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Isolation and Characterization of Bacteriocin Producing Lactic Acid Bacteria's from Fermented Milk Products of Solan District of Himachal Pradesh

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ABSTRACT : Lactic acid bacteria (LAB) *i.e. Lactococcus* AP-2 was isolated from the fermented milk products of Solan district of Himachal Pradesh. 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 isolate *Lactococcus* AP-2 exhibited highest zone of inhibition against the indicator pathogenic strains *i.e. P. aeruginosa*, *S. dysentriae* and *S. aureus*. The antimicrobial activity of *Lactococcus* AP-2 was further characterized for heat sensitivity, storage stability, pH stability and effect of surfactant. The bacteriocin produced by *Lactococcus* AP-2 stable at low temperature *i.e.* up to 60°C for 72h and with the rise in temperature its activity start decreasing and above 60°C it becomes inactive. The bacteriocin is stable at acidic pH conditions (i.e. 2-6) and can be used as a potential biopreservative for the preservation of acidic food materials.

Keywords : Lactic acid bacteria (LAB), *Lactococcus* AP-2, Bacteriocin, Biopreservative.

INTRODUCTION

The fermented dairy products constitute a significant part of human diet in various parts of world. The fermented milk and milk products are generally produced by the lactic acid bacteria (LAB). The lactic acid bacteria (LAB) are a group of Gram positive, non spore forming, cocci or rod shaped organisms which are considered as 'Generally Recognized as safe' (GRAS) organisms (Patil *et al*. 2010).

LAB have been used as a flavoring and texturing agent as well as preservative in foods for centuries and now are used as starters in food fermentation (Caplice E and Fitzgerald GF, 1999). These organisms produce antimicrobial molecules such as lactic acid, acetic acid, hydrogen peroxide and bacteriocin which are widely known to inhibit the growth of food borne pathogens and spoilage microorganisms (Patil et al. 2010, Jeevaratnam *et al*. 2005). Since chemical preservatives are being continuously questioned with regard of safety, the use of LAB and their metabolites is generally accepted by consumers as something natural and health promoting. This offers a logical explanation for the non-reducing interest of the food scientists in the particular area and the expanding trend of applications of LAB in the food industry (Papagianni and Anastasiadou, 2009). LAB from different sources have been considered safe in the form of food preservatives, since they can be degraded by gastrointestinal protease (Facklam and Elliott, 1995; Cleveland *et al*. 2001).

Bacteriocin produced from the LAB is extracellular and bactericidal in nature (Patil *et al*. 2010). The bacteriocin showed its bactericidal effect against most of gram positive bacteria but its bactericidal effect varies from species to species (Jeevaratnam *et al*. 2005). LAB, *Lactococcus* lactis produce nisin, a commercially used biopreservative, which showed bactericidal effect against large number of gram positive organisms (Rodruiguez, 1996). Earlier, LABs were isolated from grains, dairy products, fermenting vegetables and meat products (Sharma and Kapoor, 2004; Sharma *et al*. 2009; Adetunji and Adegoke, 2007 and Patil *et al*. 2010). In the present study we report the 'Isolation and characterization of bacteriocin producing LABs from fermented milk products of district Solan of Himachal Pradesh. The bacteriocin produced by isolated *Lactococcus* AP-2 exhibited very good bacteriocidal potential against the pathogenic bacteria.

MATERIAL AND METHODS

Chemicals. Analytical grade chemicals and dyes were obtained from Hi-media Laboratories, Pvt. Ltd, India and SRL India, respectively.

Preparation of LAB culture. 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.

Indicator bacterial strains. Different indicator pathogenic bacterial strains were collected from Department of Biotechnology, Himachal Pradesh University, Shimla. The indicator strains used were *Escherechia coli, Pseudomonas aeruginosa, Shigella dysenteries, Stapyhlococcus aureus and Bacillus cerus.*

Isolation and screening of LAB for antimicrobial activity

Eleven samples of fermented milk products (dahi, lassi, etc.) were collected from different areas of Solan district of Himachal Pradesh in sterilized vials. One ml of respected sample was serially diluted by using sterile saline solution. The serial dilutions of the sample in the series i.e. 10^{-1} ml to 10^{-6} ml were prepared. From each dilution (*i.e.* 10^{-1} ml, 10^{-2} ml and 10^{-6}); 100 µl was spreaded on de Mann Rogosa Sharpe agar (modified MRS agar) plates and incubated for 48h at 30°C. After sufficient growth (after 48h) the selected colonies were successively streaked on MRS agar plates for purity.

Identification and characterization of LAB

Colonies with characteristic features (such as pin pointed, whitish etc.) were randomly selected from plates and tested for Gram-stain reaction, cell morphology, catalase reaction as discussed by Kandler and Weiss, (1986).

Production of crude bacteriocin and detection of its antimicrobial activity

All the LAB strains were inoculated individually in 1000 ml Elliker's broth (pH 6.8) for 48h at 30°C in static incubator with sealed plugs. For extraction of bacteriocin, a cell-free culture supernatant was obtained after centrifugation of culture broth (10,000 'g' for 20 min at 4°C). As the bacteriocin was produced extracellularly, the pellets were discarded and supernatant was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatant through a 0.22 µm pore-size filter. The supernatant was assayed for primary antimicrobial activity against indicator bacterial strains. The antimicrobial activity of the supernatant was determined by agar well diffusion assay (Klaenhammer, 1988) on M-H agar plates which were spreaded with test organisms. 100 µl of supernatant was poured into wells (6 mm diameter) and the plates were incubated at 37°C for growth of the test organism. After 24h, the diameters (millimeters) of the growth inhibition zones were measured.

Partial purification of bacteriocin

The bacteriocin produced from the isolated LAB (*i.e Lactococcus* AP-2) was partially purified by using Ammonium sulphate precipitation method (Ogunbanwo *et al*. 2003). The ammonium sulfate precipitation table (Scopes, 1982) was followed to calculate the required amount of ammonium sulfate to be added in cell free extract. Ammonium sulfate was added with continuous stirring until the precipitates were formed and the precipitates were stored at 4°C for 45 min. The precipitated proteins were recovered by centrifugation at 10,000 g for 20 min and the pellet obtained was resuspended in 0.3 M (pH 7.0) potassium phosphate buffer. The protein estimation was carried out by following the procedure of Bradford, (1976). Assay of the bacteriocin activity was carried out at each step in both the precipitate and supernatant to know which one actually contained the bacteriocin.

Characterization of partially purified bacteriocin

The crude bacteriocin samples were characterized by studying the effect of temperature, pH, storage stability, treatment with surfactants and UV light on their activity.

Heat resistance: The bacteriocin was exposed to various temperatures: 30, 40, 50, 60, 80 and 121°C. An aliquot of each fraction were removed after 30 min and then assayed for bacteriocin activity using agar well diffusion method.

pH sensitivity: The bacteriocin was adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 with hydrochloric acid (HCl) and sodium hydroxide (NaOH), incubated for 4h at room temperature and assayed as described above.

Stability of bacteriocin during storage: The bacteriocin (1ml) was stored at -20, 4, 37, 60, 80°C and room temperature (22°C). At different time intervals, samples were taken from the stored materials to determine bacteriocin activity.

Effect of surfactant on bacteriocin activity: This was carried out by incorporating non-ionic (Triton X100, Tween-80, Tween 20) surfactants. The surfactants were added to partially purified bacteriocin at a concentration of 0.1 ml of surfactant per ml of bacteriocin solution. Their preparations were incubated at 30°C for 60 min and assayed for bacteriocin activity against indicator organisms.

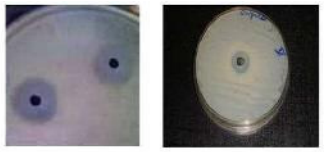
Effect of UV light on bacteriocin activity: The bacteriocin (1 ml) was placed in sterile microcentrifuge tube and exposed to UV light under laminar air flow. Time of exposure was 10 min, after that bacteriocin activity was analyzed by well diffusion method.

RESULTS

In the present study Lactic acid bacteria (LAB) from the fermented milk products from rural areas of Solan district of Himachal Pradesh were isolated. From eleven fermented milk samples only five isolates were procured. As most of the spread plates showed similar colonies thereby indicating the presence of only one population of LAB. Only five different LAB colonies are identified as *Lactococcus* sp. which were different from other LABs on the basis of their phenotypic characters, as given in Table 1. These five isolates have been characterized for their cell morphology, Gram's reaction and catalase activity. These colonies on MRS Agar plates were of small size and mostly looked like tiny dots. All the isolates were found to be gram +ve, catalase -ve, non-spore forming and spherical in shape (except AP-4). These types of typical colonies are formed by LAB (Kandler and Weiss, 1986). The characteristics selected in present study are generally used to characterize LAB (Sharpe *et al*. 1979).

Table 1: Cell morphology and other characteristics of isolated strains.

S. No.	Strains	Cell form	Cell	Gram arrangement	Catalase
1.	AP-1	Spherical	Grouped	Positive	Negative
2.	AP-2	Spherical	Paired	+	–
3.	AP-3	Spherical	Single and chains	+	–
4.	AP-4	Rod shaped	Single and chains	+	–
5.	AP-5	Small cocci	Paired or grouped	+	–



(a) (b)

Fig. 1 showing antimicrobial activity against test pathogenic strains (a) *P. aeruginosa* (b) *S. dysentriae*

Table 2: Antimicrobial activity of bacteriocin produced by various LAB isolates against test pathogenic organisms (Well diffusion assay).

S. No.	LAB	Indicator organisms	Diameter of inhibition (mm)
1.	AP-1	*P. aeruginosa*	8
		S. dysenteries	9
		S. aureus	9
2.	AP-2	*P. aeruginosa*	12
		S. dysenteries	11
		S. aureus	12
3.	AP-3	*P. aeruginosa*	10
		S. dysenteries	11
		S. aureus	11
4.	AP-4	*P. aeruginosa*	8
		S. dysenteries	10
		S. aureus	10
5.	AP-5	*P. aeruginosa*	10
		S. dysenteries	10
		S. aureus	9

Table 3: Percent increase in inhibition zone size against test indicators of partially purified bacteriocin of Lactococcus AP-2 over crude bacteriocin.

Pathogen	Bac* Zone size (mm)	Bac** Zone size (mm)	Increase%
P. aeruginosa	11	14	27.27
S. dysenteries	10	13	30.00
S. aureus	12	14	16.66

Bac* Crude bacteriocin, **Bac**** Partially purified bacteriocin

The extracts of five isolates of Lactococcus gave zone of inhibition against the indicator food borne pathogenic bacteria. The selected LABs i.e. Lactococcus sp. were used to produce extracellular bacteriocin in Elliker's broth. The bacteriocin produced by these five LAB isolates, showed inhibitory activity against the Gram-positive and Gram-negative target test strains (Fig.1a,b and Table 2). Out of these five isolates Fig. 1, 2 *Lactococcus* AP-2 showed highest zone of inhibition. The cell free extract of *Lactococcus* AP-2 was subjected to sequential ammonium sulphate saturations from 0 to 80 %. The bacteriocin of Lactococcus-AP-2 was recovered at 30-40 % ammonium sulphate saturation level and the protein was 0.08 mg/ml (Bradford, 1976). There was a significant increase in zone size of partially purified bacteriocin as compared to the crude bacteriocin (Table 3).

The effect of temperature, pH, storage time and surfactant and UV on the partial purified bacteriocin of *Lactococcus* AP-2 was determined against *P. aeruginosa*, *S. dysentriae* and *S. aureus* which were used as indicator organisms. The partially purified bacteriocin of *Lactococcus* AP-2 was exposed to various temperatures (30, 40, 50, 60, 70, 80 and 121°C). The bacteriocin produced by Lactococcus AP-2 was heat labile and the bactericidal effect goes on decreasing with rise in temperature from 30°C to 80°C (Fig. 2a).

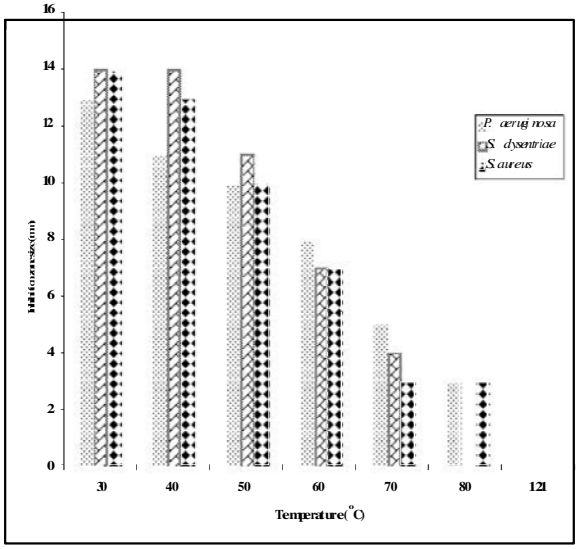


Fig. 2.a Effect of temperature

The bacteriocin activity was measured against *P. aeruginosa*, *S. dysentriae* and *S. aureus* by using well diffusion assay method. The pH of the partially purified bacteriocin of *Lactococcus* AP-2 was varied from pH 2-8 and its bacteriocidal effect was observed on the indicator strains *i.e. P. aeruginosa*, *S. dysentriae* and *S. aureus* (Fig. 2b). The bacteriocin is stable at acidic pH *i.e.* pH 2-6. Effect of storage time and temperature on bacteriocin of

Lactococcus AP-2 was studied (Table 4).

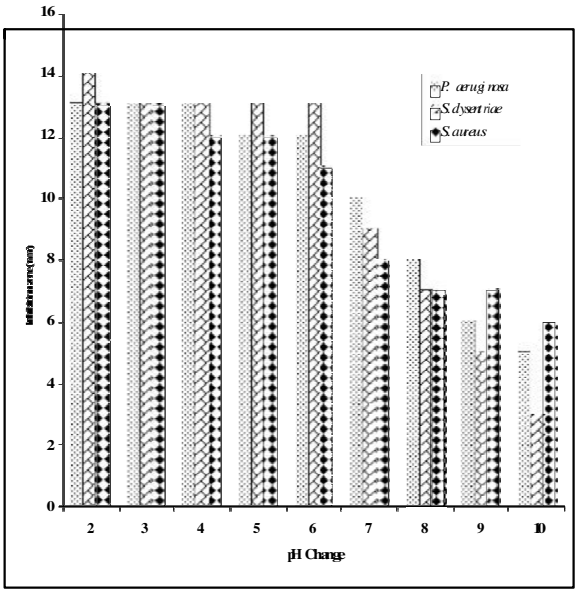


Fig. 2(b). Effect of pH on bacteriocin activity of *Lactococcus* AP-2 against *P. aeruginosa*, *S. dysentriae* and *S. aureus*.

Table 4: Effect of storage time and temperature on bacteriocin activity of *Lactococcus*-A against *P. aeruginosa*, *S. dysentriae* and *S. aureus*.

S.No	Temp (°C)(h)	Time	Zone of inhibition (mm)		
			P. aeruginosa	*S. dysentriae*	*S. aureus*
1.		12	14	13	14
		24	13	13	13
	–20	36	11	10	11
		48	9	9	10
		60	9	8	9
		72	8	7	9
2.		12	13	14	14
		24	13	12	14
	4	36	12	10	13
		48	11	10	10
		60	10	11	10
		72	9	9	8
3.		12	14	13	13
		24	12	12	13
	20	36	10	11	12
		48	9	9	10
		60	8	8	9
		72	8	7	7
4.		12	11	10	11
		24	10	9	10
	37	36	10	8	8
		48	9	8	7
		60	7	6	7
		72	5	4	6
5.	60	12	0	0	0
6.	121	12	0	0	0

The bacteriocin of *Lactococcus* AP-2 was stable at -20, 4, 20°C up to 72h of incubation whereas with the rise in temperature *i.e.* 37°C the bacteriocin activity started decreasing and at 60°C it is not stable even up to 12h of incubation. The effect of three different nonionic surfactant *i.e.* Tween 20, Tween 80 and Triton X-100 was observed on partially purified bacteriocin. The surfactant Tween 20 and 80 enhanced the bacteriocin activity up to 2 to 3 mm of inhibition zone size, whereas the Triton X-100 only rises the activity up to 1mm with reference to control (without surfactant) (Table 5). The exposure of the UV radiation to the bacteriocin does not cause any effect on its bacteriocidal effect and its activity remains similar to the control.

Table 5: Effect of surfactant on bacteriocin activity of *Lactococcus* AP-2.

S.No	Indicator strain	Surfactant	Zone of inhibition
1.	*P. aeruginosa*	Control	11
	S. dysentriae		12
	S. aureus		11
2.	*P. aeruginosa*	Tween 20	15
	S. dysentriae		14
	S. aureus		14
3	*P. aeruginosa*	Tween 80	15
	S. dysentriae		15
	S. aureus		14
4	*P. aeruginosa*	Triton X-100	12

DISCUSSION

In the present study we collected the fermented milk samples from the rural area of the Solan district of Himachal Pradesh for the isolation and characterization of bacteriocin producing lactic acid bacteria. There were about eleven different fermented milk samples from which only 5 different organisms having bacteriocin activity were isolated. These organisms exhibited the common characteristic properties of lactic acid bacteria, which are described by Sharpe *et al.* (1979).

All the 5 isolates were characterized for their cell morphology, gram characters and catalase assay. The colonies of these isolates were pin pointed and small sized which again confirms the presence of LAB's (Kandler and Weiss, 1986). All the colonies exhibited gram positive character. Earlier a large number of gram positive bacteriocin producing LAB's were also identified from milk products (Tagg et al. 1976, Vandenbergh et al. 1993, Sanni et al. 1999). The bacteriocin producing property of all isolated LAB's was determined by applying the crude extract of these organisms against the three major pathogenic strains (*P. aeruginosa*, *S. dysenteries* and *S. aureus*) out of which *Lactococcus* AP-2 showed maximum zone of inhibition.

The partial purification of bacteriocin produced by LAB's was carried out by the ammonium sulphate precipitation. The protein *i.e.* bacteriocin was precipitated at the 30-40% cut of ammonium sulphate saturation level. The protein precipitated above or below this saturation level exhibited very low or nil bacteriocin activity. The partially purified bacteriocin was heat labile in nature and when it

was exposed to different temperature (30-80°C and 121°C) its activity started decreasing with rise in temperature. At 80°C there was about 79% decrease in bacteriocin activity as compared to at 30°C (Fig. 2a). Nettles and Barefoot, (1993), studied the detailed heat stability of Lactobacillus sp. According to their research bacteriocin of L. brevis OG1 was stable for 60 min at 121°C. Temperature stability is important if the bacteriocins are to be used as a food preservative because many procedures of food preparation involve a heating step.

The pH also affects the stability and bacteriocidal property of bacteriocin produced by the *Lactococcus* AP-2 (Fig. 2b). Earlier reported bacteriocins e.g. bacteriocin from *L. plantarum* F1 and L. brevis OG1 exhibited maximum bacteriocidal activity at pH 2 to 6 and was inactive at pH 8 to 12 (Ogunbanwo et al. 2003) and bulgaricans (type of bacteriocin) isolated from the L. bulgaricus showed its highest activity at pH 2.2 to 4.0, respectively (Reddy et al. 1984, Abdel Bar et al. 1987). The bacteriocin from *Lactococcus* AP-2 is stable at lower pH range and hence it has a good potential to preserve the food materials which are acidic in nature.

CONCLUSION

A new bacteriocin producing lactic acid bacteria i.e. *Lactococcus* AP-2 is isolated and bacteriocin produced from it, is characterized. The bacteriocin from this organism 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.

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