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APPLICATION OF PURIFIED BACTERIOCIN PRODUCED BY *LACTOCOCCUS LACTIS* AP2 AS FOOD BIOPRESERVATIVE IN ACIDIC FOODS

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Abstract
Bacteriocins are biologically active proteins or protein complexes that display a bactericidal mode of action towards usually closely related species. Bacteriocin producing Lactococcus lactis AP2 was isolated from fermented milk products (dahi, lassi etc.) from the different regions of Solan district of Himachal Pradesh, India. The strain was selected and screened for their ability to produce bacteriocin by agar well diffusion method using the supernatant of centrifuged test culture. The bacteriocin had wide spectrum of inhibitory activity against test strains of pathogenic and food spoilage micro organisms. The cell-free supernatant of Lactococcus lactis AP2 inhibited the growth of Escherechia coli, Pseudomonas aeruginosa, Shigella dysenteries, Stapyhlococcus aureus and Bacillus cereus. The bacteriocin of Lactococcus lactis AP2 was recovered at 30-40 % ammonium sulphate saturation level and the protein was 0.10 mg/ml. Purified bacteriocin was found to possess a molecular weight of 12 kDa. The bacteriocin from the organism viz. Lactococcus lactis AP2 is stable at low temperature (up to 72h) and at acidic pH 2 to 6 thereby rendering it to be used in acidic foods as biopreservative. Biopreservative effect of bacteriocin i.e. purified bacteriocin expressed better results as compared to chemical preservative i.e. sodium benzoate used for the preservation of acidic food items viz. orange and mixed fruit juice.

Keywords: Acidic foods, Bacteriocin, cell-free supernatant, Biopreservative, Lactococcus lactis AP2

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1. INTRODUCTION

The single most important development permitting the formation of civilization was the ability to produce and store large quantities of food. Food storage has always been at odds with food spoilage. In 1864, Louis Pasteur proved that microorganisms in foods were the cause of food spoilage and it was the heat treatment of food which killed these microbes and the sealed containers helped to preserve food by preventing recontamination from atmospheric air (Soomro *et al.,* 2002). Now-a-days consumers are concerned about the synthetic chemicals used as preservatives in food, and there is resulting trend towards less processed food. These untreated foods can harbour dangerous pathogens which can multiply under refrigeration and without oxygen. A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms. Lactic acid bacteria (LAB) have been used as microbial preservative for thousand of years in order to extend the shelf life of foods. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity, against those bacteria which are closely related to the producer strain (Klaenhammer, 1988a; Tagg *et al.,* 1976).

Bacteriocins produced by lactic acid bacteria (LAB) have attracted special interests from the aspect of their potential use as safe and natural antimicrobials which can be applied as food preservatives, fine chemicals or post-antibiotic pharmaceuticals. Bacteriocins produced by *Lactococcus* species, especially nisin, are widely used in foods since the LAB have generally been regarded as safe (GRAS)

organisms. A large number of other LAB bacteriocins have been identified and the list is still growing (De Vuyst and Vandamme, 1994). Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (Klaenhammer and Kullen, 1999; Fooks and Gibson, 2002). Bacteriocins and bacteriocin-producing strains of lactic acid bacteria (LAB) have been the focus of extensive research in recent years due to their potential as biopreservatives (Luchansky, 1994). Bacteriocin-producing organisms have a selective advantage over their competitors in their natural environment.

The antagonistic effects of bacteriocins against food spoilage (Leroy *et al.,* 2003), is usually achieved by inhibition of *Pseudomonas*, *Staphylococcus aureus*, *Salmonella* sp. and *Listeria monocytogenes* (Chinang et al*.,* 2000) and they have great potential as biopreservatives for food (Gravenson *et al.,* 2004). The bacteriocins produced by Gram positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative (Galvez et al., 2008) and therapeutic (Jack et al., 1995) potentials. In order to use bacteriocin as food biopreservative and as a therapeutic agent, large-scale production is required with high level of activity. Bacteriocin producing LAB have the "generally recognized as safe" (GRAS) status and can be administrated to animals to strengthen the barrier function of the gut microflora and/or for a non specific enhancement of the immune system (Tome et al., 2008). In the present investigation, an attempt has been made to observe the preservative effect of bacteriocin produced by *Lactococcus lactis* AP2 with special emphasis on antagonistic activity against serious food borne pathogens.

2. MATERIAL AND METHOD

Bacterial isolate and bacteriocin preparation

Lactic acid bacteria producing bacteriocin were isolated from fermented milk products (dahi, lassi etc.) from the different regions of Solan district of Himachal Pradesh, India. The indicator strains used were *Escherechia coli, Pseudomonas aeruginosa*, *Shigella dysenteries*, *Stapyhlococcus aureus* and *Bacillus cereus.* The inhibitory spectrum was tested by bit disc and well diffusion assay (Barefoot and Klaenhammer, 1983; Kimura *et al.,* 1998). The bacteriocin produced from the isolated LAB (i.e *Lactococcus* AP-2) was purified by using ammonium sulphate precipitation method (Ogunbanwo *et al.,* 2003), followed by gel exclusion chromatography (Pratush et al., 2011).

Application of purified bacteriocin of *Lactococcus lactis* AP2 as food bio-preservative

The effect of bacteriocin as preservative was checked on orange juice and mixed fruit juice. The juice samples were procured from local juice corner. The experiment was carried in three sets of three bottles (A, B and C) each containing 50 ml of juice samples inoculated with test strain i.e. *Stapyhlococcus aureus* @8.14 log CFU/ml. First bottle (A) labelled as 'Control' (without any preservative), second bottle (B) (with purified bacteriocin), labelled as 'Test' and third bottle (C) having Sodium Benzoate. Purified bacteriocin and sodium benzoate were added @2,000 ppm and 600 ppm, respectively in each pathogen treated food sample to observe preservative effect of different preservatives against test indicator. The storage studies for stability were done at interval of 0, 1, 2, 3…12 days and log CFU/ml was noted.

Effect of bacteriocin on preservation of orange juice

The orange juice sample locally made (500 ml) was taken in sterilized glass bottles and pasteurized by keeping it in hot water at 72^{o}C for 2 min. The juice sample was then brought to room temperature. The test strain viz. *S. aureus* @ 8.10 log CFU/ml was used for inoculating different set of orange juice sample. The purified bacteriocin was added @ 2000 ppm in each of orange juice sample. Similarly chemical preservative – sodium benzoate (600 ppm) was added in other orange juice tubes for comparative studies while control was kept as such i.e. without addition of any

preservative. These samples were kept at refrigerated temperature for further studies. The colony count for each of the sample was noted by pour plate method in petri plate on day 0, 1, 2, 3…12 and log CFU/ml was calculated

Effect of bacteriocin on preservation of mixed fruit juice

The mixed fruit juice sample locally made (500 ml) was taken in sterilized glass bottles and pasteurized by keeping it in hot water at 72°C for 2 min. The juice sample was then brought to room temperature. The test strain viz. *S. aureus* @ 8.10 log CFU/ml was used for inoculating different set of mixed fruit juice. The purified bacteriocin was added @ 2000 ppm in each of mixed fruit juice sample. Similarly chemical preservative – sodium benzoate (600 ppm) was added in other fruit juice tubes for comparative studies while control was kept as such i.e. without addition of any preservative. These samples were kept at refrigerated temperature for further studies. The colony count for each of the sample was noted by pour plate method in petri plate on day 0, 1, 2, 3……12 and log CFU/ml was calculated

3. RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) from the fermented milk products from rural areas of Solan district of Himachal Pradesh were isolated (Pratush *et al.,* 2011). The bacteriocin of *Lactococcus lactis* AP2 was recovered at 30-40 % ammonium sulphate saturation level and the protein was 0.10 mg/ml (Bradfoerd, 1976). Purified bacteriocin was subjected to SDS PAGE which showed a band of 12 kDa. The bacteriocin from the organism viz. *Lactococcus lactis* AP2 is stable at low temperature (up to 72h) and at acidic pH 2 to 6 thereby rendering it to be used in acidic foods as biopreservative. In the present investigation comparative biopresevative effect of purified bacteriocin and sodium benzoate was observed against *S. aureus* to enhance the shelf life of acidic foods viz. orange juice and mixed fruit juice, respectively.

Table 1 exhibits the record on the storage studies showing comparative study of purified bacteriocin and sodium benzoate for mixed fruit juice (4°C) inoculated with *S. aureus* (8.10 log cfu/ml and 1.0 OD). There was a decline in log CFU/ml after storage for one day and was noted to be 6.12 for purified bacteriocin and 6.95 for sodium benzoate while it reached 8.39 for control in *S. aureus*. The mean was 7.15. The 2nd day log CFU/ml for purified bacteriocin, sodium benzoate and control were 5.85, 6.34 and 9.07, respectively. The mean was 7.08. The log CFU /ml was lowest for purified bacteriocin of 3th day and was found to be 5.63. The log CFU/ml for purified bacteriocin, sodium benzoate and control were 6.01, 6.23 and 10.05 on 5th day and the mean was found to be 7.43.

There was an increase of 11.12, 6.74 and 7.07 log CFU/ml on 7th day for control, purified bacteriocin and sodium benzoate, respectively. The mean was 8.31. The log CFU/ml for 9th day was 13.31, 9.26 and 9.75 for the respective comparative studies with a mean of 10.71. The 12th day showed high increase in log CFU/ml for all three treatments viz. purified bacteriocin, sodium benzoate and control, respectively. It was found to be 15.21, 12.17 and 12.52 with a mean of 13.30. The overall mean for purified bacteriocin was found to be 7.48, for sodium benzoate it was 7.87 and 10.57 for control. It was found that among both the preservatives used to enhance the shelf life of mixed fruit juice, purified bacteriocin proved to be better preservative having mean value 7.48 log CFU/ml as compared to sodium benzoate with mean value 7.87 log CFU/ml at 4°C. Maximum spoilage was found in control having highest mean value i.e. 10.57.

Table 2 showed the record on the storage studies showing comparative study of biopreservative (purified bacteriocin and sodium benzoate for orange juice inoculated with *S. aureus* (8.10 log CFU/ml; 1.0 OD). Initial log CFU/ml was 8.10 for purified bacteriocin, sodium benzoate and for control having no preservative.

Table 1:A comparative study to use biopreservative (Purified bacteriocin) with chemical preservative (sodium benzoate) against *S. aureus* to enhance storage of mixed fruit juice (4°C)

Days	Biopreservative (Purified bacteriocin) (log cfu/ml)	Chemical preservative (sodium benzoate) (log cfu/ml)	Control (no preservative) (log cfu/ml)	Mean
0	8.10	8.10	8.10	**8.10**
1	6.12	6.95	8.39	**7.15**
2	5.85	6.34	9.07	**7.08**
3	5.63	6.06	9.54	**7.07**
5	6.00	6.23	10.05	**7.43**
7	6.74	7.07	11.12	**8.31**
9	9.26	9.75	13.13	**10.71**
12	12.17	12.52	15.21	**13.30**
Mean	**7.48**	**7.87**	**10.57**	

Table 2: A comparative study to use biopreservative (Purified bacteriocin) with chemical preservative (sodium benzoate) against *S. aureus* to enhance storage of orange juice (4°C)

Days	Biopreservative (Purified bacteriocin) (log cfu/ml)	Chemical preservative (sodium benzoate) (log cfu/ml)	Control (no preservative) (log cfu/ml)	Mean
0	8.10	8.10	8.10	**8.10**
1	6.35	6.91	8.32	**7.19**
2	6.08	6.13	9.11	**7.10**
3	5.81	5.95	9.53	**7.09**
5	6.23	6.34	10.00	**7.52**
7	6.90	7.03	11.22	**8.38**
9	9.43	9.92	13.34	**10.89**
12	12.34	12.71	15.32	**13.45**
Mean	**7.65**	**7.97**	**10.61**	

The mean for initial h was 8.10. The means for control, purified bacteriocin and sodium benzoate were 10.16, 7.65 and 7.97, respectively. A decrease of 6.35 and 6.91 log CFU/ml for purified bacteriocin and sodium benzoate, respectively was observed on first day. There was an increase of 8.32 log CFU/ml in case of control. The mean for the first day was 7.19.

The log CFU/ml was found 9.11, 6.08 and 6.13 for control, purified bacteriocin, and sodium benzoate on the second day. The mean for second day was 7.10. The log CFU/ml decreased to 5.81 and 5.95 for purified bacteriocin and sodium benzoate, respectively on third day. The mean for third day was 7.09. After third day there was a continuous increase in log CFU/ml which was recorded to be 10.00, 6.23 and 6.34 for control, purified bacteriocin and sodium benzoate, respectively on fifth day. There was much increase in microbial count on seventh day. The log CFU/ml was found to be 11.22, 6.90 and 6.34 for control, purified bacteriocin and sodium benzoate, respectively. The mean was 8.38. The log CFU/ml reached to 15.32, 12.34 and 12.71 on 12th day for control, purified bacteriocin and sodium benzoate, respectively.

It was found that among both the preservatives used to enhance the shelf life of orange juice, purified bacteriocin proved to be better preservative having less mean value 7.65 log CFU/ml as compared to sodium benzoate with higher mean value 7.97 log CFU/ml at 4°C. Maximum spoilage was found in control having highest mean value i.e. 10.61. Less mean value for mixed fruit juice viz. 7.48, 7.87 and 10.57 as compared to orange juice viz. 7.65, 7.97 and 10.61 showing comparatively more effectiveness of bacteriocin in mixed fruit juice than in orange juice. Statistically compared results for biopreservative i.e. purified bacteriocin expressed better results than chemical preservative i.e. sodium

benzoate. Among the food items used results for mixed fruit juice were better than the orange juice.

Joshi *et al.* (2006), also observed the biopresevative 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 %. Galvez et al. (1998) tested the preservative potential of bacteriocin produced by *Enterococcus faecalis* A48-32 in glucose-MRS broth, fresh made apple juice and two commercial apple ciders. It was found that a concentration of 0.5 mg/ml of enterocin AS-48 was enough to inactivate *B. licheniformis* LMG19409 in glucose-MRS broth while 3 mg/ml was necessary to obtain the inhibition in fresh apple juice.

Alpas and Bozoglu, (2000) studied the combined effect of high hydrostatic pressure, heat and bacteriocin (5000 AU/ml sample) on inactivation of *S. aureus*, *L. monocytogenes*, *E. coli* 0157:H7 and *Salmonella* in orange juice. For orange juice samples there was more than 8 log cycle reduction for all the bacterial species studied by use of high hydrostatic pressure, heat and bacteriocin. Enterocin AS-48 bacteriocin was used as food biopreservative for fruit and vegetable juices. It showed variable interaction with fruit and vegetable juices, with complete, partial or negligible loss of activity. The loss of activity was ameliorated by increasing the bacteriocin concentration, diluting the juice or applying a heat pretreatment. In fresh made fruit juices and mixed fruit juices, AS-48 was stable for 15 days at 4^{o}C. The bacteriocin activity was slight as detectable after 30 days of storage (Grande *et al*., 2007).

4. CONCLUSION

The purified bacteriocin from *Lactococcus lactis* AP2 is very much effective in the preservation and enhancement of the shelf life the acidic foods such as fruit juices i.e orange and mixed fruit juice. The purified bacteriocin from *Lactococcus lactis* AP2 has shelf life of 72 h at low temperature and acidic pH 2-6. Due to its stability at acidic pH this bacteriocin can be used for the preservation of acidic food products. It is observed in various studies that the contamination in processed food products takes place at very slow rate and to overcome this contamination problem the purified bacteriocin of *Lactococcus lactis* AP2 can be a safe and easier way to enhance shelf life of fruit juices for long time because of its strong preservative properties.

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