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BACTERIOCIN PRODUCING LACTIC ACID BACTERIA FROM FERMENTED GREEN GRAM BATTER - BIOLOGICAL PRESERVATIVE AGENT IN SWEET LIME AND LEMON JUICES

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ABSTRACT

Isolation of bacteriocin producing lactic acid bacteria (LAB) and to identify its biopreservative property in sweet lime and lemon juices. Bacteriocins are proteins produced by bacteria which inhibit the growth of similar or closely related bacterial strains. LAB are predominantly present in the fermented foods are well known for producing bacteriocins. LAB were isolated from fermented green gram batter using de Man, Rogosa and Sharpe (MRS) media. Total of four isolates were isolated from the green gram (GG) batter and were named as GG1, GG2, GG3 and GG4. The GG1 and GG2 isolates were identified as Lactobacillus casei. The GG3 and GG4 isolates were identified as Streptococcus species according to Bergey's Manual of Systemic Bacteriology. Antimicrobial activity of the bacteriocin was studied at pH 5 and pH 7 by agar well diffusion method. The bacteriocin produced by these organisms inhibited the growth of Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Klebsiella sps. The bacteriocin of all the four isolates exhibited highest antibacterial activity at pH5 except in case of bacteriocin produced by GG2 showed highest antibacterial activity at pH 7. The biopreservative efficacy of bacteriocins of all four isolates was observed in sweet lime and lemon juices. The minimum inhibitory concentration (MIC) of bacteriocins for all the four isolates was found to be 100 ul in sweet lime. The MIC of bacteriocins of GG1 was 25 µl; GG2 and GG3 was 50 ul, whereas bacteriocin of GG4 isolate could not inhibit the growth even at 100 µl in lemon juice.

KEY WORDS

Bacteriocin, Biopreservative, Lactic acid bacteria

INTRODUCTION

Biopreservation is natural antimicrobials of preserving food and extending its shelf life [1]. The LAB has antagonistic properties which make them particularly useful as biopreservatives. When LABs compete for nutrients, their metabolites often include active antimicrobials such as lactic and acetic acid, hydrogen peroxide and peptide bacteriocins. LABs are harmless to humans [2].

The LAB bacteriocins are used as an integral part of hurdle technology. Using them in combination with other preservative techniques can effectively control food spoilage bacteria and other pathogens, and can inhibit the activities of a wide spectrum of organisms, including inherently resistant Gram-negative bacteria [1]. Chemical preservatives have been used in food products to inhibit microbial growth which lead to serious health problems, thus challenging the

food scientists for providing safer and healthier food. However consumer demand for faster, healthier and ready to eat products have strongly demanded the use of more natural preservatives instead of chemical preservative. Although many types of bacteriocins such as subtilin, cerein, thuricin, plantaricin etc have been isolated and characterized, so far only one bacteriocin, Nisin, has been given the status of biopreservative to be added in food items commercially [3,4,5]. The present study was aimed at isolating bacteriocin producing lactic acid bacteria from fermented green gram batter and identifying its biopreservative efficacy in sweet lime and lemon juices. Prior to arriving to certain level of concentrations to be used in the apple juice and coconut water, experiments were carried out in our laboratory to decide the appropriate concentrations of bacteriocin.

MATERIALS AND METHODS

Sample collection and preparation

Green gram was collected, cleaned, soaked in water for 8 hrs and batter was prepared. It was allowed to ferment at room temperature overnight (O/N).

Isolation and identification of bacteriocin producing LAB

The fermented batter (sample) of 1 ml was added to 9 ml of distilled water, serial dilutions were performed up to 10^{-7} and plated on MRS agar. The plates were incubated at 37°C for 24 hrs. After incubation, pure culture was made on MRS agar and tested for bacteriocin production [6]. Then the strains were Gram stained and examined microscopically. Based on Bergey's Manual of Systemic Bacteriology [7], isolates (strains) were tested for their ability to ferment glucose and mannitol. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide and Oxidase by using oxidase disc.

Maintenance and propagation of pure cultures

Isolates and the indicator strains were streaked and re-streaked on MRS agar medium and Nutrient agar medium (containing 0.6% yeast extract) respectively at frequent intervals of time. The stock cultures were preserved in a refrigerator at 4˚C [8, 9].

Production of bacteriocin

The strain was grown in MRS broth at 37°C for 48 hrs. After incubation, the broth was centrifuged at 5000 rpm for 10 min and the cells were separated out [10]. The cell free supernatant was used as crude bacteriocin.

Confirmation of cultures

The LAB isolates as well as indicator bacterial strains were checked for their purity by performing Gram staining and microscopic examination at intervals of time [9, 11].

Antimicrobial activity against indicator strains

The antibacterial activity was tested against Gram positive organism *Bacillus subtilis*, *Staphylococcus aureus* and Gram negative organisms *Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris* and *Klebsiella sps*.

Agar well diffusion method

Antimicrobial activity of bacteriocin against pathogenic microorganisms was determined by well diffusion method [12] at pH 5 and pH 7. The pH of the bacteriocin was adjusted to pH 5 and pH 7 using 1M NaOH and 1N HCl. Agar plates were inoculated with 100 ul of each indicator microorganisms after growing them in a nutrient broth and diluting appropriately. The inhibitory activity against all pathogenic microorganisms was tested on nutrient agar .Wells (6 mm) were cut in agar plate and 100 ul of cell free culture supernatant (crude bacteriocin) of the isolated strains was added into each well. Plates were incubated at 37oC for 24 hrs. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

Biopreservative efficiency of bacteriocin in Sweet lime juice and Lemon juice

Sweet lime juice and lemon juice were obtained aseptically in the laboratory. To a set of four tubes 5ml of sweet lime juice was added to each tube. To another set of four tubes 5 ml of lemon juice was added to each tube.

In both the sets of first tube (control) crude bacteriocin was not added. To rest of the three tubes of first set (second, third and fourth tubes containing sweet lime juice) the crude bacteriocin of different concentrations of 25 ul, 50 ul and 100 ul was added. Similarly, to the other set of three tubes (second, third and fourth tubes containing lemon juice) the bacteriocin of 25 ul, 50 ul and 100 ul was added. The four tubes of both the sets are left at room temperature.

Processing tubes containing Sweet lime juice with Bacteriocin

On the zero minute (immediately after the addition of bacteriocin to the sweet lime juice) 100 ul of sample from all the four tubes (first tube without bacteriocin and three tubes with different concentrations of bacteriocin) was placed on nutrient agar (NA) and incubated O/N (overnight) at 37˚C. The plates were observed for microbial colonies.

On the first day (After 24 hrs of incubation of sweet lime juice with bacteriocin), 100 ul of sample from each tube (first tube without bacteriocin and three tubes with different concentrations of bacteriocin) was placed on NA and incubated at 37˚C, after O/N plates were observed for microbial colonies.

Processing tubes containing Lemon juice with Bacteriocin

On the zero minute (immediately after the addition of bacteriocin to the lemon juice) 100 ul of sample from all the four tubes (first tube without bacteriocin and three tubes with different concentrations of bacteriocin) was placed on nutrient agar (NA) and incubated O/N at 37˚C. The plates were observed for microbial colonies.

On first day (After 24 hrs of incubation of lemon juice with bacteriocin), 100 ul of sample from each tube (first tube without bacteriocin and three tubes with different concentrations of bacteriocin) was placed on NA and incubated at 37˚C, after O/N plates were observed for microbial colonies.

RESULTS AND DISCUSSIONS

The bacteriocins were isolated from four different bacterial isolates from the fermented green gram batter samples which were named as GG1, GG2, GG3 and GG4. Table 1 shows that the bacterial isolates GG1 and GG2 were identified as *Lactobacillus casei*, strains GG3 and GG4 were identified as *Streptococcus sps* based on their physiological and biochemical characteristics. The strains GG1 and GG2 were Gram-positive rods, Catalase negative, acid production in glucose and mannitol fermentation, and no hemolysis having smooth round colonies on MRS agar medium [13]. The strains GG3 and GG4 were Gram positive cocci, Catalase negative, no hemolysis The LAB is considered as “food grade” organisms, show special promise for selection and implementation as protective cultures. In addition, some LAB exhibit potent antimicrobial activities in the form of small, heat-stable, antimicrobial peptides called bacteriocins [14].

Table 1: General properties of isolated strains

S.No.	Sample	Isolates	General properties	Identified organism
1.	Green gram	GG1	Gram positive rods Catalase negative, No hemolysis, Acid production in glucose And mannitol fermentation	*Lactobacillus casei*
2.	Green gram	GG2	Gram positive rods Catalase negative, No hemolysis, Acid production in glucose And mannitol fermentation	*Lactobacillus casei*
3.	Green gram	GG3	Gram positive cocci Catalase negative, No hemolysis	*Streptococcus sps*
4.	Green gram	GG4	Gram positive cocci Catalase negative, No hemolysis	*Streptococcus sps*

Table 2: Antibacterial activity of bacteriocins

Strain isolates	Zone of inhibition (mm)											
	E.coil		*B.subtilis*		*P. aeruginosa*		*P. vulgaris*		*S. aureus*		*Klebsiella sps*	
	pH5	pH7	pH5	pH7	pH5	pH7	pH5	pH7	pH5	pH7	pH5	pH7
GG1	12	-	-	-	-	-	-	-	8	10	12	-
GG2	06	-	-	-	20	-	-	-	10	12	10	-
GG3	-	-	-	-	11	21	-	-	-	-	12	-
GG4	13	-	-	-	6	-	-	-	12	-	7	-

(-): Indicates no zone of inhibition

The Table 2 indicates the antibacterial activity of isolated strains at a temperature of 37˚C, at a pH 5 and pH 7. An agar well diffusion method was used for testing the antibacterial activity of bacteriocins with six indicator organisms. Out of the six indicator organisms, the growth of four indicator organisms was inhibited by bacteriocins isolated by *Lactobacillus casei* and *Streptococcus sps*. The three susceptible Gram negative indicator organisms to the crude bacteriocin isolated from *Lactobacillus casei* and *Streptococcus sps* were *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella sps. Bacillus subtilis* and *Proteus*

vulgaris were not inhibited by the activity of the crude bacteriocin. The effect of pH played an important role in the antibacterial activity of bacteriocins. The maximum zone of inhibition was measured at pH 5 (20 mm) and pH 7 (21 mm) with *P. aeruginosa*. The bacteriocin of isolates, GG1 and GG4, were effective at pH5. LABS are more tolerant to acidic pH and other organisms are inhibited at low pH and most of the LAB thrive best at a pH < 4.5 [15, 16, 17]. The bacteriocin of isolates, GG2 and GG3 showed inhibitory activity at pH 5 and also at pH 7.

The plate counts were obtained on MRS agar for strain isolates of GG1, GG2, GG3 and GG4. The counts were recorded in colony forming units per ml (CFU/ml). At 24 hrs the microbial counts were 1.4 x 10^5, 1.6 x 10^6, 1.8 x 10^8 and 1.9 x 10^5 CFU/ml for GG1, GG2, GG3 and GG4.

The results of Table 3 indicate the antagonistic property of bacteriocin with different concentrations (25 ul, 50 ul and 100 ul) of GG1, GG2, GG3 and GG4 isolates in sweet lime juice at zero minutes (immediately after the addition of bacteriocin) and after one day (24 hrs). No inhibitory activity was observed at 0 min with 25 ul, 50 ul and 100 ul of bacteriocin. At 24 hrs, there was no inhibition with 25 ul and 50 ul; inhibitory activity (no microbial growth), minimum inhibitory concentration (MIC) was observed with 100 ul of bacteriocin of the all the four isolates. Hence, bacteriocin of high concentration of all four isolates showed biopreservative property in sweet lime juice. The bacteriocins can be added to foods in the form of concentrated preparations as food preservatives and can be used as shelf-life extenders [18].

Table 3: Bio-preservative efficiency of bacteriocin in Sweet lime juice at zero minutes (0 min) and after one day (24 hrs)

Stain isolates	Bacteriocin concentration in microlitres (ul)							
	In the absence of Bacteriocin		25 ul		50 ul		100 ul	
	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs
GG1	+	+	+	+	+	+	+	-
GG2	+	+	+	+	+	+	+	-
GG3	+	+	+	+	+	+	+	-
GG4	+	+	+	+	+	+	+	-
GG1	+	+	+	+	+	+	+	-

(+): Indicates presence of microbial colonies, (-): Indicates absence of microbial colonies

In Table 4 the MIC of bacteriocin was observed as 50 ul for all the four isolates (GG1, GG2, GG3 and GG4) at 24 hrs in lemon juice. The bacteriocin of all strains didn't showed inhibitory activity at zero minutes. Therefore, the bacteriocin of all four isolates was effective in inhibiting the microbial flora at low concentration in lemon juice as compared to sweet lime juice (high concentration of bacteriocin was required). Application of Enterocin AS-48 to citrus based juices was studied by Abriouel *et al* and he reported that Enterocin AS-48 had variable interactions with fruit juices, with complete, partial, or negligible loss of activity [19, 20, 21]. The hurdle approach of bacteriocin enhances microbial inactivation [22]. Several bacteriocins show additive or synergistic effects when used in combination with other antimicrobial agents, including chemical preservatives, natural phenolic

compounds, as well as other antimicrobial proteins [23].

Table 4: Bio-preservative efficiency of bacteriocin in Lemon juice at zero minutes (0 min) and after one day (24 hrs)

Stain isolates	Bacteriocin concentration in microlitres (ul)							
	In the absence of Bacteriocin		25 ul		50 ul		100 ul	
	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs	0 min	24 hrs
GG1	+	+	+	+	+	+	+	-
GG2	+	+	+	+	+	+	+	-
GG3	+	+	+	+	+	+	+	-
GG4	+	+	+	+	+	+	+	-

(+): Indicates presence of microbial colonies, (-): Indicates absence of microbial colonies

CONCLUSIONS

The present study revealed that bacteriocin from *Lactobacillus casei* and *Streptococcus sps.* isolated from fermented green gram batter posses a wide spectrum of inhibitory activity against *E.coli, P.aeruginosa, S.aureus* and *Klebsiella sps*. In the presence of bacteriocin there was a reduction in the microbial population in sweet lime juice and lemon juice during storage at room temperature. The biopreservative efficacy of bacteriocins of GG1, GG2, GG3 and GG4 isolates was observed as 100 ul (MIC) in sweet lime and 50 ul (MIC) in lemon juices. Therefore, bacteriocins from these bacteria can be used for biopreservation of citrus fruit juices. Further research into synergistic effects of these natural preservatives in combination with advanced hurdle technologies could result in replacement of chemical preservatives and could allow in maintaining the quality of food.

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