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Study of Physical and Cultural Parameters on the Bacteriocins Produced by Lactic Acid Bacteria Isolated from Traditional Indian Fermented Foods

Sourav Bhattacharya and Arijit Das
Jain Institute of Vocational and Advanced Studies,
127/3, Bull Temple Road, Chamarajpet, Bangalore-560019, Karnataka, India

Abstract: Lactic Acid Bacteria (LAB) predominates the micro flora of fermented products. They produce metabolites that inhibit the growth of food-borne pathogens and spoilage microorganisms. The objectives of the present study were isolation, identification of LAB from traditional Indian fermented foods and study of physical and cultural parameters on the bacteriocins produced by them. Seven isolates of bacteriocin producing LAB were isolated from curd, dosa batter and idli batter and were identified as species of *Lactobacillus*. The culture supernatants of the seven isolates were evaluated for their antimicrobial activity against pathogens like *Staphylococcus aureus* and *Pseudomonas* sp. The stability of the bacteriocins was tested at different temperatures, pH, presence of bile salt like sodium deoxycholate and storage period at 4°C. The diameters of the inhibitory zones ranged between 9 and 12 mm for *Staphylococcus aureus*, with no effect on *Pseudomonas*. The bacteriocins produced by the isolates were stable at temperatures ranging between 30 to 80°C and over a wide range of pH from 2 to10, with the highest activity at pH 6. It was also found that the bacteriocins were stable at different concentrations of the bile salt used and remained active even after a storage period of 30 days at 4°C. Sodium Dodecyl Sulphate polyacrylamide gel electrophoretic analysis of the partially purified bacteriocins suggested their apparent molecular weights between 16.5 to 48 kDa. These bacteriocins may have a potential use as food biopreservatives and may help in improving the gut environment by combating several pathogenic microorganisms.

Key words: *Lactobacillus*, *Pseudomonas* sp., *Staphylococcus aureus*, bacteriocins, Lactic Acid Bacteria (LAB)

INTRODUCTION

In India, a wide variety of traditional fermented foods made from ingredients like milk, cereals, pulses and vegetables have been developed for the benefit of human health from ancient times. The primary microorganisms responsible in bringing about the desirable attributes in the final products are those belonging to Lactic Acid Bacteria (LAB). Amongst the few alleged benefits are modulation of intestinal health and the immune system, as well as anti-carcinogenic, anti-diarroheal and hypocholesterolaemic effects (Papamanoli *et al*., 2003; Itsaranuwat *et al*., 2003; Talarico and Dobrogosz, 1989; Tannock, 1997). The LAB exert

Corresponding Author: Sourav Bhattacharya, Jain Institute of Vocational and Advanced Studies, 127/3, Bull Temple Road, Chamarajpet, Bangalore-560019, India
Tel: +91 98869 19383

strong antagonistic activity against many microbes including food spoilage organisms and pathogens by producing various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins or bacterial peptides during lactic acid fermentation (Vandenbergh, 1993; Vossen *et al*., 1994; Zhennai, 2000).

Innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using bio-therapeutic agents such as live bacterial forms or their products (Ray and Bhunia, 2007). In food preservation and safety, the indigenous microflora has advantages in suppressing undesirable microorganisms (Vescovo *et al*., 1995). The LAB cultures were used effectively against gram positive pathogens and coliforms. Few of the bacterial forms inhibited are *S. aureus*, *Bacillus cereus*, *Salmonella typhi* and *Listeria monocytogens*.

Antimicrobial proteinaceous compounds produced by bacteria that are active against other bacteria, despite varying greatly in chemical nature, mode of action and specificity have traditionally been defined as bacteriocins (Jack *et al*., 1995). Bacteriocins form a heterogeneous group with respect to the producing bacterial species, molecular size, antimicrobial spectrum, stability and physical and chemical properties and mode of action (Campos *et al*., 2006; Tagg *et al*., 1971) and have been detected in all genera of Lactic Acid Bacteria. Research on bacteriocins from LAB has expanded during the last decades. The use of these substances in extending shelf life of vegetables and milk or its products has provided successful results (Schuenzel and Harrison, 2002).

Considering the bio-preservative effect of LAB, it was proposed to screen a few species of LAB from natural habitat and study their antimicrobial activity against human pathogens like *S. aureus* and *Pseudomonas*. Although, lactobacilli show a high impact on effective protection to human health, there is obvious evidence that lactobacilli from different origins possess antimicrobial properties at different levels (Haller *et al*., 2001). Thus, the present study was undertaken to study the inhibitory activity of partially purified bacteriocins of the LAB isolates from three traditional Indian fermented foods-curd, idli (a fermented steamed product with soft and spongy texture), dosa (a fermented pan cake product) on human pathogens and also to evaluate the antimicrobial activity of these bacteriocins under different physical and cultural conditions like pH, temperature, presence of bile salt and storage time.

MATERIALS AND METHODS

The present study was conducted during the period from 12-02-2009 to 09-07-2009 at Jain Institute of Vocational and Advanced Studies, Chamarajpet, Bangalore, India. All the media used during the course of the study were obtained from HiMedia Laboratories Pvt. Limited (A-406, Bhaveshwar Plaza, Mumbai-400086, India). Each of the tests in the determination of antimicrobial activity of the bacteriocins was conducted in triplicates.

Isolation of LAB

Samples of fermented products like curd, dosa batter and idli batter were procured from retail markets of Bangalore city in India. Samples were kept at refrigerated condition until analysis. For isolation of LAB, the serial dilutions of the samples were inoculated into De Man Rogosa Sharpe's (MRS) agar (De Man *et al*., 1960) by pour plate method and incubated in anaerobic condition at 37°C for 48 h for the colonies to develop. Following incubation, 10 colonies for each sample were randomly selected from the MRS agar plates. The colonies were propagated on the same media until the pure cultures were obtained.

Purification of the cultures was confirmed by Gram's staining. Pure colonies were again cultured on MRS agar slants and broth (in duplicates) and stored at 4°C until used.

Antimicrobial Activity and Bioassay

The antimicrobial activities of these isolates were studied by the disc diffusion procedure (Tadese *et al*., 2005; Hernnadez *et al*., 2005). A loopful of each of the LAB isolates from the MRS agar slants was inoculated into tubes containing 10 mL of sterile MRS broth. These broth cultures were incubated at 37°C for 48 h. After incubation, the cultures were centrifuged (5000 rpm for 35 min at 4°C) to obtain the Culture Free Supernatant (CFS). The pH of the CFSs was adjusted to pH 7 with 1 M NaOH to exclude antimicrobial effects of organic acids (Sharpe *et al*., 1979). Control for each tube was prepared using un-inoculated MRS broth. Sterile cotton swabs were dipped into the cultures of the test (indicator) microorganisms (previously propagated in Brain Heart Infusion (BHI) broth for 24 h at 37°C) and inoculated by swabbing over the entire surface of the pre-set Mueller-Hinton agar plates. Care was taken to evenly distribute the test pathogens throughout the entire surface of the plates. Sterile filter paper discs of 6 mm diameter were prepared from Whatman No. 1 filter paper. Each disc was impregnated with the respective culture supernatant, air dried and placed on a 150 mm plate, within 5 to 15 min after swabbing the test pathogens. After 18 to 24 h of incubation at 37°C each plate was examined for the zone of inhibition. The diameters of the inhibitory zones were measured including the diameters of the discs to the nearest whole number.

Identification of Lactic Acid Bacteria

Identification of the selected isolates (with the desired antimicrobial activity) was carried out using morphological and biochemical methods. The identification of the isolates was performed according to the criteria of Bergey's Manual of Determinative Bacteriology (7th Edn.) and using Sharpe *et al.* (1979) criteria. The studies included motility, catalase test, Gram's staining, growth at 15 and 45°C, growth in media with 4.0 and 6.5% NaCl (Harigon and MacCane, 1976), growth in the presence of 0.1 and 0.3% methylene blue added to milk, acidification of sugars (sucrose, maltose, mannitol, lactose, fructose) and CO_2 production from glucose.

Effect of Heat Treatment on Inhibitory Substances

The CFSs of the isolates (grown in MRS broth for 48 h) were exposed to various heat treatments. The culture supernatants were incubated for 30 min at 3, 50 and 80 and at 121°C for 15 min, 15 lbs pressure in an autoclave. A control was maintained by incubating the CFSs at 37°C.

Effect of pH on Inhibitory Substances

The sensitivity of the active substance to different pH was estimated by adjusting the pH of the culture supernatants to pH 2 to 12 with 1 M NaOH or 1 M HCL (Hernnadez *et al*., 2005; Vignolo *et al*., 1993). After incubation for 1 h and before plating, the pH treated samples were neutralized to pH 6.5-7. The antimicrobial activity was then determined as described earlier.

Evaluation of Antimicrobial Activity During Storage

The culture supernatants were stored at 4°C for different time intervals of 7, 10 and 30 days to assess the stability of the antimicrobial compound under shelf life condition (Khalil *et al*., 2009).

Bile Tolerance Test

The screening for the bile tolerance was carried out by growing the isolates in MRS broth containing 0.3% of Sodium deoxycholate for 24 h at 37°C. Culture broths with turbidity more than 0.5 units at 600 nm were classified as bile tolerant. These isolates were selected for exposure to broths containing higher concentrations of 0.5 and 1.0% (w/v) of Sodium deoxycholate (Srikanjana *et al*., 2008).

Effect of Proteolytic and Other Enzymes

Proteolytic enzymes including pepsin, trypsin and non proteolytic enzymes such as lipase, amylase and catalase were dissolved in 0.002 M HCl (pH 7), 40 mM Tris-HCl (pH 8.2), 0.1 M potassium phosphate (pH 6.0), 0.1 M potassium phosphate (pH 7.0) and 10 mM potassium phosphate (pH 7.0), respectively to a final concentration of 0.5 and 1 mg mL^{-1}. Enzyme solutions were sterilized by passing them through Millipore membrane filters (0.2 μm). Filtrates were incubated with 500 and 1000 mg of each enzyme for 1 h at 37°C, except for samples containing trypsin and catalase, which were incubated at 25°C. All the vials after the enzyme treatment were subsequently heated in boiling water for 5 min to inactivate the enzymes and finally assayed for antimicrobial activity (Bizani and Brandelli, 2002; Lewus *et al*., 1991).

Partial Purification and Molecular Weight Determination

The isolates were grown in MRS broth for 48 h at 37°C. Following incubation, the cultures were centrifuged at 5000 rpm for 35 min at 4°C, after which the bacteriocins were precipitated from the supernatant with 45% saturated ammonium sulphate (Aktypis *et al*., 1998) and kept overnight at -20°C for precipitation. Following precipitation, centrifugation of the supernatants resulted in the formation of pellets, which were collected and stored in phosphate buffer (pH 7.0). The molecular weights of the bacteriocins were determined using SDS-PAGE. Molecular weight markers ranging from 6.5 to 175 kDa was used. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue (s.d. Fine-Chem, Mumbai). The apparent molecular weights of the samples were determined by comparison with the mobility of the standard markers (New England BioLabs, UK).

RESULTS

A total of 30 bacterial strains were isolated from 3 types of fermented foods. Microscopic identification could determine the rod shaped cells. Gram's staining and catalase test could support the characterization of lactobacilli. After taking these criteria into account, 10 strains were found to be gram positive, rod shaped, non-spore forming and catalase negative, which indicated the typical basic characteristics of lactobacilli. Among these 10 strains, five (A1, A2, B1, B2 and F1) were isolated from curd, three (C1, C2 and E1) from idli batter and two (F2 and F3) were obtained from dosa batter.

The antimicrobial activity of the 10 isolates of LAB and their degree of inhibition against the test pathogens were studied. From a total of 10 lactic acid bacteria, the culture supernatants of 7 isolates yielded zones of inhibition when tested against the indicator strains and the values are represented in Table 1.The diameters of the inhibition zones ranged from 9 to 12 mm. The highest diameter (12 mm) was recorded for the culture supernatants of C2 and F2 on *S. aureus* and the smallest of 9 mm for E1 and F3 on *S. aureus*. No zone of inhibition was observed against *Pseudomonas*.

Table 1: Zone of inhibition (mm) for 7 bacteriocin-producing isolates against *S. aureus* and *Pseudomonas*

	Isolates						
Pathogens	A1	B2	C2	E1	F1	F2	F3
S. aureus	10	9	12	9	10	12	9
Pseudomonas	-	-	-	-	-	-	-

Table 2: Biochemical characterization of the bacteriocin producing LAB isolates

Tests	A1	B2	C2	E1	F1	F2	F3
Motility	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-
Growth at 15°C	-	-	+	+	-	+	+
Growth at 45°C	+	+	-	+	+	-	+
Growth in 4% NaCl	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	-	+	+	-	+
Growth in milk with 0.1% methylene blue	+	+	+	+	+	+	+
Growth in milk with 0.3% methylene blue	+	+	+	+	+	+	+
Growth in presence of 0.3% sodium deoxycholate	+	+	+	+	+	+	+
Growth in presence of 0.5% sodium deoxycholate	+	+	+	+	+	+	+
Growth in presence of 1.0% sodium deoxycholate	-	-	+	++	-	+	++
Sugar fermentation							
Sucrose	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Maltose	+/-	+/+	+/-	+/+	+/-	+/-	+/+
Mannitol	+/+	+/+	+/+	+/-	+/+	+/+	+/-
Lactose	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Fructose	+/-	+/-	+/+	+/+	+/-	+/+	+/+
Glucose	+/+	+/+	+/-	+/-	+/+	+/-	+/-

+: Good growth, ++: Luxuriant growth, -: No growth, +/-: Acid and no gas, +/+: Acid and gas

Table 3: Effect of temperature and pH on the zones of inhibition (in mm) of bacteriocins

		Temperature °C			pH					
Isolates	Pathogen	30	50	80	2	4	6	8	10	12
A1	*S. aureus*	10.00±0.00	9.5±0.70	9.25±0.35	9.00±0.00	10.0±0.000	10.0±0.00	10.00±0.00	9.00±0.35	-
B2		9.25±0.35	9.0±0.00	9.00±0.00	9.50±0.35	9.8±0.035	10.0±0.00	10.00±0.00	9.00±0.35	-
C2		12.00±0.00	11.8±0.25	10.25±0.35	9.25±0.35	11.3±0.350	12.0±0.00	11.80±0.35	9.25±0.35	-
E1		9.00±0.00	9.0±0.00	9.25±0.35	9.00±0.00	9.0±0.000	9.5±0.70	9.25±0.35	9.00±0.00	-
F1		9.30±0.35	9.0±0.00	9.50±0.70	9.00±0.00	10.0±0.000	10.0±0.00	10.00±0.00	9.00±0.35	-
F2		12.00±0.00	12.0±0.00	11.30±0.35	9.00±0.00	11.3±0.350	12.0±0.00	11.30±0.35	9.00±0.00	-
F3		9.0±0.000	9.0±0.00	9.00±0.00	9.00±0.00	9.0±0.000	9.0±0.00	9.50±0.70	9.00±0.00	-

-: No inhibition of growth

The results of different biochemical tests for the 7 bacteriocin producing LAB strains (A1, B2, C2, E1, F1, F2 and F3) are presented in Table 2. These results showed that among the 7 strains, 3 (A1, B2 and F1) comprised of *Lactobacillus acidophilus,* 2 (C2 and F2) were of *L. plantarum* and 2 (E1 and F3) belonged to *L. fermentum.*

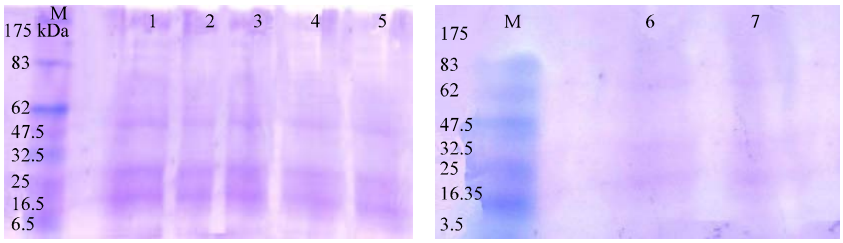
The effects of different temperature and pH on the inhibitory activity of the bacteriocins produced by various LAB isolates have been outlined in Table 3. The antimicrobial substances produced by the isolates were relatively stable during heat treatments at 30, 50 and 80°C for 30 min. Treatment of the culture supernatants at these temperatures did not show significant difference from the control. However, the thermal stability of our bacteriocins was markedly lost upon autoclaving.

The antimicrobial activity of the culture supernatants after treatment at different tested pHs was not significantly affected. The bacteriocin characterized showed better antimicrobial activity at the acidic pH than the basic pH. The highest zone of inhibition was obtained for C2 and F2 (12 mm) at pH 6 and lowest (9 mm) at pH 2 and pH 10. No zone of inhibition was recorded for any of the isolates at pH 12.

Exposure of the bacteriocins to different storage periods of 7, 10 and 30 days at 4°C resulted in an insignificant decrease in activity.

Table 4: Effect of different enzymes on the zones of inhibition (mm) of bacteriocins of the isolates

		Enzymes				
Isolates	Pathogen	Pepsin	Trypsin	Amylase	Catalase	Lipase
A1	*S. aureus*	0	0	9.5±0.35	9.5±0.35	9.0±0.00
B2		0	0	9.5±0.35	9.0±0.00	9.5±0.35
C2		0	0	12.0±0.00	12.0±0.00	11.8±0.25
E1		0	0	9.0±0.00	9.0±0.00	9.5±0.35
F1		0	0	9.0±0.00	9.5±0.35	9.5±0.35
F2		0	0	12.0±0.00	12.0±0.00	11.8±0.25
F3		0	0	9.0±0.00	9.5±0.35	9.5±0.35



(a) (b)

Fig. 1: (a, b) Protein patterns after SDS PAGE of ammonium sulphate precipitates of the whole cell- free extracts of the LAB isolates. At the top of gel, the numbers 1, 2, 3, 4, 5, 6, 7 indicate the protein profile for A1, B2, C2, E1, F1, F2 and F3, respectively. Lane M indicates the molecular weights of the protein marker in kDa

In this study, 0.3, 0.5 and 1.0% concentrations of bile salts were used, since, the physiological concentration of bile salts in the small intestine varies between 0.2 and 2.0%. All the isolates showed growth in 0.3 and 0.5% bile salt solutions. Significant reduction in growth of majority of the isolates was seen when they were subjected to 1.0% bile salt concentration. Only E1 and F3 grew luxuriantly in the presence of 1.0% of bile salt, growth was moderate for C2 and F2, whereas no growth was observed for A1, B2 and F1.

Antimicrobial compounds produced by the isolates were inactivated by all the proteolytic enzymes (pepsin and trypsin). No reduction in the zone of inhibition was encountered when the bacteriocins were treated with amylase, catalase and lipase (Table 4).

The apparent molecular weights of the bacteriocins were determined by SDS-PAGE. Coomassie Brilliant Blue stained gel showed several bands ranging between 16.5 to 48 kDa (Fig. 1a, b).

DISCUSSION

Alternate methods for controlling pathogenic bacteria by the production of antimicrobial peptides called bacteriocins are now highly considered. Bacteriocins from gram positive organisms, such as lactic acid bacteria have attracted much attention and have been the subject of intensive investigation due to their ability to act as a biopreservative agent, which led to their incorporation into foods, particularly in the dairy foods and also in human therapeutics.

In this study, strains producing antimicrobial compounds were isolated from local fermented foods like curd, idli and dosa batter and these antimicrobial compounds were partially purified. The result obtained in our study regarding the production of antimicrobial

compounds against the human pathogens is in complete agreement with the works done by other workers (Tadese *et al*., 2005; Cadirci and Citak, 2005). They found varying degrees of inhibition using various indicator microorganisms, although, inhibitory substances produced by the lactic acid bacterial strains act differently on different indicator strains (Savadogo *et al*., 2004). The production of organic acid and hydrogen peroxide by lactobacilli was reported to inhibit both gram positive and gram negative bacteria (Olasupo *et al*., 1997). Inhibition caused by hydrogen peroxide and organic acids were ruled out as the isolates were cultured anaerobically and the Cell Free Supernatants (CFSs) were neutralized before checking the antimicrobial activity. The reason for *Pseudomonas* showing no sensitivity to the antimicrobial compounds of the LAB isolates may be attributed to the resistance of Gram negative bacteria due to the nature of their cell wall. Pediocin (bacteriocins produced by *Pediococcus acidilactic*) interacts with lipoteichoeic acids that are absent in Gram negative bacteria (Bhunia *et al*., 1991; Albano *et al*., 2007). However, contrary to other researchers, bacteriocins of LAB against gram negative bacteria are also reported by Vignolo *et al*. (1993). Earlier reports (Daeschel and Klaenhamner, 1985; Sanni *et al*., 1999; Tagg *et al*., 1971) have shown that some bacteriocins produced by gram positive bacteria have a broad spectrum of activity. These variations of sensitivity are due to the characteristics of the indicator strains like presence or absence of receiving sites or immunoproteins.

The interesting feature of heat stability at 80°C for 30 min supports the fact that it might constitute an advantage in view of its potential use as a food additive in processes like pasteurization and drying. The loss of the antimicrobial activity at autoclaving condition was probably due to the prolonged time of exposure to heat and pressure. These results are in accordance with the previous works, which described that the activity of bacteriocin produced by lactobacilli was completely lost at 121°C for 15 min (Hernnadez *et al*., 2005; Vinod *et al*., 2006). A similar result was reported for thuricin 7 produced by *Bacillus thuringiensis* BMG 1.7 (Cherif *et al*., 2001).

Every microorganism has a minimal, a maximal and an optimal pH for growth and metabolism. Microbial cells are significantly affected by the pH of their immediate environment because they apparently have no mechanism for adjusting their internal pH. Thus, studying the effect of pH on the antimicrobial compounds produced by our isolates was an important criterion of this study. The results obtained in our study regarding the pH tolerant bacteriocins are consistent with other reports (Karaoglu *et al*., 2003). Such wide range of pH tolerance is an extremely important feature, since, the isolates have the ability to survive, grow and produce their antimicrobials both under acidic and alkaline conditions. Retention of the antimicrobial activity by the strains indicates that cold temperature does not significantly decrease the potential of these active compounds and hence can be applied to foods and therapeutics when kept under cold conditions for considerable time duration.

Besides the strong acid media in the stomach, the probiotic microorganisms taken orally have to defend against the bile salt in the gastrointestinal tract. Hence, bile tolerance is considered to be one of the important properties required for high survival and as a consequence for a probiotic activity (Papamanoli *et al*., 2003). There is no consensus about the precise concentration to which the selected strain should be tolerant. Among the isolates, some grew well in the presence of different concentrations of the bile salt whereas growth was significantly reduced or completely inhibited for the rest. This decrease or inhibition of growth might be attributed to the increase in permeability of the bacterial cell membrane which is composed of lipids and fatty acids (Gunn, 2000).

The activity of the different enzymes on the antimicrobial compounds indicates that the inhibitory compounds are proteinaceous in nature, a general characteristic of bacteriocins.

The enzymes like amylase and lipase did not show any effect on the bacteriocins, suggesting the absence of glycosylated and lipid moieties in the bacteriocins. Similar observations were made by many researchers (Talarico and Dobrogosz, 1989; Delmar *et al*., 2005).

The apparent molecular weight determination of the bacteriocins was done by SDS-PAGE. Present result is in agreement with those obtained from the SDS-PAGE assays of other bacteriocins such as pediocin PA-1 produced by *Pediococcus acidilactici* where the molecular weight was estimated to be 16.5 kDa (Gonzalez and Kunka, 1987). The characterization of the bacteriocins revealed unique properties that emphasize on their applications in food industry as biological control of pathogenic and spoilage microorganisms. The antimicrobial properties of these bacteriocins against other food-borne pathogens are currently been investigated.

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