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Research Paper

Biopreservative activity of bacteriocin-producing lactic acid bacteria isolated from fermented green gram batter

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Abstract

The biopreservative property of bacteriocin produced by lactic acid bacteria (LAB) from fermented green gram batter was investigated in apple juice and coconut water.LAB was isolated from fermented green gram batter using Man, Rogosa and Sharpe (MRS) media. A total number of four isolates G1, G2, G3 and G4) were isolated from fermented green gram batter. G1 and G2 were identified as *Lactobacillus casie* and G3 and G4 were identified as *Streptococcus* species according to Bergeys Manual of Systemic Bacteriology. Antimicrobial activity of the bacteriocin produced by these strains 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* species. The biopreservative efficacy of bacteriocins of G1, G2, G3 and G4 was found both in apple juice and coconut water. The bacteriocins of G1, G3, and G4 exhibited highest antagonistic activity at pH 5 and G2 at pH 7. In apple juice, Minimal Inhibitory Concentrational *lactobacillus*, *streptococcus* (MIC) bacteriocin was found to be 25 ul and in coconut water MIC of bacteriocin was found to be 400 ul.

Keywords: Bacteriocin, Lactic acid bacteria, Biopreservative, lactobacillus streptococcus.

Biological preservation implies a novel scientific approach to improve the microbial safety of foods, which refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy the undesirable microorganisms in foods. The biological preservation of foods is considered to be safe and provide substantial health-benefits to man (Adams, 1999). A number of factors have been identified to contribute to the antimicrobial activity of LAB, that have attracted attention in recent years because of their generally regarded as safe (GRAS) status and efficacy as natural biopreservatives which can find applications in the food and cosmetic industries (Cleveland *et al*., 2001; Daeschel, 1993; Riley and Wertz, 2002). These bacteria produce different antimicrobials, such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins.

Bacteriocins play a potential role as novel food preservatives and received greater attention as most of them are heat stable and amenable to proteolytic inactivation, hence extending the shelf-life and enhancing the safety of food products (Holzapfel *et al*., 1995; Aymerich, 2000). Many bacteriocins have been isolated and characterized, and found to exhibit antibacterial activity against a range of pathogenic and food spoilage bacteria (Sakala *et al*., 2002; Cintas *et al*., 1995; O'Keeffe and Hill, 1999; Cleveland *et al*., 2001). Nisin, produced by *Lactococcuslactis*, is the most thoroughly studied bacteriocin to date and has been applied as an additive to certain foods worldwide (Delves-Broughton *et al*., 1996).

Other bacteriocins such as pediocin, may also have potential applications in foods, though they are not currently approved as antimicrobial food additives (Naghmouchi *et al*., 2007).

Fresh fruits harbor various microorganisms, some of them may be pathogenic and spoilage that are capable of growing at room temperature. Therefore, it is important to seek biopreservatives that could control these microorganisms. Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products (Ibourahema *et al*., 2008). The present study involved the isolation, identification of LAB from fermented green gram batter and biopreservative property of bacteriocin with different concentrations in apple juice and coconut water. 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 preparation

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

Isolation and identification of bacteriocin producing LAB species

Serial dilutions were performed upto 10^{-7} and plated on the MRS agar and incubated at 37°C for 24 hrs. After incubation, pure culture was made on MRS agar and tested for bacteriocin production (Federal, 1988). Then, the strains were Gram stained and examined microscopically. The Gram staining and microscopic examination of LAB isolates at regular intervals was performed according to the method described (Dhewa *et al*., 2010b). Based on Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986; Clevelan*det al*., 2001) strains were tested for their ability to ferment glucose and mannitol. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide and oxidase test was performed using oxidase disc (Ravi *et al*., 2011; Dhewa, 2011).

Maintenance of LAB 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 (Sharma *et al*., 2006, Cherif *et al* 2001).

Preparation of culture supernatant

The strains were grown in MRS broth at 37°C for 48 hrs. The broth was centrifuged at 10,000 rpm for 10 min and the bacterial cells were separated out (Maldonado *et al.,* 2003). The cell free supernatants of all the four isolates were used as crude bacteriocins.

Determination of antimicrobial activity of bacteriocin

The bacteriocin was used for testing inhibitory activity against indicator organisms. The indicator organisms include Gram positive bacteria, *Bacillus subtilis, Staphylococcus aureus* and Gram negative bacteria *Pseudomonas aerosginosa, Escherichia coli*, *Proteus vulgaris* and *Klebsiella species*.

Agar well diffusion method

Antimicrobial activity of bacteriocin against pathogenic microorganisms was determined by well diffusion method at pH 5 and pH 7. The pH of the bacteriocin was adjusted to pH 5 and pH 7, using 1N HCl and 1M NaOH. 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 37°C for 24 hrs. The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the wells.

Biopreservative efficiency of bacterioicn in apple juice and coconut water

Apple juice and coconut water were obtained aseptically in the laboratory.

Group A: Testing the efficacy of bacteriocin in apple juice: Four tubes were taken and 5 ml of apple juice was added to each tube. The bacteriocin was not added to the tube 1, but 25 ul , 50 ul and 100 ul were added to the tube 2, tube 3 and tube 4, respectively.

Group B: Testing the efficiency of bacteriocin in coconut water: Four tubes were taken and 5 ml of coconut water was added to each tube. The bacteriocin was not added to the tube 1, but 100 ul, 200 ul and 400 ul were added to the tube 2, tube 3 and tube 4 respectively.

All the above mentioned eight tubes of group A and B were incubated at room temperature. .

Processing of tubes containing apple juice withbacteriocin

At zero time (immediately after the addition of bacteriocin to the apple juice) and at 24 hrs of incubation, 100 ul of sample from all the four tubes was plated (spread plate method) on nutrient agar (NA) and incubated over night at temperature of 37°C. The plates were observed for microbial colonies.

Processing of tubes containing coconut water with bacteriocin

At zero minute and at 72 hrs of incubation, 100 ul of sample from all the four tubes was plated on nutrient agar (NA) and incubated over night at a temperature of 37°C. The plates were observed for microbial colonies.

Results and Discussion

Characterization of isolated strains

A total of four isolates were isolated and named as G1, G2, G3, and G4. The isolates G1 and G2 were gram-positive rods, catalase negative, acid production in glucose showed fermentation of mannitol, no haemolysis on blood agar, Isolated colonies morphologically appeared as smooth round colonies, circular, 2 mm in size, creamish white in color on MRS agar medium (Sablon *et al*., 2000). The strains G1 and G2 were identified as *Lactobacillus casie*, G3 and G4 were identified as *Streptococcus* species based on their physiological and biochemical characteristics as per the data is presented in Table 1.

Table 1. Identification of isolated LAB

S. No	Sample	Abbre-viation of isolates	General properties	Identified organism
1	Green gram	G1	Gram positive rods, catalase negative, no hemolysis, acid production in glucose and mannitol fermentation	Lactobacillus casie
2	Green gram	G2	Gram positive rods, catalase negative, no hemolysis, acid production in glucose and mannitol fermentation	Lactobacillus casie
3	Greeen gram	G3	Gram positive cocci, catalase negative, no hemolysis	Streptococcus species
4	Green gram	G4	Gram positive cocci, catalase negative, no hemolysis	Streptococcus species

Sharpe (2009) reported 8.7% bacteriocinogenic strains among 92 LAB isolated from fresh-cut vegetable products, whereas Sezer and Guven (2009) screened 12,700 LAB isolates from milk and meat products and found only 35 exhibited bacteriocin production. Therefore, the choice of food source and media are important for the successfully isolation of bacteriocinogenic LAB. The strains were isolated from fermented green gram batter (Table 1).

Determination of antibacterial activity of bacteriocins

Table 2 shows results of, antibacterial activity of bacteriocins of G1, G2, G3 and G4 isolated strains at pH 5 and pH 7. An agar well diffusion method was used for testing the antagonistic property of bacteriocins with indicator organisms. Out of the six indicator organisms, the antibacterial activity of bacteriocins was observed with *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella sps*. Inhibitory activity was not observed against *Proteus vulgaris* and *Bacillus subtilis*. The maximum inhibitory activity was shown by G2 against *P.aeruginosa* and least activity was seen against *E.coli*. The maximum inhibition zone was measured at pH 5 (20 mm) and pH 7 (21 mm) with *P. aeruginosa*. . Therefore the effect of pH played an important role in the antagonistic activity of bacteriocins. Earlier, the bacteriocin produced by *Lactococcus lactis* D53 and 23 was active over a wide pH range with the highest activity shown at low pH range of 3–5 (Schillinger and Lucke, 1989), as was the case with the bacteriocin from *Pediococcus*sp. (Jamuna and Jeevaratnam, 2004). Earlier, the bacteriocin produced by a newly isolated *Bacillus* species strain 8A was found active over a pH range of 5–8 (Bizani and Brandelli, 2002). Liu and Hansen (1990) reported that the Nisin is 228 times more soluble at pH 2 than at pH 8. The antibacterial activity of bacteriocin obtained from *Lactobacillus casie* and *Streptococcus species* was maximum at pH 5 (Table 2).

The plate counts were obtained on MRS agar for strain isolates of G1, G2, G3 and G4. The counts were recorded in colony forming units per ml (CFU/ml). No growth was detected on MRS agar at zero minutes. At 24hrs the microbial counts were 1.4×10^5, 1.6×10^6, 1.8×10^8, 1.9×10^5 CFU/ml for G1, G2, G3 and G4. At 72 hrs the microbial counts were 1.5×10^6, 1.7×10^7, 1.9×10^9, 1.9×10^9 CFU/ml for G1, G2, G3 and G4.

Biopreservative property of bacteriocin in apple juice

Table 3: At zero minutes - Biopreservative efficiency of bacteriocin in apple juice

Strain isolates	Bacteriocin concentration in ul			
	In absence of bacteriocin	25 ul	50 ul	100 ul
G1	+	+	+	-
G2	+	+	-	-
G3	+	+	-	-
G4	+	+	+	-

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

The bacteriocins from isolates G1, G2, G3 and G4 were tested for the preservative effect in apple juice (Table 3) at zero time (immediately after the addition of

Table 2. Antibacterial Activity of Bacteriocins at pH 5 and pH 7

Strain isolates	Zone of inhibition (mm)											
	E.coli		*B.subtilis*		*P.aeruginos*		*P.vulgaris*		*S.aureus*		*Klebsiella* sps	
	pH 5	pH 7	pH 5	Ph 7	pH 5	pH 7	Ph 5	pH 7	pH 5	pH 7	pH 5	pH 7
G1	12	-	-	-	-	-	-	-	-	-	12	-
G2	6	-	-	-	20	-	-	-	10	12	10	-
G3	-	-	-	-	11	21	-	-	-	-	12	-
G4	13	-	-	-	6	-	-	-	-	-	7	-

(-): Indicates absence of inhibition

bacteriocin). The minimum inhibitory concentration (MIC) was 50 ul for the bacteriocins of G2 and G3. The MIC was 100 ul for the bacteriocins of G1 and G4. Vescovo *et al*., (1995) observed a reduction in high initial bacterial loads of ready-to-use mixed salads on addition of bacteriocin producing LAB. Komitopoulou *et al*., (1999) studied the growth of *A. acidoterrestris* in fruit juice and its sensitivity to heat treatment and nisin (Grande *et al*., 2005a). In our study, the preservative effect of bacteriocins of all the four isolates in apple juice, increased with the increase in the concentration of bacteriocins.

Table 4. After one day (24 hours) - Biopreservative efficacy of bacteriocin in apple juice

Strain isolates	Bacteriocin concentration in ul			
	In absence of Bacteriocin	25 ul	50 ul	100 ul
G1	+	-	-	-
G2	+	-	-	-
G3	+	+	-	-
G4	+	+	-	-

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

The inhibitory activity of bacteriocins of four isolates (G1, G2, G3 and G4) in apple juice was observed after storage period of one day (24 hours) at room temperature (Table 4). It was observed that there was absence of microbial colonies with 25 ul of bacteriocin of G1 and G2 isolates. The bacteriocin of G3 and G4 showed complete inhibition with 50 ul. The results revealed that the preservative efficacy of bacteriocin of strains G1 and G2 was higher than G3 and G4. Previous studies have demonstrated the nisin effectiveness in promoting lag phase increase and growth rate reduction (Chihib *et al*., 1999) as well to inactivate contaminants in beer (Galvagno *et al*., 2007), indicating the potential use of this bacteriocin. The results of table 4 indicate, the MIC of bacteriocins of G1, G2 was 25 ul. The MIC of bacteriocins of G3 and G4 was 50 ul.

Biopreservative property of bacteriocin in coconut water

Table 5. After three days (72 hours) - Biopreservative efficacy of bacteriocin in coconut water

Strain isolates	Bacteriocin concentration in ul			
	In absence of bacteriocin	100 ul	200 ul	400 ul
G1	+	+	+	-
G2	+	+	+	-
G3	+	+	+	-
G4	+	+	-	-

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

At zero time, in the absence (without bacteriocin) or presence (different concentrations) of bacteriocin in the fresh coconut water didn't show any bacterial growth and appears to be sterile. At 72 hrs of incubation at room temperature (storage period) with lower concentrations of 25, 50 and 100 microlitres of bactoriocin (Data not shown) the microbial growth was not inhibited. Therefore, the biopreservative activity of bacteriocin in coconut water was observed at higher concentrations for 72 hrs. The results (Table 5) suggest, MIC of bacteriocin of G4 was 200 ul, and MIC of G1, G2 and G3 was 400 ul. Applications of protective cultures and co-cultures are considered as additional safety factors warranting the microbiological stability of the resulting foods reducing risks of growth and survival of food-borne pathogens and food spoilage organisms (Joshi *et al* 2006). Esayas *et al*., (2008) also observed the bio-preservative potential of purified bacteriocin from isolate CA44 against *B.cereus* and found that the preservative effect in fruit juice increased with the increase in the concentration of bacteriocin. Eighty seven per cent reduction of *Bacillus cereus* population was observed in juice at a concentration of 0.5 %. Hence, the preservative property of bacteriocin of strain G4 was better than bacteriocins of strains G1, G2 and G3 (Table 5).

Conclusion

The study revealed that bacteriocins produced by *Lactobacillus casie and Streptococcus sps* isolated from fermented green gram batter possess wide spectrum of inhibitory activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella sps.* The bacteriocins were effective at acidic pH. In the presence of bacteriocin, there was a reduction in the microbial population in apple juice and coconut water. Therefore, the bacteriocins of *Lactobacillus casie* and *Streptococcus sps* naturally occur in fermented green gram batter, have been found to play a defining role in the control of undesirable flora in apple juice and coconut water. The low concentration of bacteriocin (25 ul, 50 ul and 100 ul) in apple juice and high concentration (200 ul and 400 ul) was required as biopreservative agent in coconut water. It can be concluded that bacteriocins of these two species holds a potential for extension of shelf-life and improvement of microbiological safety in food industry.

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