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Full Length Research Paper

Heat stable bacteriocin from *Lactobacillus paracasei* subsp. *tolerans* isolated from locally available cheese: An *in vitro* study

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Lactic acid bacteria (135) were isolated and screened for bacteriocin production by agar overlay method. Among them, *Lactobacillus paracasei* subsp. *tolerans* isolated from locally available cheese showed maximum zone of inhibition (24-26 mm) against food borne (*Listeria monocytogenes, Staphylococcus aureus*) and human pathogens *(Escherichia coli, Salmonella typhi*). Bacteriocin was found to be proteinaceous in nature, heat stable (121^0 C for 15 min) and active over a wide pH range of 4.0-8.0. It showed stability (60 %) for 30 days at room temperature (30- 32^{0}C). Addition of surfactants (EDTA, SDS, hexadecyl trimethylamonium bromide) up to 1% to crude bacteriocin showed increase in antibacterial activity where as metal ions (calcium chloride, zinc sulphate and mercuric chloride) in low concentration (0.5-1 mgl^{-1}) decreased the activity. The bacteriocin was purified to its homogeneity by ammonium sulfate precipitation, gel filtration chromatography and HPLC. Molecular weight of bacteriocin was found to be 8.6 and 8.3 KDa by SDS PAGE and LC/MS respectively. Maximum Bacteriocin production was obtained at 35ºC, pH 7.0, NaCl (0.2%) after 28h of incubation. Addition of L-Ascorbic acid increased bactreiocin production. Our present study demonstrates the possibility of using *L. paracasei* subsp. *tolerans* as a biopreservative in dairy industry.

Key words: Lactic acid bacteria, *Lactobacillus paracasei* subsp. *tolerans,* Antibacterial activity, Bacteriocin, heat stability

INTRODUCTION

In spite of modern advances in technology, the preservation of foods is still a debated issue, not only in developing countries (where implementation of food preservation technologies are clearly needed) but also in the industrialized world. The empirical use of microorganisms for the preservation of foods (biopreservation) has been a common practice in the history of mankind. The lactic acid bacteria (LAB) produce an array of antimicrobial substances such as bacteriocins, organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin and antifungal peptides (Rajaram et al., 2010).

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Bacteriocins are antimicrobial peptides synthesized by ribosomes of bacteria which have the property of inhibiting other bacteria, either of the same species (narrow spectrum) or across genera (Cotter et al., 2005). Some of them are inhibitory towards food spoilage and food-borne pathogenic bacteria including *Bacillus*, *Clostridium*, *Staphylococcus* and *Listeria*. Therefore, bacteriocins of LAB are of particular interest because of their existing and potential applications as natural preservatives in foods. The first bacteriocin to appear on both the European food additive list and the United States FDA list was nisin, which was intended for use in the production of pasturized processed cheese (Forouhandeh et al., 2010).

Bacteriocins have been grouped into four main classes based on their chemical and genetic properties (Kaiserlian et al., 2005; Dimitrijevic et al., 2009) class I,

the lantibiotics; class II, the non-lantibiotic peptides, which are divided into the subgroups IIa: peptides active against *Listeria*, the characteristic representants are pediocin PA-1 and sakacin P, IIb: bacteriocins whose activity depends on the complementary action of two peptides, and IIc *sec*-dependent secreted bacteriocins; class III, large, heat-labile protein bacteriocins. The class IV bacteriocins are a group of complex protein, associated with other lipids or carbohydrate moieties. They are relatively hydrophobic and heat stable (Z'Graggen et al., 2005). Bacteriocin-producing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality. Most of the bacteriocins from LAB have been isolated from species of the genus *Lactobacillus*, probably because of the diversity of its species and habitats.

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (i) they are generally recognized as safe substances, (ii) they are not active and nontoxic on eukaryotic cells, (iii) they become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) they are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation.

The main objective of the present study is to evaluate the nature of the bacteriocin and optimization of cultural condition for production of bacteriocin from *L. paracasei* subsp*. tolerans* isolated from home made cheese.

MATERIALS AND METHODS

Bacterial indicator strain and media

Indicator strains such as *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* were maintained in Brain heart infusion agar for 24h at 37ºC before experimental use. De Mann Rogosa Sharpe (MRS) and Brain Heart Infusion (BHI) agar plates were prepared by the addition of 1.5% agar. BHI soft agar was prepared by adding 0.75% agar to broth media.

Isolation and identification of LAB

LAB was isolated from different food (cheese, paneer, sausages, etc.) and environmental waste samples (vegetable waste, sewage waste) by standard spread plate technique. Samples were serially diluted (up to 10^5) 0.1 ml was spread on to MRS agar. Plates were incubated at 35 °C for 24 h. Identification was done by studying the morphological and biochemical characterization (based on carbohydrate fermentation profile, catalase, gelatinase, arginine hydrolysis and motility test). MRS agar without yeast extract was used for the study of carbohydrate fermentation with phenol red as indicator (Bettache and Mebrouk 2004). Further identification of the selected isolate was confirmed by VITEK 2 system and 16S rDNA analysis (Biomeriux, USA).

Screening for antibacterial compound

The isolated LAB were screened for the production of antibacterial compound by testing them against food borne (*L. monocytogenes, S. aureus*) and human pathogens *(E. coli, S. typhi*) by agar overlay method. Strains of LAB were spot inoculated on MRS agar, incubated at 35°C for 22 – 24 h. Pathogenic organisms (the growth always maintained at 0.5 OD at 660nm) were inoculated into Brain Heart Infusion soft agar and overlaid on these LAB cultures. Plates were incubated at 37°C for 22 h and zone of inhibition was measured. Organism that showed maximum inhibition was selected for further study (Alvarado et al., 2006).

Quantitative determination of antibacterial compound

A well diffusion assay method was followed (Rammelsberg and Radler, 1990). The isolate was subcultured twice at 35 °C in MRS broth (pH-6.0) without Tween 80. Inoculum (2% v/v) from over night culture was added to MRS broth, incubated at 35 °C for 18 – 20 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4 °C). The cell-free supernatant was adjusted to pH 6.5 with NaOH and used as crude antibacterial compound for further study. Crude antibacterial compound was serially diluted with sterile distilled water. Aliquot from each dilution was placed in wells of 7mm (diameter) in plates seeded with food borne pathogens and human pathogens. Plates were kept at 4 °C for 8 h. Plates were then incubated at 35 °C for 22 h and checked for zone of inhibition. The antibacterial titer defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed as units per ml (AU mL^{-1}) (Ogunbanwo et al., 2003).

Characterization of antibacterial compound

The cell free supernatant was characterized and tested for antibacterial activity by agar well diffusion method against pathogens. Effect of proteolytic enzyme trypsin on activity of crude antibacterial compound was studied. Three types of preparation were taken *i.e.,*1) 0.15ml of phosphate buffer + 0.15ml cell free supernatant + 0.15ml trypsin (0.25mg ml^{-1}); 2) 0.15ml phosphate buffer + 0.15ml cell free supernatant; 3) 0.15ml phosphate buffer and 0.15ml trypsin (0.25mg ml^{-1}). The effect was studied by agar well diffusion method (Neha and Nivedita, 2009). Temperature sensitivity was assessed by heating samples of supernatant to various heat treatment (15, 30, 45, 60, 75, 90, 100, 121 °C) in boiling water bath for 60 min and every 15 min of time interval; an aliquot of crude sample was removed to determine the activity. To study the pH stability active culture supernatant were adjusted to different pH (2.0 – 10.0) incubated for 4 h to check the activity (Ogunbanwo et al., 2003). Culture supernatant was treated with different detergents (Tween– 20, 80, SDS, EDTA, hexadecyl trimethylamonium bromide) concentration ranging from 0.05 – 1.0% incubated at 35°C for 3 h, and activity was determined (Mojgani et al., 2009). They were stored at different temperatures (4, 32 and -20 °C). Each day the activity was checked for a period of 30 days

(Tenbrink et al., 1994). Crude supernatant was treated with metal ion such as calcium chloride, copper sulphate, zinc sulphate, ferrous sulphate and mercuric chloride at concentration ranging from 0.05 – 1.00 mg L^{-1} (Nusrat et al., 2009).

Purification of bacteriocin

Ammonium sulphate precipitation

For extraction of bacteriocin, the strain was cultivated for 28 h in MRS Broth at 35 $^{\circ}$C**.** The crude bacteriocin sample was treated with ammonium sulphate at different percentage of saturation (0-40, 40-60, 60-80) for 24h at 4°C with gentle stirring. Protein precipitated at each saturation was extracted by centrifugation at 10,000g for 30 min. The obtained protein was dissolved in phosphate buffer (0.1M, pH-7.0) and checked for antibacterial activity (Liliana et al., 2008).

Gel filtration

The protein sample showing bacteriocin activity in ammonium sulphate precipitation was purified by Sephadex G75 gel filtration chromatography. Column was equilibrated by phosphate buffer of 0.1M (pH 7.2) for 30 min and protein was eluted with the same buffer at a rate of 2ml min^{-1}.The protein concentration was measured at 280nm. Each fraction was again tested for bactericidal activity in petriplate by well diffusion assay. Concentration of protein at each step of purification was determined as described by Lowry et al. (1951) using BSA (300 µg ml^{-1}) as standard (Liliana et al., 2008).

HPLC

Active fractions from gel filtration were further checked for homogeneity by HPLC (Thermo Finnigan Surveyor) using C18 column. The bacteriocin was eluted with acetonitrile and 0.2 % formic acid (v/v, 1:1). The flow rate was maintained at 0.2 ml min^{-1} throughout the run, and 0.2 ml fractions were collected and tested for their antibacterial activity against pathogens (*L. monocytogenes and S. aureus*) using the agar-well diffusion method (Esther et al., 2008).

Molecular weight determination by SDS PAGE

Purified bacteriocin was analyzed by SDS PAGE with 20 % and 4 % concentration of acrylamide in separating and stacking gel respectively. Active fractions were pooled and loaded into the well along with a broad range marker protein (3– 200 kDa). The gel was run at constant current 25mA until the tracking dye (bromophenol blue) had migrated to the end. After running, gel was stained overnight with coomassie blue stain. Destaining process was done to visualize the protein band (Yang et al., 1992).

Bacteriocin activity on PAGE

Bacteriocin activity was checked on SDS PAGE with 20% and 4% concentration of acrylamide in separating and stacking gel respectively. After running, the gel was washed with distilled water for 4 h and then placed on Nutrient agar prepoured plate. The gel was overlaid with BHI soft agar seeded with pathogen such as *L. monocytogenes* and *S. aureus*. Plates were kept at 4 ^{0}C for 4 h for diffusion and incubated at 35 ^{0}C for 16 h, observed for the zone of inhibition (Yang et al., 1992).

Molecular weight determination by LC/ESI-MS

Molecular weight detection of bacteriocin was determined by LC/MS using C18 column (Thermo LCQ Deca XP MAX). The bacteriocin was eluted with acetonitrile and formic acid (0.2 %) in 50: 50 ratio (v/v) and detection was performed from 10 min to 35 min of the elution programme. The flow rate was maintained at 0.2 ml min^{-1} throughout the run. The condition ESI-MS detection was performed from 10 min to 35 min of the elution programme. The conditions of ESI-MS were as follows: ionization mode, positive; desolvation temperature, 260°C; needle voltage, 2000 V; orifice voltage, 75 V; ring lens voltage, 10 V. The total ion chromatograms were taken in a mass range from *m/z* 50 to 2000 (Zendo et al., 2008).

Effect of environmental parameters on optimum production of Bacteriocin

The isolate was selected for optimization of growth condition in MRS broth without Tween 80. The procedure adopted for optimization of various parameters influencing bacteriocin production was to evaluate the effect of independent parameters keeping others as constant and to incorporate it at the optimized level in the next experiment. Growth of the isolate was checked at 660nm and bacteriocin activity was determined after adjusting the pH to 6.5 by NaOH. All experiments were done in triplicate and the mean values are calculated.

Effect of temperature

Growth and bacteriocin production were estimated at various temperatures (20, 25, 30, 35, 40 and 45°C). Culture was inoculated to MRS broth at pH 6.0, incubated for 24h (Ogunbanwo et al., 2003).

Effect of pH

Growth and bacteriocin production were estimated at different pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0). Media was incubated at 35°C for 24h. The optimum pH obtained was used for subsequent experiments (Ogunbanwo et al., 2003).

Effect of NaCl

Effect of salt on growth and activity of bacteriocin was determined. Sodium chloride concentration ranged from 0.2 to 5.0% in the media. Flasks were incubated at 35°C, at pH 7.0 (Ogunbanwo et al., 2003).

Effect of incubation period

Effect of incubation time (12 to 48 h) on growth and bacteriocin

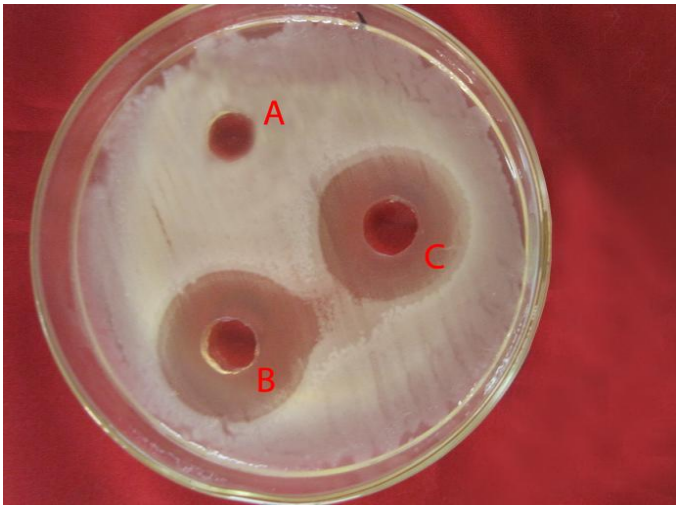

Figure 1. *L. paracasei* subsp. *tolerans* showing bacteriocin activity against *L. monocytogenes* A: control, B and C: Culture supernatant (300µl) showing bacteriocin activity

production was determined. Flasks were incubated at 35°C at pH of 7.0, for every 2 h of incubation period, growth and activity was determined (Ogunbanwo et al., 2003).

Effect of carbon source

Different carbon sources such as monosaccharides (Glucose, Fructose), disaccharides (Mannitol, Lactose. Sucrose) and polysaccharides (Trehalose, Xylose, Arabinose, Raffinose, Ribose, Rhamnose, Starch; at 2% (w/v) were added to MRS broth, incubated at 35°C for 28h and bacteriocin activity was determined (Von Mollendorff et al., 2009).

Effect of vitamin

Thiamine, riboflavin and ascorbic acid were added at 1mg l^{-1} concentration into the growth medium and incubated at 35 °C. Growth and bacteriocin activity were determined (Von Mollendorff et al., 2009).

Statistical analysis

All experiments were carried out in triplicate. Results were expressed with average and standard deviation (MS Excel 3). One way and Multivariate ANOVA test (SPSS 16) has been applied for the analysis of results. Mean which is significantly different are expressed in different alphabetical superscripts ($P<0.05$).

RESULTS

Isolation and Identification

Based on the morphological and microscopical characteristics 135 isolates of LAB were found to be cocci, coccobacilli and rods. Biochemical characterization of these isolates showed that they belong to the genera *Lactobacillus, Lactococcus, Pediococcus* and *Bifidobacterium*. The selected *Lactobacillus* was identified upto species level as *Lactobacillus paracasei* by VITEK 2 system. The identification was further corroborated with studies on its 16S rDNA gene sequencing carried out by Accugenix, USA. The isolate was confirmed as *Lactobacillus paracasei* subsp. *tolerans.* The boot strapped unrooted tree was structured by the neighbour - joining method from the distance data generated by multiple alignment of the nucleotide sequence.

Screening of LAB for antibacterial activity

All the isolated LAB (135 in number) were screened for antibacterial activity. Among them 75% showed positive antibacterial activity. The diameter of inhibition ranged from 14 – 26mm. The *L. paracasei* subsp. *tolerans* showed maximum zone of inhibition i.e 24-26 mm in diameter against *L. monocytogenes, S. aureus, E. coli* and *S. typhi* (Figure 1).

Characterization of crude antimicrobial compound

The activity of the antibacterial compound was tested under conditions which eliminate the possible effect of organic acids by adjusting the pH of the cell free supernatant to 6.5. Trypsin completely inactivated the antibacterial activity, suggesting that the inhibitor agent studied contained proteinacious part, identified as

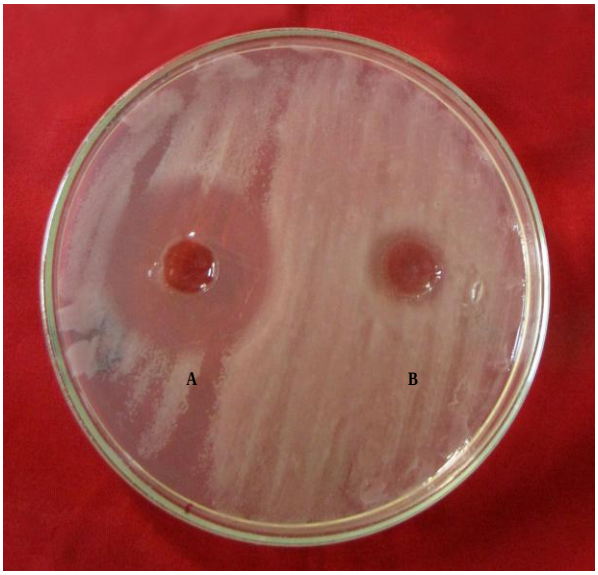

Figure 2. Effect of Trypsin on stability of bacteriocin activity; A: Control; B: Bacteriocin treated with trypsin

Table 1. Effect of heat on stability of bacteriocin

Time interval (min)	15 ºC	30 ºC	45 ºC (Zone of inhibition in mm)	60 ºC	75 ºC	90 ºC	100 ºC
15	10.56 [a]	10.00 [a]	9.80 [a]	7.86 [b]	6.33 [a]	5.33 [a]	4.57 [b]
30	10.23 [a]	10.30 [a]	9.70 [a]	7.80 [b]	6.26 [a]	5.30 [a]	4.50 [b]
45	10.23 [a]	10.00 [a]	9.83 [a]	7.06 [a]	5.87 [a]	4.97 [a]	4.00 [a]
60	10.50 [a]	10.00 [a]	10.00[a]	6.90 [a]	5.80 [a]	4.97 [a]	3.90 [a]

Scheffe post hoc test: Means sharing different alphabetical superscripts in a column significantly different (P<0.05). Means of the groups homogenous subsets are displaced.

bacteriocin (Figure 2) and found to be heat stable up to 100ºC for 60min and 121 ºC for 15min (Table 1). It was also found to be stable at broader pH range of 4.0- 8.0 (Figure 3). To detect the hydrophobic nature, the bacteriocin was treated with a group of detergents. The addition of anionic detergent (SDS) increased antimicrobial effect of the bacteriocin, while the addition of nonionic detergents (Tween 20 and 80) did not affect the antibacterial activity. Treatment with all tested detergents such as SDS, EDTA and hexadecyl trimethylamonium bromide resulted in increase in antibacterial activity whereas Tween 20 and 80 decreased the activity (Table 2). It was found to be more stable at -20 ^{0}C and 4 $^{\circ}$C for 30days. Activity decreased at 32 $^{\circ}$C after 20 days (Figure 4). Increase in the concentration of metal ions tested had reciprocal effect on activity of Bacteriocin; however, the activity was lost with mercuric chloride at less concentration of 0.5mg ml^{-1} (Figure 5).

Purification of bacteriocin

A significant increase in the yield and purification fold of the bacteriocin was observed during different purification stage (Table 3). The bacteriocin activity was found at 80% of ammonium sulphate precipitation. Recovered active proteins in the form of pellets were dissolved in phosphate buffer (0.1M, pH- 6.5) and then fractionated by gel filtration. Fractions from 12 to 16 showed bacteriocin activity against *L. monocytogenes* and *S. aureus*. HPLC analysis of these active fractions showed a single peak at retention time of 12.0 to 13min indicating the homogeneity of protein (Figure 6).

Molecular weight

The active fraction showed a single band with the estimated molecular weight of 8.6 KDa in SDS PAGE

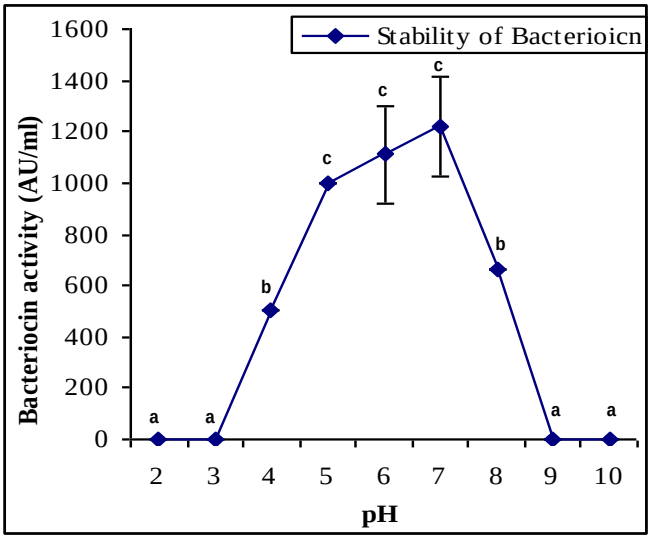

Figure 3. Effect of pH on stability of Bacteriocin by *L. paracasei* subsp. *Tolerans* Scheffe post hoc test: Means sharing different alphabetical superscripts is significantly different (P<0.05).

Table. 2 Effect of detergent on antibacterial compound from *L. paracasei* subsp. *Tolernas*

Concentration of Surfactant	EDTA	SDS	TWEEN 20	TWEEN 80	HDTDM
0.00	9.833 ^{bc}	10.277 ^{ab}	10.266 ^{c}	9.500 ^{c}	10.383 ^{cd}
0.2	6.166 ^{a}	9.583 ^{ab}	3.833 ^{a}	2.777 ^{a}	6.233 ^{a}
0.4	8.833 ^{b}	7.776 ^{a}	3.777 ^{a}	5.000 ^{ab}	7.750 ^{ab}
0.6	10.333 ^{cd}	8.277 ^{ab}	4.333 ^{a}	6.666 ^{b}	9.000 ^{bc}
0.8	11.667 ^{de}	11.333 ^{b}	3.888 ^{b}	6.566 ^{b}	10.667 ^{d}
1.0	12.833 ^{e}	10.777 ^{ab}	3.777 ^{a}	7.000 ^{bc}	14.667 ^{e}

* Zone of inhibition expressed in mm
Scheffe post hoc test: Means sharing different alphabetical superscripts in a column significantly different (P<0.05).

(Figure 7). Incubation of the electrophoresed gel on the agar seeded with *S. aureus* and *L. monocytogenes* further confirmed the inhibitory nature of the bacteriocin.

LC/ESI-MS: A peak at 12.0 - 13 min could be identified as a potent bacteriocin-derived peak. The mass spectrum at this retention time showed that this molecule was detected as *m/z* 1043.50 which correspond to $(M + 8H)^{8+}$. Therefore, the molecular mass of the bacteriocin was calculated to be 8.3 KDa (Figure 8).

Effect of environmental parameters

Influence of temperature, pH and NaCl concentration on *L. paracasei* subsp. *tolerans* was studied. It was found that the growth and bacteriocin activity was optimum at 35 $^{\circ}$C, pH 7.0 and 0.2% of NaCl the growth and bacteriocin activity was optimum. Optimum yield of cell mass was obtained at 24 – 28 h and bacteriocin was obtained at an incubation period of 28 h. Monosaccharides and disaccharides such as D-glucose, fructose, mannitol, lactose and sucrose were found to be the best carbon sources and addition of L -ascorbic acid at concentration of 1mg L^{-1} increased the growth and bacteriocin activity (Figure 9a – f).

DISCUSSION

The result of our initial study to select a potential organism for production of antibacterial compound with the 135 LAB isolates revealed that 80% of isolates was effective against pathogenic organisms tested. Among all the isolates tested, *L. paracasei* subsp. *tolerans* showed maximum zone of inhibition against pathogens tested. The antibacterial activity was more effective towards

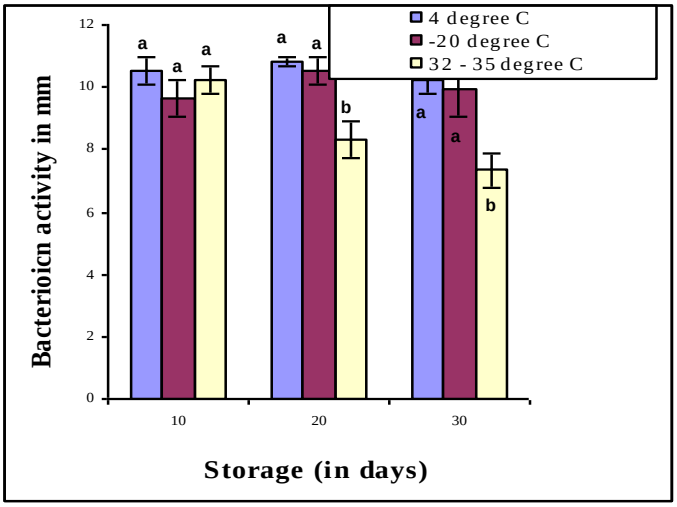

Figure 4: Effect of storage on stability of bacteriocin
Scheffe post hoc test: Means sharing different alphabetical superscripts is significantly different (P<0.05).

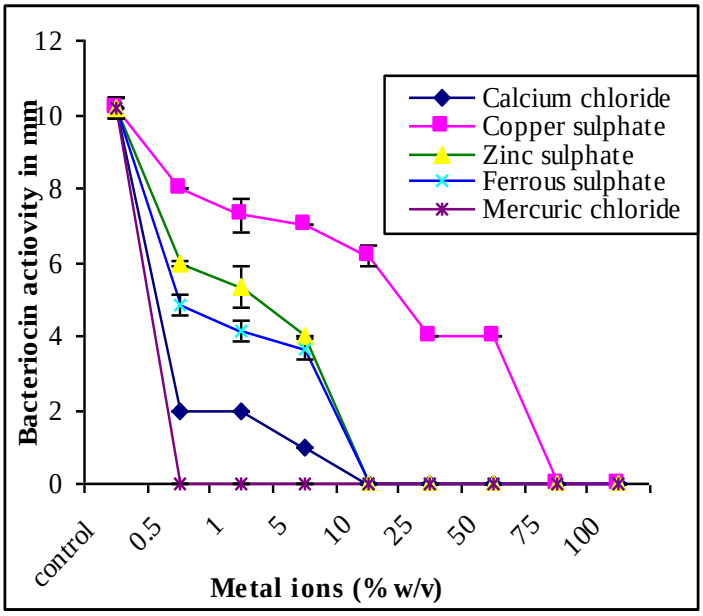

Figure 5: Effect of metal ion on stability of Bacteriocin SD in error bars

gram positive than gram negative bacteria. The sensitivity of gram positive bacteria can be attributed to the susceptible nature of the lipoteichoic acid in cellular envelop to antibacterial compound. Experimental evidence in *Pediococcus acidilacticin* reveals that lipoteichoic acid is susceptible to Bacteriocin (Bhunia et al., 1991). So far, Bacteriocin has reported to inhibit only closely related strains. Villani et al. 1995 reported a bacteriocin from *Streptococcus thermophilus* active against *Listeria*, but not against *E. coli.* Activity against *E. coli* and other Gram-negative bacteria was rarely reported for bacteriocin producers (Skytta et al., 1993). Antibacterial efficacy of bacteriocin from *P. acidilactici, P. pentosaceous* and nisin was evaluated individually and in combination against several gram positive and gram negative food spoilage and pathogenic organisms like *C.*

Table 3. Purification of bacteriocin from *L. paracasei* subsp. *tolernas*

Purification stage	Bacteriocin activity (§)	Protein concentration (†)	Specific activity (*)	Purification factor (††)
Culture supernatant	666.6	380	2.0	1.0
Ammonium sulphate Precipitation (80%)	1333.3	260	5.5	2.7
Gel filtration	4000	120	31.25	5.6

§ Bacteriocin activity (AU mL^{1})
† Protein concentration (µg mL^{1})
* Specific acitivity is the activity unit divided by the protein concentration
†† Purification factor is the increase in the initial specific activity

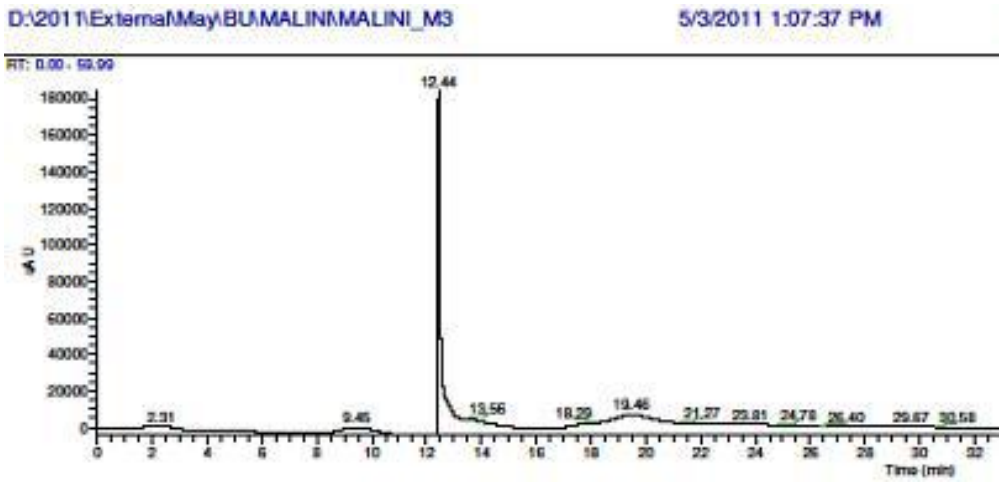

Figure 6: Bacteriocin showing a single peak at retention time of 12.0 to 13.0

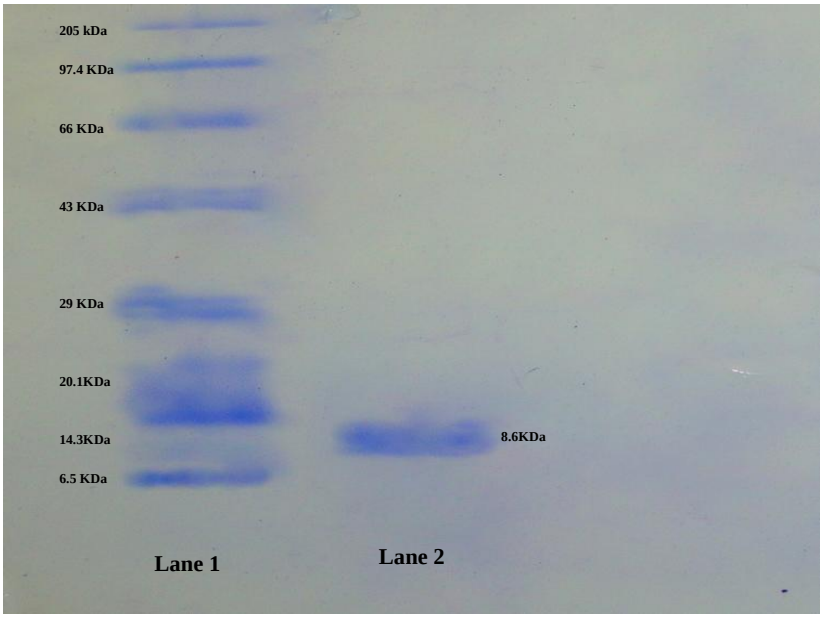

Figure 7: Purified bacteriocin showing homogeneity. Lane 1: Broader range Marker protein, Lane 2: Bacteriocin

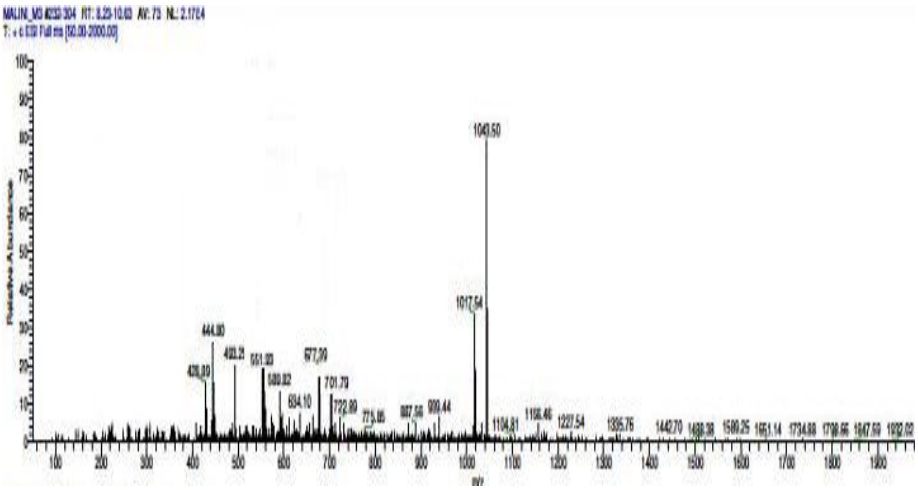

Figure 8: Mass spectrum of bacteriocin was detected as $(M+8H)^{8+}$ (m/z 1043.50) ions.

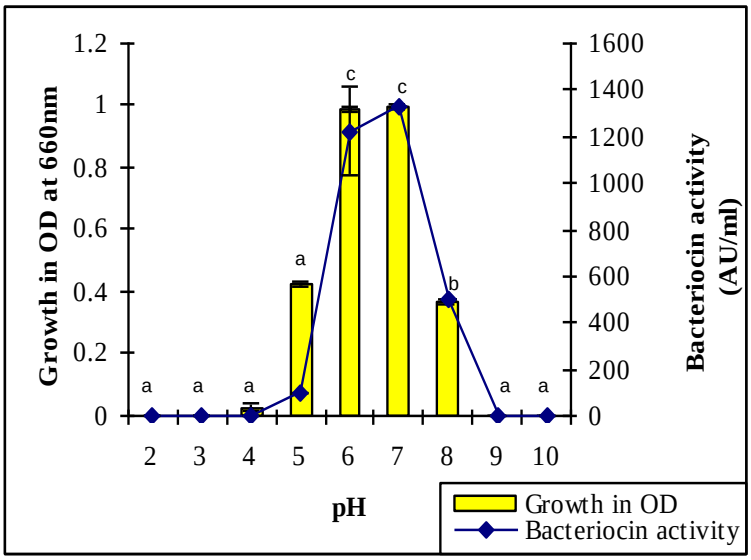

Figure 9a: Effect of pH on growth and bacteriocin production
Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

sporogenes, *L. monocytogenes*, *S. aureus* and *E. coli* in liquid medium (Jamuna and Jeevaratnam, 2009). In the present study, the bacteriocin from *L. paracasei* subsp. *tolerans* active against food borne pathogens such as *L. monocytogenes*, *E. coli* and *S. aureus*. The application of such a broad spectrum bacteriocin or the producer strain could result in improvement of the starter cultures of dairy products and also in food preservation.

The antibacterial activity produced by *L. paracasei* subsp. *tolerans* was tested under condition eliminating the effects of organic acids (Fricourt et al., 1994; Mojgani et al., 2009). As demonstrated by trypsin inactivation, the inhibitory substance was shown to be protein moiety. Temperature stability is an important factor if the bacteriocins are to be used as a food preservative as many procedures of food preparation involve a heating step. The phenomenon of heat stability of LAB bacteriocin have been reported earlier in Lactocin RN 78 (Mojgani and Amirinia, 2007), Plantaricin C19 (Audisio et al., 1999) and a bacteriocin produced by *L.brevis* OGI (Ogunbanwo et al., 2003). Our observations of bacteriocin from *L. paracasei* subsp. *tolerans* are in agreement with the above reports as heat stable showing tolerance at 121^0C for 15min.

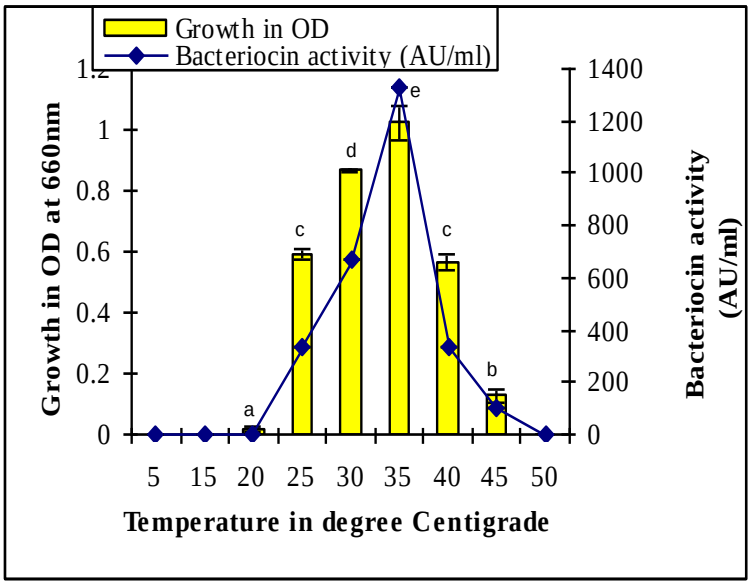

Figure 9b: Effect of Temperature on growth and bacteriocin production Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

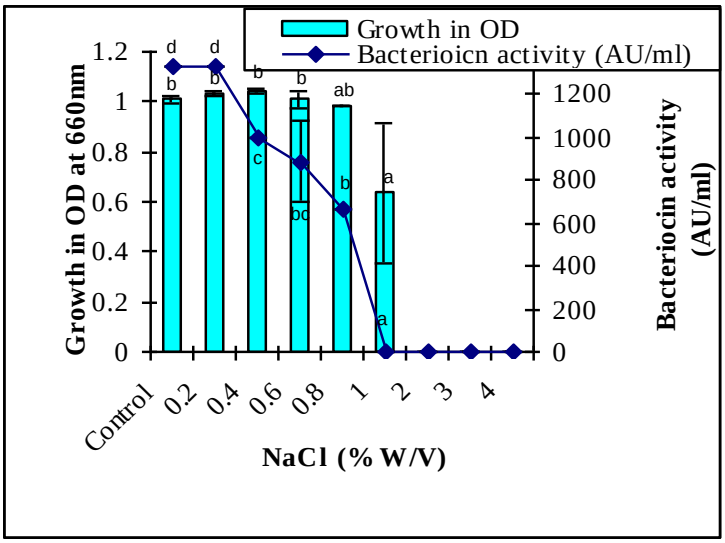

Figure 9c: Effect of NaCl on growth and bacteriocin production Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

Ogunbanwo et al. (2003) and Mojgani and Amirinia (2007) reported that bacteriocin from *L. brevis* OG1 and from *L. brevis* NM24 respectively exhibit highest activity in acidic pH range of 2.0 to 6.0. However, the bacteriocin from *L. paracasei* subsp. *tolerans* showed stability at a broader pH range of 4.0 – 8.0. Exposure of the bacteriocin samples to surfactants like anionic and cationic resulted in an increase in bacteriocin titers. It gives indirect information about the structure of the active molecule. Ivanova et al. (2000) reported that anionic detergent SDS are known to unfold proteins by complexing to the interior hydrophobic core of their native structure thus affecting their three dimensional conformation. In our findings, addition of SDS detergent

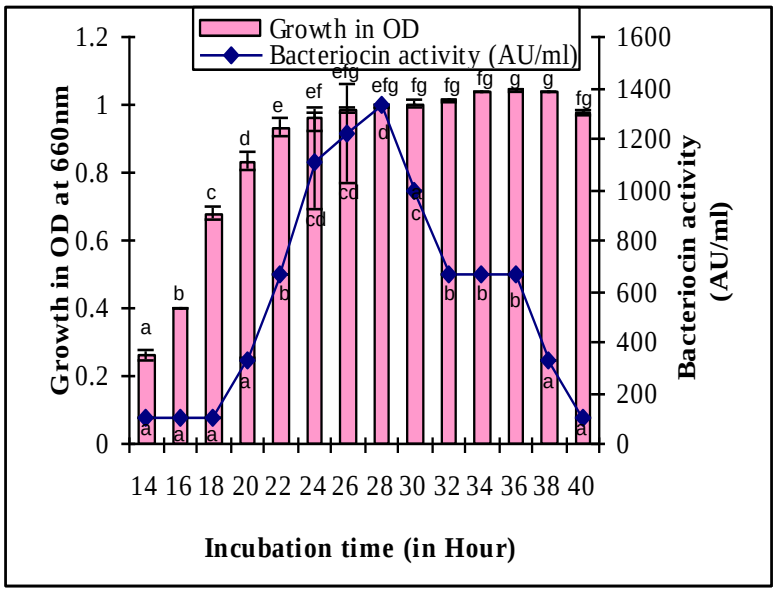

Figure 9d: Effect of Incubation period on growth and bacteriocin production Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

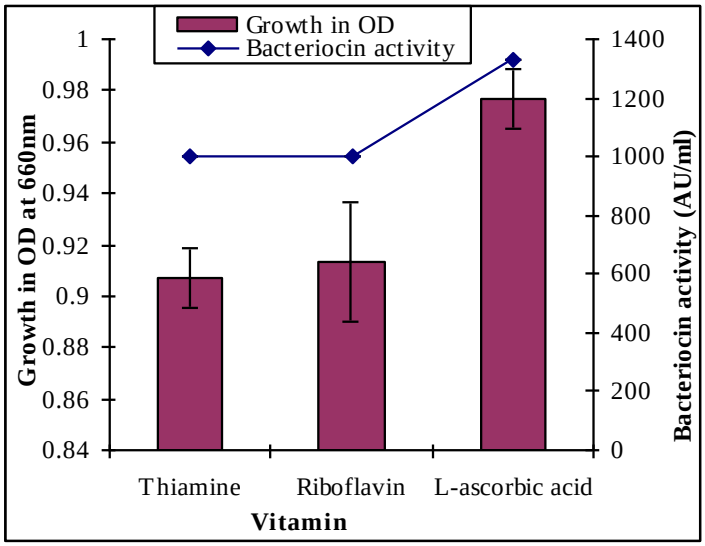

Figure 9E: Effect of Vitamin on growth and bacteriocin production Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

resulted in an increased bacteriocin effect, which might be due to the solubilization of insoluble aggregates. We observed that the addition of nonionic detergents such as Tween 20 and 80 reduced the bacteriocin activity which is in contradictory to the statement of Sahar et al. (2007) who reported increase in activity with tween 20. However, cationic detergent hexadecyl trimethylammonium bromide increased bacteriocin activity. This is due to the dispersion of the bacteriocin complex into active subunits which results in more lethal hits and consequently enhance the activity (Muriana and Klaenhammer, 1991).

In our study, there was a decrease in the activity of

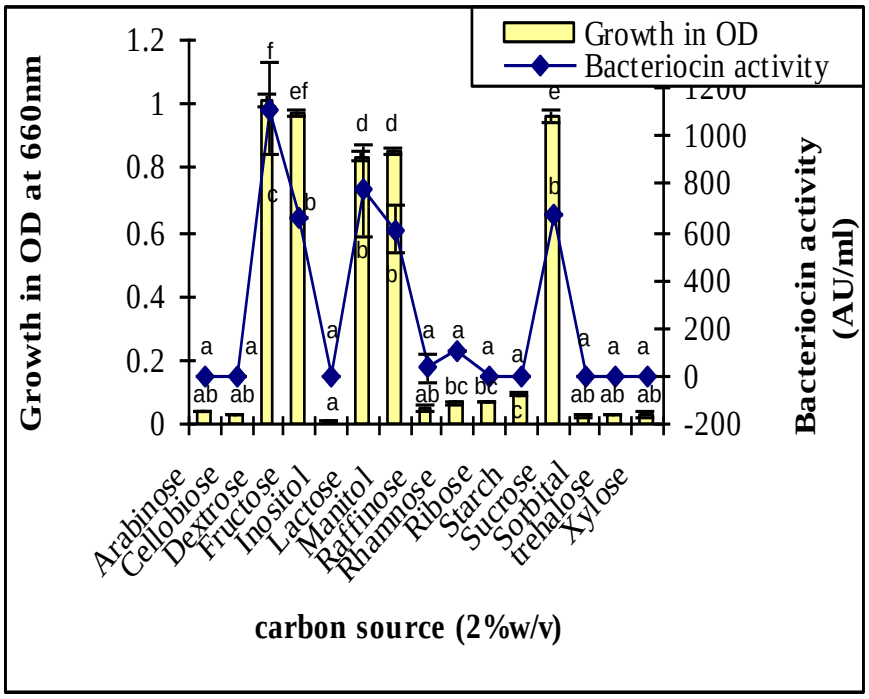

Figure 9f: Effect of Carbon source on growth and bacteriocin production
Scheffe post hoc test: Means sharing different alphabetical superscripts (a, b, c) is significantly different (P<0.05). SD shown in error bars

bacteriocin when the concentration of metal ions increased. Similar such observation was done by Graciela et al. (1995) in *L. casei*. Mercuric chloride affected the activity of bacteriocin even at low concentration of 0.5mg L^{-1}.Activity of bacteriocin in *L. paracasei* subsp. *tolerans* was found to be more stable after storage for 30days at -20 ^{0}C and 4^{0}C but the activity decreased within 20 days of storage at 32^{0}C, indicating that cold temperature is the most appropriate preservation technique.

Purification of bacteriocin from *L. paracasei* subsp. *tolerans* revealed homogeneity on SDS PAGE and molecular weight was estimated as 8.6 kDa. Mass spectrum at the retention time showed that the bacteriocin was detected as $[M+8H]^{8+}$ (*m/z* 1043.50) and calculated mass is 8348 Daltons, which is approximately corresponding to the molecular weight determined by SDS page *i.e* 8600.0 Daltons. Zendo et al. 2008, used acetone precipitation method for higher resolution of bacteriocin by LC/MS analysis. However, the structure has to be determined to derive the exact molecular weight of the bacteriocin in *L.paracasei* subsp.*tolerans*.

Maximum growth and bacteriocin production was found to be 30^{0}C and 37^{0}C in *L. acidophilus* AA11and *L. lactis* respectively as reported by Abo-Amer and Aly (2010) and Rajaram et al (2010). However, in our present study, it was found that the growth and production of bacteriocin from *L. paracasei* subsp. *tolerans* was optimum at 35 ^{0}C with 0.2 % Sodium Chloride and pH of 7.0. Abo-Amer and Aly (2010) have reported that an initial pH of 6.5 is required for optimal growth of *L. acidophilus* AA11 as well as for bacteriocin production. Similar results were reported for bacteriocins ST23LD produced by *L. plantarum* ST23LD (Todorov and Dicks, 2006), bacteriocin ST13BR produced by *L. plantarum* ST13BR (Todorov et al., 2004) and bacteriocin bacST202Ch by *L. plantarum* bacST202Ch (Todorov et al., 2010), all of which recorded maximum activity at an initial medium pH of 6.5 which is in contradictory to our findings. According to Parente and Ricciardi (1999) bacteriocin production in LAB is growth associated and maximum production occurs through out the growth phase and ceases at the end of the exponential phase. However, detectable amount of bacteriocin from *L. paracasei* subsp. *tolerans* was recorded from 14 h onwards and up to 48 h of incubation period. Production increased during the logarithmic growth phase and decreased at the early stationary phase. Maximum growth of the isolate was found at 24 – 28 h and maximum bacteriocin production was at early stationary phase (28 h). Of the 15 carbon sources tested for bacteriocin production glucose, fructose, sucrose, mannitol and lactose were suitable for bacterial growth; however, glucose was found to be the best for bacteriocin production compared to other carbon sources. Growth and production was less in raffinose, ribose, inositol and cellobiose. Such observation was also

reported by Cheigh et al. (2002) during the production of nisin-like bacteriocin by *Lactobacillus lactis.* Addition of L ascorbic acid increased the growth and bacteriocin production of the organism. Von Mollendroff et al. (2009) has reported similar such findings in *L. plantarum* and *L. fermentum*.

Conclusion

The bacteriocin identified from *L. paracasei* subsp. *tolerans* which was isolated by us from locally available cheese posses significant characters such as heat resistance, acid and alkaline tolerance and storage stability at room temperature for a longer period of time. Based on these characters, this isolate could be recommended as a biopreservative in dairy industry. The stability of bacteriocin in low concentration (0-0.5mg L^{-1}) of metal ions could also be considered as a significant factor since in recent days bacteriocin is incorporated as one of the ingredients in cosmetics.

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