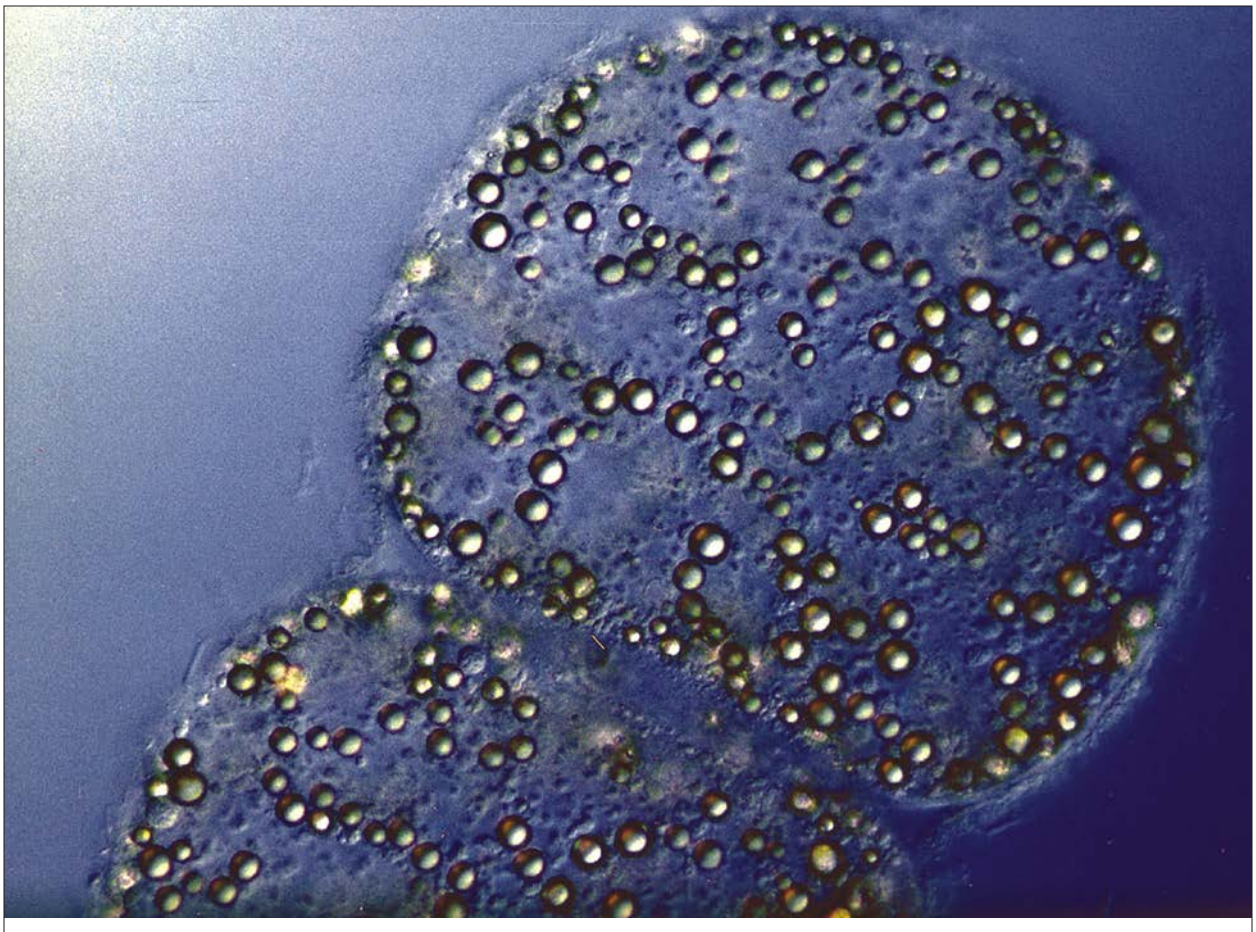

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Isolation, Screening and Evaluation of Antimicrobial Activity of Potential Bacteriocin Producing Lactic Acid Bacteria Isolate

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ABSTRACT

In recent years, antimicrobial agents represent the greatest advantage especially as preservatives in food items. Bacteriocin, one of the antimicrobial substances is ribosomal-synthesized antimicrobial compounds, proteinaceous in nature and mostly acts against closely related species. The present work reports screening of microorganisms for bacteriocin producers obtained from milk, curd, paneer and other fermented food items. Out of the 125 bacteriocin producing lactic acid bacteria isolated from various food items and tested for antagonistic properties, forty bacteria were found to have activity against different gram positive and gram negative bacteria including certain pathogenic and food spoiling microorganisms. Lactic acid bacteria strain BS13, a gram-positive, aerobic, homofermentative, cocci produced highest antimicrobial activity (27306 AU mL^{-1}) with wide range of inhibition. This bacteriocin could potentially be used as food preservatives.

Key words: Biopreservative, homofermentative, catalase, fermented foods, probiotic

INTRODUCTION

Technologies on processing and preservation of food product, which help in maintaining its nutritional values besides ensuring safety issues, are the area of current food research. Many chemicals are being used for inactivation of food borne pathogens so as to preserve food products for longer duration. Chemical preservatives have some of the undesirable side effects like alteration in the constituents, nutritional and organoleptic properties of the food and toxic effect on human health (Sharma *et al*., 2006). Despite all these measures, food borne diseases by pathogenic bacteria do occur frequently (Adak *et al*., 2002).

Ever increasing demand of consumers for faster, healthier and ready-to-eat products without use of chemical preservatives have evolved new techniques of using preservatives of biological origin i.e. biopreservatives. It offers the possibility of extending storage life of high quality foodstuffs without the use of artificial chemical preservatives (Oguntoyinbo *et al*., 2007; Stoyanova *et al*., 2007). Among biopreservatives, bacteriocin has caught the attention of food researchers and industries. The bacteriocins are peptides of proteins having an antimicrobial activity against closely related microorganisms. These could be used as a natural food

biopreservative due to its antimicrobial activity against food spoilage and pathogenic bacteria. Majority of the research on biopreservatives including bacteriocin focused on using lactic acid bacteria.

In recent years, the major research interest is shown globally towards production of bacteriocin from Lactic Acid Bacteria (LAB). Lactic Acid Bacteria (LAB) are comprised of at least ten genera: *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagococcus* (Yildirim, 2001). Most representatives of this group have been consumed for thousands of years, do not pose any health risk to man and are designated as GRAS (generally recognized as safe) organisms. Lactic acid bacteria play important role in majority of foods (dairy, vegetable, meat and fish) fermentation (Gulahmadov *et al*., 2009), preservation and flavor development and acts as protective culture in various food systems, so, they can be selected and implemented in the food products. LAB exerts strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens (Sawa *et al*., 2009). They are also known to have a probiotic effect on human health (Saxelin *et al*., 2005). Probiotic organisms alter the composition of the gastrointestinal flora. Bacterial pathogens like *E. coli, Streptococcus, Clostridium, Bacteriodes* and *Salmonella* are inhibited by probiotics. Their additional benefits include production of mucosal nutrients, elimination of toxins and reduction of faecal ammonia to decrease mucosal toxicity (O'Sullivan, 2001).

India is rich in ancient traditional foods and the various fermented products like milk products, vegetables, fruits, cereals, meat are few of the examples (Thapa *et al*., 2004; Jeyaram *et al*., 2009). In addition to preservation, fermented foods can have the added benefits of enhancing flavor, increasing digestibility, improving nutritional value and pharmaceutical values. Fermented foods are associated with a unique group of microflora which increases the level of proteins, vitamins, essential amino acids and fatty acids, thereby, helping in solving malnutrition problems in population. Therefore, the present study is aimed at exploitation of LAB from fermented foods for the production of bacteriocin for their possible application in food.

MATERIALS AND METHODS

The experimentation for the present investigation has been carried during the period of May 2010 to January, 2011.

Bacterial strains, media and culture conditions: Different samples of food items were collected from the local market. All lactic acid bacteria isolates were cultivated in MRS broth (Himedia) at 37°C. The indicator strains and reference strains including *P. acidilactici* (LB 42), *L. sake* (DSM 100017), *L. monocytogenes* (MTCC 657), *S. aureus* (NCDC 110), *E. faecium* (DSMZ 20477) were obtained from Institute of Micobial Technology, Chandigarh and National Dairy Research Institute, Karnal, Haryana.

Strain isolation: The 1 mL or 1 g of each sample was taken and added into 9 mL of normal saline. After homogenization, serial dilutions were prepared with sterile 0.85% (w/v) sodium chloride upto 10^{-7} and were plated on MRS agar plates. The plates were incubated at temperature 37°C for 24 h. Different colonies were picked depending on their morphological differences. The colonies were then sub-cultured to purity in MRS broth. Stock cultures were maintained in glycerol stocks at -20°C.

Bacteriocin assay

Agar spot assay: The isolates were spotted on three different MRS plates and the plates were incubated at 30°C for 24 h. The soft agar seeded with *Enterococcus faecium* DSMZ 20477, *Pediococcus acidilactici* LB42 and *Lactobacillus brevis* MTCC 1750 were spread on these plates.

Well diffusion assay: The 1 mL of culture was taken and was centrifured in the eppendroffs. The culture supernatant was neutralized and 75 μL of it was added in to the wells of MRS plates seeded with *Enterococcus faecium* DSMZ 20477, *Pediococcus acidilactici* LB42 and *Lactobacillus brevis* MTCC 1750 as indicator organisms. The isolates showing the zone of inhibition in both the assays and for all the indicator organisms were selected.

Antimicrobial activity: Gram positive, homofermentative and catalase negative forty selected isolates were tested for their anti-microbial spectrum against *Staphylococcus aureus* NCDC 110, *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 118, *Bacillus subtilis* MTCC 441 and *Listeria monocytogenes* MTCC 657. Agar spot assay as well as diffusion assay was performed for analyzing the inhibiting potential against food borne pathogens. The isolates showing the wide spectrum of inhibition were selected for further analysis.

Bacteriocin activity assay: Comparative analysis was then performed with the standard culture for the four selected strains for determining their bacteriocin activity. Dilutions were prepared from neutralized culture supernatant and well diffusion assay was performed for the selection of the potential bacteriocin producing LAB isolate. The antimicrobial activity of the bacteriocin was estimated as the reciprocal of the highest dilution indicating inhibition of the indicator lawn and was expressed in activity units per mL (AU mL^{-1}) (Graciela *et al.*, 1995).

RESULTS AND DISCUSSION

Samples were collected from 50 different samples of food items collected from local market and screened for the production of bacteriocins. The 125 lactic acid bacteria were isolated and their antimicrobial activity was estimated against *Enterococcus faecium* DSMZ 20477 and *P. acidilactici* LB 42 by both agar spot assay and well diffusion estimation method. Clearance zone diameter was measured in millilitre and the organisms were selected on the basis of the diameter of the zone. Raja *et al.* (2009) reported the production of bacteriocin of *Lactobacillus lactis cremoris* from kefir and control the food spoilage bacteria. Screening of 2242 strains randomly isolated from different foods for the bacteriocin production and active against *L. monocytogenes*. where 95% of the bacteriocin positive isolates were from dairy isolates (Benkerroum *et al.*, 2007).

The results of antimicrobial activities against different indicator strains for 40 positive isolates are shown in Table 1. The screening was against different strains including three sensitive *E. faecium* DSMZ 20477, *P. acidilactici* LB 42, *L. sake* DSM 100017 and two pathogenic strains *S. aureus* NCDC 110, *L. monocytogenes* MTCC 657. Bacteriocin production assay revealed that the antimicrobial activity against at least two of 5 indicator /pathogenic strains was found in all the selected strains, while 19 out of 40 strains (47.5%) showed activity against all the five reference strains. Fricourt *et al.* (1994) demonstrated that lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity and many of these agents are bacteriocins which are proteinaceous in nature while others are non-protein agents (Piard *et al.*, 1992; Lash *et al.*, 2005). It is well known phenomena that bacteriocin producing organism had no inhibitory effect on the organism producing it.

Table 1: Inhibition of different target organisms by different isolated strains using agar spot assay (ASA) and well diffusion assay *(WDA)

	P. acidilactici (LB 42)		*L. sake* (DSM 100017)		*L. monocytogenes* (MTCC 657)		*S. aureus* (NCDC 110)		*E. faecium* (DSMZ 20477)	
Sample No.	ASA (mm)	WDA (mm)	ASA (mm)	WDA (mm)	ASA (mm)	WDA (mm)	ASA (mm)	WDA (mm)	ASA (mm)	WDA (mm)
BS09	12	-	-	-	12	-	20	12	24	11
MK17	14	-	-	-	12	-	15	-	25	-
BS13	18	9	18	10	15	14	24	-	25	13
C12	15	-	-	-	12	-	20	-	14	-
BS08	10	-	-	-	14	-	22	-	18	-
DS02	-	-	-	-	14	-	20	-	20	-
BS14	-	-	-	-	18	14	29	14	24	10
BS02	16	12	10	08	18	12	24	13	24	10
BS15	11	-	-	-	15	-	36	-	25	-
BS07	15	-	12	-	16	-	24	12	22	-
DS03	15	-	10	-	14	-	12	-	14	-
PN07	-	-	-	-	-	-	20	-	21	-
MK21	14	12	13	-	16	-	20	-	24	-
PN02	25	-	-	-	12	-	40	-	24	-
MK09	-	-	-	-	-	-	18	-	14	-
YH01	-	-	-	-	12	-	24	-	32	-
DS04	12	-	15	-	16	-	24	-	18	-
YH02	15	8	14	-	14	-	26	-	18	-
PN11	-	-	24	-	16	-	12	-	25	-
BS16	15	-	11	-	13	-	24	10	20	-
LS02	12	-	10	-	11	-	20	-	-	-
MK22	-	-	-	-	12	-	22	-	16	-
BS17	12	-	12	-	15	-	20	11	18	-
PN10	-	-	14	-	-	-	18	-	14	-
PN14	10	-	-	-	14	-	20	-	18	-
JB04	22	10	10	-	24	10	29	12	12	10
PN13	12	-	-	-	16	-	26	14	20	-
BS18	14	-	10	-	12	-	20	-	18	-
BS06	20	-	-	-	16	-	23	12	16	-
LS05	-	-	-	-	16	-	18	-	28	-
BS05	11	-	10	-	16	-	24	11	16	-
BS19	12	-	12	-	12	-	25	14	-	-
BS20	16	-	16	-	12	-	22	-	15	-
JB05	22	-	18	-	28	-	15	12	32	-
C14	12	-	10	-	12	-	21	18	18	-
MK24	-	-	-	-	20	-	25	12	25	-
BS21	-	-	08	-	10	-	08	-	14	-
LS07	08	-	12	-	18	-	14	-	12	-
JB06	12	-	-	-	-	-	10	-	16	-
PN15	-	2	12	2	08	-	12	-	18	-

*6 mm diameter well, 18 h incubation of culture supernatant, (-) means no zone

Both agar spot assay and well diffusion assay was performed for determination of antagonistic properties of these 40 isolates and 4 isolates with wide range of range pathogenic and activity was measured (Table 1, 2).

Table 2: Comparison of action of bacteriocins against different pathogenic strains using agar spot assay

	Zone of clearance (mm)								
Isolate No.	*P. acidilactici* LB 42	*L. lactis* MTCC 440	*E. faecium* DSMZ 20477	*L. brevis* MTCC 1750	*P. pentosaceus* MTCC 3817	*S. aureus* MTCC 737	*E. coli* MTCC 118	*B. subtilis* MTCC 441	*L. sake* DSM 100017
BS02	16.0	12.0	24.0	16.0	11.0	24.0	18.0	12.0	10.0
BS13	18.0	15.0	25.0	18.0	18.0	24.0	24.0	16.0	13.0
JB04	22.0	10.0	12.0	12.0	8.0	29.0	14.0	10.0	--
BS14	--	11.0	24.0	14.0	9.0	29.0	14.0	11.0	--

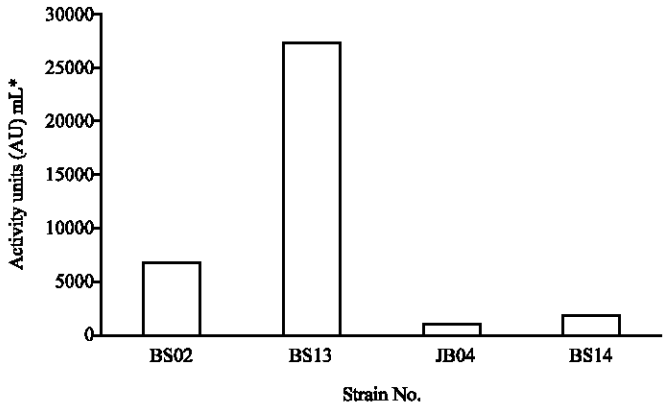

Fig. 1: Bacteriocin production by different selected isolates. Different dilution of bacteriocin from different strains were estimated from the clear zone obtained and activity units were estimated as per formula * AU mL^{-1} = Higher dilution producing a distinct zone of inhibition×1000 μL vol $well^{-1}$

All the bacteriocin produced by the four selected isolates inhibited *Lactococcus lactis* MTCC 440, *Enterococcus faecium* DSMZ 20477, *Lactobacillus brevis* MTCC 1750, *Pediococcus pentosaceus* MTCC 3817, *Staphylococcus aureus* NCDC 110, *Escherichia coli* MTCC 118 and *Bacillus subtilis* MTCC 441. Isolate BS14 do not showed any inhibition against *P. acidilactici* LB 42 and *Lactocaillus sake* DSM 100017 while JB04 showed antagonistic activity against all except *Lactobacillus sake* DSM 100017. Production of bacteriocin by strain BS02 and BS13 gave wider spectrum of inhibition as compared to the bacteriocin producing strains by JB04 and BS14 as they further inhibited the growth of *L. sake* DSM 100017 and *P. acidilactici* LB 42. The potential of these bacteriocins to inhibit the food pathogens such as *E. coli* MTCC 118, *S. aureus* MTCC 737 and *B. subtilis* MTCC 441 makes them suitable for preservation of food items especially in processed foods where risk of food pathogens and spoilage is very high. *Lactococcus lactis* CCSULAC1 bacteriocin showed broad antimicrobial spectrum against *E. coli*, *Enterobacter* sp., *Pseudomonas* sp., *S. aureus*, *B. polymyxa*, *S. typhii*, *Micrococcus* sp. and *B. subtilis* (Sharma *et al*., 2010). Bacteriocin plantaricin UG1 from *L. plantarum* UG1 cells producing resulted inactivation of the monocytogenes cells (Enan, 2006), while Leuconocin, produced by *Leuconostoc lactis* isolate from raw cattle milk was inhibitory against *Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis* (Thakur and Utpal, 2009). Basic morphological properties of these strains were determined (Table 3). All these Gram positive and catalase negative isolates except JB04, which having short chain rods, were cocci and arranged in chains. A number of earlier reports have

Table 3: Basic characteristics of the selected strains

Isolate No.	General properties
BS02	Gram positive, cocci, chains and bunches, catalase-ve,
BS13	Gram positive, cocci, diplococcus and very few chain, catalase-ve
JB04	Gram positive, rods, short chains, catalase-ve
BS14	Gram positive ,cocci, chains, catalase-ve

shown the production of some bacteriocin by gram-positive bacteria having a broad spectrum of activity (Sanni *et al.*, 1999).

Activity of bacteriocin production by different strains has been given in Fig. 1. Isolate BS13 produced maximum activity of 27306 AU mL^{-1} which is quite high as compared to that of other selected strains (853-6826 AU mL^{-1}). The activity of 6400 and 3200 AU mL^{-1} in *L. plantarum* F1 and *L. brevis* OG1, respectively (Ogunbanwo *et al.*, 2003), while activity of only 1250 AU mL^{-1} in MRS and *Lactococcus lactis* subsp. *lactis* CCSULAC1 (Sharma *et al.*, 2010) has been reported. Strain BS13 showed largest clear zone as compared to other selected strains for the production of bacteriocin and selected for carrying our further production and purification process.

CONCLUSION

Isolate BS-13 was found to be most potential bacteriocin producing strain showing wide range of anti microbial activity against food pathogenic micro organisms like *Staphylococcus aureus* NCDC 110, *Staphylococcus aureus* MTCC 737, *Escherichia coli* MTCC 118, *Bacillus subtilis* MTCC 441 and *Listeria monocytogenes* MTCC 657. Further, characterization of the screened isolate is being carried out.

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