistically at par with chemical preservative – sodium benzoate with log CFU/ml 9.41 and 9.42, respectively followed by partially purified bacteriocin and nisin with log CFU/ml 9.48 and 9.52, respectively. Maximum spoilage was found in control having highest mean i.e. 10.71. Effect of preservatives on orange juice inoculated with *C. perfringens* MTCC1739 (8.18 log CFU/ml and 1.0 OD) was also observed for 0, 2, 5, 7 and 10 days. Initial log CFU/ml was 8.18 for control, partially purified bacteriocin, purified bacteriocin nisin and sodium benzoate. The means for control, partially purified bacteriocin, purified bacteriocin nisin and sodium benzoate were 10.15, 9.25, 9.20, 9.33 and 9.24, respectively (Figure 5). A decrease of viable count of *C. perfringens* MTCC1739 was observed on day 2 (1.02, 1.12, 0.98 and 1.03 log CFU/ml for partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively) while an increase of 1.07 log CFU/ml was observed in case of control. Increase by1.08, 1.08, 1.12, 1.13 and 1.06 log CFU/ml was noted for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate on day 5. Then there was a continuous increase by 0.12, 2.06, 2.09, 2.08 and 2.09 log CFU/ml for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. Increase in microbial count was higher on day 10; the increase was by 2.11, 2.14, 2.07, 2.12 and 2.13 log CFU/ml was found for control, partially purified bacteriocin, purified bacteriocin, nisin and sodium benzoate, respectively. The CD value was found to be 0.05. The T value was 0.0116 and D value was 0.0015 and the TxD value was 0.0037. During the preservation studies, purified bacteriocin possessed a preservative effect statistically at par to chemical preservative-sodium benzoate (9.42 and 9.45 mean log CFU/ml) followed by partially purified bacteriocin and nisin (9.47 and 9.54 mean log CFU/ml) in orange juice inoculated with *Clostridium perfringens*

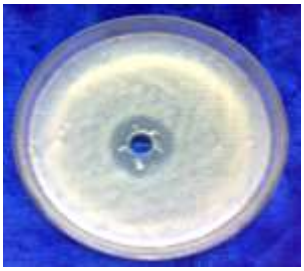
MTCC1739. Maximum spoilage was found in case of control having highest mean value. As the orange juice was deliberately inoculated with high number of tested strains (>8 log CFU/ml) to evaluate the antagonistic effect of bacteriocin, the pathogenic bacterial count has been increased to an extensive high number. Inhibition of the large number of test pathogens by bacteriocin used in this study rendered it safe to be used as bio preservative to enhance the shelf life and safety of the food items. Bacteriocin produced by *Brevibacillus borstelensis* AG1 has been found to be effective against gram positive test indicators viz. *L. monocytogenes* MTCC839, *B. subtilis* CRI and *C. perfringens* MTCC1739. From the above study, it is observed that preservative potential of purified bacteriocin was comparable to commercially used chemical preservative –Sodium benzoate. Purified bacteriocin was found quite effective in preserving and enhancing shelf life of foods having low pH viz. orange juice. Reduction in CFU/ml of pathogenic bacteria is due to the bactericidal action of the bacteriocin. Thermostable nature is one of the desirable characteristic for application of bacteriocin as biopreseravative. The bacteriocin produced by *B. borstelensis* has also been found to be thermostable i.e. it is able to resist heat and thus can be used as a potential biopreservative. Inactivation of purified bacteriocin with proteolytic enzyme reflects its protein nature and thus suggests its break down in digestive tract of human beings rendering it completely harmless[16]. Purified bacteriocin has been found active in low pH range i.e. upto 3.0, so it can be used as a biopresevative in acidic food items. Since, in the present study, test pathogens had been added in very higher concentration to observe antimicrobial potential of different preservatives and therefore samples under study could be kept safe for short period only. But in nature, contamination of processed food items takes place at very low pace; therefore it can be stated strongly that bacteriocin produced by *B. borstelensis* AG1 would prove very effective to enhance shelf life of acidic foods i.e. orange juice for long time because of its strong preservative attributes. Similar reports regarding the biopreservation and shelf stability of fruits juices have also been observed by many workers. Grande *et al*. (2005)[17] studied the effect of enterocin AS-48 in fruit juice inoculated with *A. acidoterrestris*. Vegetative cells of *A. acidoterrestris* DSMZ2498 were inactivated by 2.5 µg/ml of enterocin AS-48 in natural orange and apple juices incubated at 37^{o}C. No growth was detected in both juices until the 15th day of observation. Pei et al. (2014)[18] also studied the inhibitory effects of a newly discovered bacteriocin- bificin C6165 in diluted apple juice and found it effective against *Alicyclobacillus acidoterrestris* strains. Bificin C6165 was found to be a strong bacteriocin against both vegetative cells and spores thus preventing contamination of fruits products and juices with serious pathogens. Lucas et al. (2006)[19] incorporated enterocin AS-48 (6 µg/mL) in low-acid vegetable canned foods (tomato paste, syrup

from canned peaches, and juice from canned pineapple) and observed that the bacteriocin caused complete/partial inactivation of *B. coagulans* cells. The bacteriocin was also highly effective against thermophilic endospore formers in canned foods. Similarly, in canned corn and peas samples inoculated with a cocktail of two *Geobacillus stearothermophilus* strains, enterocin AS-48 (7µg/g) was found to reduce the viable cell counts below detection levels during storage of samples at 45°C for 30 days[20].

Table 2: Percent increase in inhibition zone size (mm) against test indicators of partially purified and purified bacteriocin of *Brevibacillus borstelensis* AG1 over culture supernatant

Indicator	Bac*	Bac** - Bac*		Bac*** - Bac*	
	Zone size (mm)	Zone size (mm)	% increase	Zone size (mm)	% increase
L. monocytogenes	10	12	20	18	80
B. subtilis	10	12	20	14	40
C. perfringens	8	11	37.5	13	62.5
Bac* -	Culture supernatant				
Bac** -	Partially purified bacteriocin				
Bac*** -	Purified bacteriocin				
% increaseBac**	$\frac{\text{Bac**-Bac*}}{\text{BAc*}}$				
% increaseBac***	$\frac{\text{Bac***-Bac**}}{\text{BAc*}}$				

***L. monocytogenes* MTCC 839**



***B. subtilis* CRI**

***Clostridium perfringenes* MTCC 1739**

Figure 1: Antimicrobial activity of bacteriocin produced by *Brevibacillus borstelensis* AG1 against *L.monocytogenes* MTCC 839, *B. subtilis* CRI and *Clostridium perfringenes* MTCC 1739.

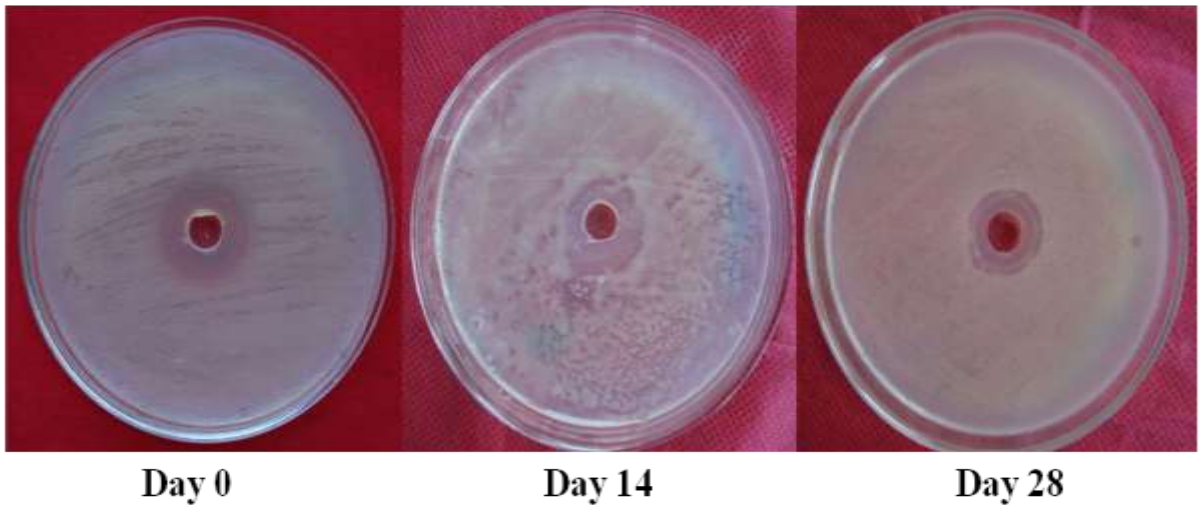


Figure 2: Effect of storage on activity of purified bacteriocin against *L. momocytogens.*

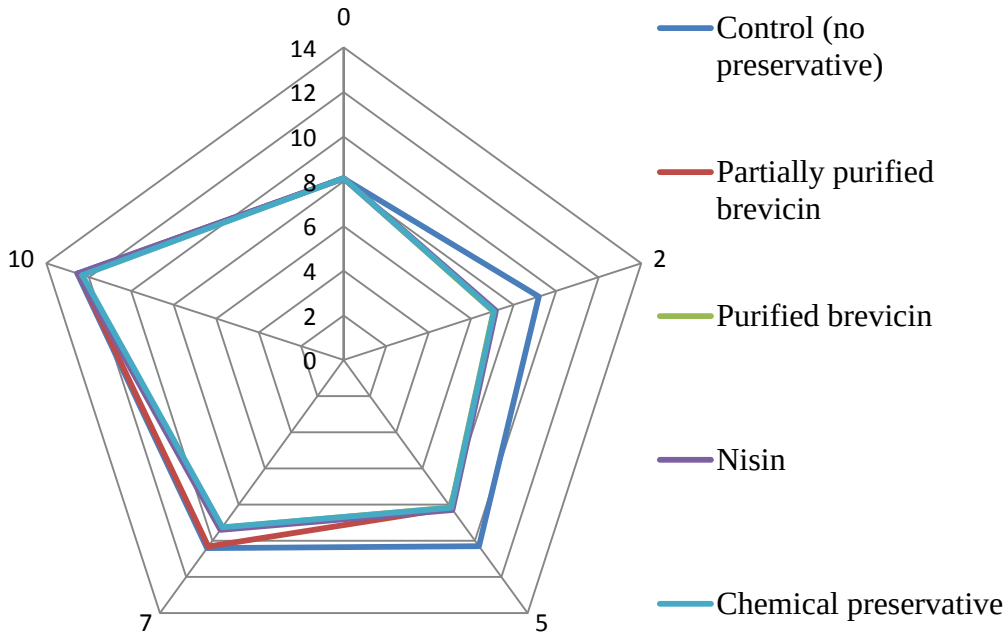


Figure 3:A comparative study to use natural biopreservative (partially purified brevicin and purified brevicin), nisin and sodium benzoate against *L. monocytogenes* to enhance shelf life of orange juice.

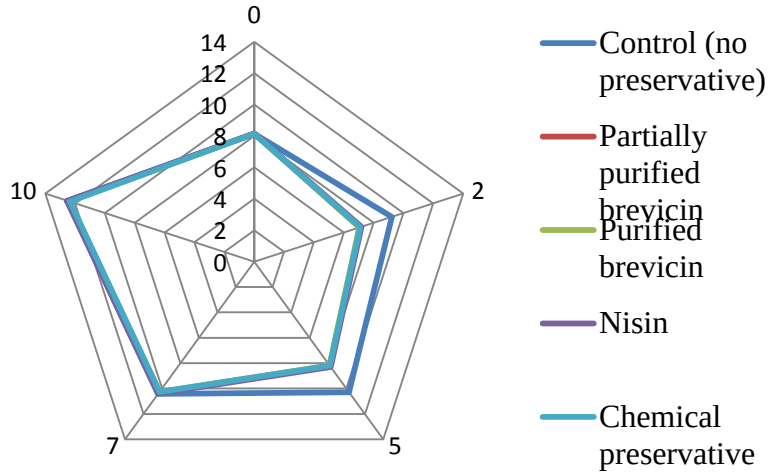


Figure 4: A comparative study to use natural biopreservative (partially purified brevicin and purified brevicin), nisin and sodium benzoate against *B. subtilis* to enhance shelf life of orange juice.

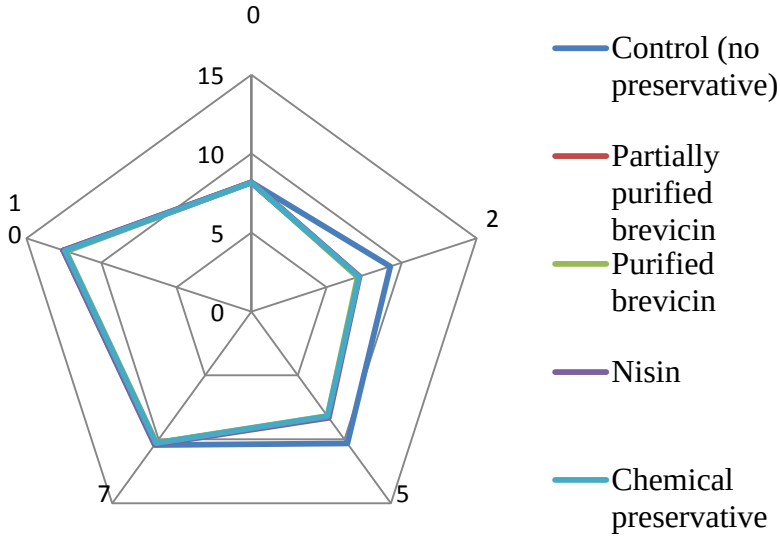


Figure 5: A comparative study to use biopreservative (partially purified brevicin and purified brevicin), nisin and sodium benzoate against *C. perfringens* to enhance shelf life of orange juice.

CONCLUSION

The purified bacteriocin secreted from *B. borstelensis* AG1 was found antagonistic to the most challengeable and serious food pathogens i.e. *L. monocytogenes*, *B. subtilis* and *C. perfringens* that required to be controlled in any food industry. The use of purified bacteriocin as food biopreservative in orange juice had been found very effective. Antimicrobial effect shown by

purified bacteriocin was found about at par with commercial chemical preservative i.e. sodium benzoate leading to a conclusion that bacteriocin secreted from *B. borstelensis* AG1 was an effective natural preservative to enhance safety and shelf life of acidic food items.

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