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Production and Characterization of Bacteriocin by Lactic Acid Bacterium- *Pediococcus pentosaceus* NKSM1 Isolated from Fermented 'Appam' Batter

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Bacteriocins have attracted much attention in the field of biopreservation and human therapeutics. Therefore a study was carried out to isolate a baceteriocin producing lactic acid bacteria from the fermented appam batter using MRS medium. The bacteriocin produced by the isolate was active against *Listeria monocytogenes* MTCC657 and *Acinetobacter baumannii* MTCC 1425 among the test organisms examined. The bacteriocin producing organism was identified as *Pediococcus pentosaceus* by standard microbiological methods and 16s rRNA sequencing. Media optimization was carried out by altering the initial pH, time and temperature employed in the production of bacteriocin. In this study, enhanced bacteriocin production was observed at pH 6.0, temp 30 °C and 24 h. The concentration of major carbon and nitrogen sources were also studied for their influence on bacteriocin production. It was found that the bacteriocin activity was increased when the carbon source –dextrose concentration was increased in MRS medium. However, nitrogen source did not have significant impact on bacteriocin production. This indicates the organism *P.pentosaceus* produced bacteriocin and exhibited significant growth inhibition against the indicator organisms. Therefore, the bacteroicn can be either used as a biopresrvative or as an antibiotic to treat clinical pathogens.

Keywords: Appam batter, Lactic acid bacteria, *P.pentosaceus,* Bacteriocin, peptide antibiotics.

The microbiology of many fermented foods is quit complex and yet to be explored in a greater way. The microbial fermentation process improves the shelf life, texture, taste and aroma of the final products. These properties aid as bio-preservatives in the food industries (Blandino *et al.*, 2003). Indigenous fermented foods have been prepared and consumed for thousands of years. They are strongly linked to culture, traditions and reveal the intellectual richness of indigenous people of the country in terms of their ability to prepare microbial products for varied purposes in addition to food and beverages (Sekar and Mariappan, 2007). However, the preparation of indigenous or "traditional" fermented foods and beverages remain as a household art even today (Larry and Beuchat, 2008).

Lactic Acid Bacteria (LAB) have been used to ferment or culture foods for at least 4000 years. A wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery products. One of the most important contributions of these microorganisms is the extended shelf life of the fermented product by comparison to that of the raw substrate. Growth of spoilage and

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pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter-derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins (Ray and Daeschel, 1992).

Bacteriocins become one of the weapons against microorganisms due to the specific characteristics of large diversity of structure and function, natural resource, and being stable to heat. Therefore, bacteriocins may become a potential drug candidate for replacing antibiotics in order to treat multiple drugs resistance pathogens in the future (Yang *et al.*, 2014). These bacteriocins are antibacterial proteins vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. These substances are produced by various gram- positive and gram-negative bacteria (Moreno *et al.*, 2000). During the past years, antimicrobial compounds produced by Lactic Acid Bacteria (LAB) have been subjected to intensive study because of their potential use for the manufacture of wide variety of traditional fermented foods (Nettles and Barefoot, 1993).

Increasing number of reports on new/novel bacteriocins with unique properties indicates that there is still a large scope to learn about this family of peptide antibiotics. These antimicrobial peptides have huge applications in food preservation and in next-generation antibiotics targeting the multiple-drug resistant pathogens. The unique properties like thermal stability, pH tolerance and no reports on the development of resistant bacteria, made bacteriocins a potential molecule (Perez *et al.*, 2014). Even though bacteriocins are good in controlling the food-borne pathogens, they are naturally safe by losing their activity due to the cleavage of bacteriocins due to gastrointestinal (GI) tract protease (Saavedra *et al.*, 2004).

Many fermented food items are associated with the life style of the people of southern states in India. The present study aims to isolate bacteriocin producing lactic acid bacteria from 'appam' batter and optimization of process parameters for enhanced production of bacteriocins with antibacterial activity.

Appam is a type of pancake made from fermented rice batter and coconut milk. It is a common food in the South Indian states of Kerala and Tamil Nadu. This dish is made by steam-cooking the batter, and is eaten most frequently.

MATERIALS AND METHODS

Chemicals

de Mann Rogosa Sharpe (MRS) broth, Nutrient broth, Muller Hinton Agar (MHA), Agar agar type I, Peptone, Tween 80, SDS, EDTA, Ammonium sulphate and Cellulose nitrate membrane were procured from Hi-Media , Mumbai. All the other chemicals and reagents used in the study were of highest purity available.

Microorganisms

The microorganisms used in this study were *Acinetobacter baumannii* MTCC 1425, Methicillin-resistant *Staphylococcus aureus* MTCC 1430 *(*MRSA*)* identified as hospital pathogens. *Listeria monocytogenes* MTCC657 a food pathogen, Lactococcus *lactis* subsp.*lactis* MTCC440 and *Enterococcus faecalis* MTCC3159 were well known bacteriocin producers. All these cultures were obtained from Microbial Type Culture Collection and Genebank (MTCC), IMTECH, Chandigarh.

Collection and processing of appam batter

'Appam' batter was collected from a local market in Malappuram, Kerala and transported to the laboratory in an aseptic container by maintaining low temperature and processed within 24 h. One gram of 'appam' batter sample was diluted into 100 ml of 0.1% (w/v) peptone water and incubated at 37 °C under shaking conditions at 100 rpm for 1- 2 h. Later the contents were serially diluted (tenfold serial dilution) with 0.1 % (w/v) peptone water. 100 microlitres of aliquot from 10^{-7} dilution was spread onto sterile MRS agar plates and incubated at 37 °C for 48 h under anaerobic condition. The colonies grown on MRS agar plates were propagated further separately on fresh MRS agar plates by quadrant streaking. This process was repeated thrice to obtain pure culture. The well grown isolates on MRS agar plates were labeled as AB1, AB2, and AB3 and so on for further references. The labeled individual isolates were stored at 4 °C on MRS slants for further studies.

Preparation of cell-free culture filtrates (CCFs)

The selected isolates were inoculated into 100 ml of sterile MRS broth in 250 ml Erlenmeyer flasks and incubated at 37 °C using shaking incubator at 125 rpm for 24 h. The fermented broth was centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatant collected was adjusted to pH

6.5 either using 1 N NaOH or 1 N HCl , and filtered through 0.45 ¼m filter, known as cell-free culture filtrate (CCF) which would be used for further studies.

Preliminary screening of LAB for antagonistic activities

The CCFs of individual LAB isolates were screened for antagonistic activity against the indicator organisms *Acinetobacter baumannii* MTCC 1425, Methicillin-resistant *Staphylococcus aureus* MTCC 1430 *(*MRSA*), Listeria monocytogenes* MTCC657, *Lactococcus lactis* subsp.*lactis* MTCC440 and *Enterococcus faecalis* MTCC3159 using MHA plates by well diffusion method as described by Vignolo (1993). The MH agar plates were prepared and seeded with individual indicator organisms. The wells with 6 mm diameter were made on the agar plates, loaded the 50 ¼l of CCF and incubated at 37 °C for 24 h. The diameter (in mm) of zone of inhibition was measured and the bacteriocin activity was determined. The LAB isolate that showed highest antagonistic activity was selected for further studies.

Examination of CCF for antibacterial activity and bacteriocin assay

The selected LAB isolate was inoculated into 100 ml of sterile MRS broth and the production of bacteriocin was carried out. Then the CCF was processed accordingly.

The assay of bacteriocin activity was carried out as described by Usmiati and Marwati, (2009) by the agar well method using the indicator organisms. Fifty µl of CCF from the selected LAB isolate was loaded into 6 mm diameter wells in Mueller-Hinton agar plates previously seeded with indicator organisms like *L. monocytogenes* MTCC 657 and *A. baumannii* MTCC 1425. After 24 h of incubation at 37 °C, the plates were measured for the zone of growth inhibition of indicator organisms. In subsequent assays, we selected *L. monocytogenes* (food-borne pathogenic bacteria), and *A. baumannii* (human pathogens) to evaluate the antibacterial effects of CCF's of *P. pentosaceus*. The bacteriocin activity was expressed as arbitrary units per milliliter (AU/ml). One AU of bacteriocin was defined as a unit area of inhibition zone per unit volume of bacteriocin added, in this case mm^2 / ml. The bacteriocin activity (AU / ml) was calculated using the following formula:

$$\text{Arbitrary Unit/ml} = {}^{Lz-Ls}/_{V}$$

Lz = clear zone area (mm^2)
Ls = well area (mm^2)
V = volume of sample (ml)

All the assays were carried out in triplicates for individual indicator strains *L. monocytogenes* MTCC 657 and *A. baumannii* MTCC 1425.

Morphological characteristics of the bacteriocin producing LAB culture

The selected bacteriocin producing LAB isolate was examined by morphological, physiological and biochemical characteristics according to the criteria of Bergey's manual of Determinative Bacteriology (1994), and the results were recorded.

Identification of bacteriocin producing LAB isolate by 16S rRNA sequencing and phylogenetic relationship

Genomic DNA from the selected LAB isolate was isolated by the method described by Galvez *et al.*, (2007). The 16S rRNA was amplified using both forward and reverse primers (16S1 : 5'-GCTCACCCTTAACCC-3' and 16S2: 5'ACCTTCCAAGGGCCTAC-3') of genomic DNA. The assay was performed by using *Taq* DNA polymerase and buffers in the thermo cycler for 30 cycles comprising 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C for extension for 45 s. The PCR amplified rRNA product was purified using the quick PCR purification kit. The analysis of alignment, homology and the construction of phylogenetic tree was performed. The nucleotide sequences determined in this study have been submitted to GenBank for assigning the Accession No.

Optimization of process parameters for bacteriocin production

The selected LAB isolate was used as a starter culture for the optimization of bacteriocin production by conventional method.

Effect of temperature on bacteriocin production

The selected LAB isolate was inoculated into a 250 mL Erlenmeyer flask containing 100 ml of sterile MRS broth (initial pH 6.5±0.2). The flasks were maintained at different temperatures

viz 25, 30, 35, 40 and 45 °C for 24 h in a shaker cum incubator (125 rpm). All other parameters like medium components, incubation time and pH were kept constant. After the incubation, the CCF was prepared and subjected to antibacterial activity.

Effect initial pH on bacteriocin production

The influence on initial pH of MRS medium was analysed for bacteriocin production. MRS medium was prepared and the initial pH was checked and adjusted to 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 using either 1 N NaOH or 1 N HCl. The selected LAB isolate was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of sterile MRS broth and incubated at 37 °C for 24 h for the production of bacteriocin under shaking conditions (125 rpm) in a shaker cum incubator. All other parameters like medium components, incubation time and temperature were kept constant. After the incubation, the CCF was prepared and subjected to antibacterial activity.

Effect of incubation time on bacteriocin production

Briefly, 100 mL of MRS broth was prepared, inoculated with the selected LAB and incubated at different time intervals like 0, 12, 24, 36, 48, 60 and 72 h. After the respective time intervals, an aliquot of CCF was collected by centrifugation to measure the optimum time of bacteriocin activity.

Effect of carbon and nitrogen sources on bacteriocin production

The effect of carbon and nitrogen sources on the production of bacteriocin was evaluated (Hoda *et al.*, 2013). An experimental setup was prepared for the production of bacteriocin by varying the concentrations of carbon and nitrogen sources in the MRS medium (table 1). The MRS broth served as control in this experiment, whereas three sets of run were carried out by varying the concentration of carbon and nitrogen constituents (protease peptone, beef extract, yeast extract and dextrose). All the experiments were carried out by inoculating the selected bacteriocin producing isolate at 37°C in an incubator cum shaker (125 rpm) for 24 h. The CCFs obtained were tested for antimicrobial activity against the indicator organisms *L. monocytogenes* MTCC 657 and *A. baumannii* MTCC 1425.

Partial purification of bacteriocin from CCF by ammonium sulphate precipitation and dialysis

The CCF obtained from the selected LAB isolate was saturated with 60, 70 and 80 % solid ammonium sulphate with continuous stirring until dissolving the salt. Then the contents were kept undisturbed at 4 °C overnight with an occasional stirring (Yang *et al*., 1992). Later the contents were centrifuged at 16,500 rpm at 4 °C for 30 min. The pellet and supernatant were separated. The pellet was reconstituted with sterile water and both supernatant and reconstituted pellet sample were dialysed against 10 mM sodium phosphate buffer (pH 6.5) using a tubular cellulose membrane dialysis bag. The buffer was changed 3-4 times in an interval time of 6-7 h. at 10 °C. Bacteriocin assay was performed in all the fractions after dialysis.

Protein Determination

The amount of protein present in the crude bacteriocin samples like CCFs and the fractions obtained after partial purification process were determined by Lowry's method (Lowry *et al*., 1951*)* using bovine serum albumin fraction-V (BSA) as a standard.

Characterization of partially purified bacteriocin

The partially purified bacteriocin sample from the selected LAB isolate was evaluated for its antimicrobial activity with respect to the influencing factors like temperature, pH, and susceptibility to denaturation by enzymes, surfactants, metals and different concentrations of NaCl (Shiba *et al.*, 2013).

Effect of temperature on bacteriocin activity

Ten milliliters of partially purified bacteriocin sample obtained from the selected LAB isolate was exposed to various temperatures like 20, 35, 50, 65, 80, 95 and 110 °C for 2 h and the fractions from each sample was examined for bacteriocin activity against the indicator organisms by agar well diffusion method.

Effect of pH on bacteriocin activity

Ten milliliters of partially purified bacteriocin sample obtained from the selected LAB isolate was adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 by adding either sodium hydroxide (1N NaOH) or hydrochloric acid (1N HCl) and incubated for 2 h at room temperature. Residual bacteriocin activity of each of the samples was

determined against the indicator organisms by agar-well diffusion method (Rattanachaikunsopon and Phumkhachorn, 2006).

Effect of surfactants on bactericidal activity

The effect of surfactants on the activity of bacteriocin was examined by adding non-ionic (Tween 20: 0.5 % (v/v); Tween 80: 0.5 % (v/v)) and ionic (SDS 0.1 % (w/v), CTAB 0.1 % (w/v)) surfactants to the partially purified bacteriocin. The samples were incubated at room temperature for 2 h and assayed for antimicrobial activity against indicator organisms by well diffusion assay as described by Hoda *et al.,* (2013). The MRS broth with the same concentration of surfactants served as control and the zone of inhibition was measured.

Effect of enzymes on bacteriocin activity

Sensitivity of partially purified bacteriocin was examined by treating the bacteriocin sample with enzymes like trypsin, papain and α-amylase at a final concentration of 1 mg/ml (in phosphate buffer at pH 6.0). The contents were incubated at 37 °C for 2 h and the remaining antimicrobial activity was determined against the indicator organisms (Shiba *et al.*, 2013).

Table 1. Experimental setup for screening the influence of carbon and nitrogen sources for the production of the bacteriocin

S. No	Modified ingredient in MRS broth	Concentration (g/l)
1	Protease peptone (g/l)	5.0
		10.0
		15.0
2	Beef Extract (g/l)	5.0
		10.0
		15.0
3	Yeast Extract (g/l)	2.5
		5.0
		7.5
4	Dextrose (g/l)	10.0
		20.0
		30.0
5	MRS broth	—

Effect of metal ions on bacteriocin activity

The impact of metal ions on bacteriocin activity was analysed by adding $MgSO_4$ and $CuSO_4$ at 0.5 % (w/v) level to the partially purified bacteriocin and the samples were incubated at room temperature for 2 h. The bacteriocin activity of each sample was determined against the indicator organisms by agar well diffusion assay (Rushdy and Gonnaa*.,* 2013).

Effect of NaCl on bacteriocin activity

The influence of sodium chloride concentration at different levels on bacteriocin activity was performed by adding 2, 4, 6 and 8 % (w/v) NaCl to partially purified bacteriocin samples. After 2 h of incubation at room temperature, the samples were examined for bacteriocin activity against the indicator organisms by agar well diffusion method (Hoda *et al.,* 2013).

Molecular mass determination

The molecular weight of partially purified bacteriocin was determined by tricine-SDS-PAGE gel electrophoresis (Hailer *et al.*, 2007). Ten µg of partially purified bacteriocin mixed with sample loading buffer (15 mM Tris HCl pH 6.8, 2.3 % SDS, 10 mM 2- mercaptoethanol, 20 % glycerol, 1 % bromophenol blue) was loaded in to the wells and was run on 10 % tricine SDS PAGE. After the run, the gel was removed and cut into half. The half containing sample and molecular weight marker were stained with coomassie brilliant blue R 250 (Sambrook *et al.*, 1989). The other half containing the samples was processed as described by Mirhosaini *et al.*, (2006). Then the gel was placed in a Petri dish and overlaid with 7 ml of 0.6

Table 2. Antimicrobial activity (AU/ml)* of CCFs of 6 LAB isolates against the different indictor organisms

Indicator strain	LAB isolates from fermented appam batter					
	AB1	AB2	AB3	AB4	AB5	AB6
Listeria monocytogenes MTCC657	-		5105	-	-	-
Acinetobacter baumannii MTCC 1425	-	-	3974	-	-	-
Methicillin-resistant *Staphylococcus aureus* MTCC 1430 *(*MRSA*)*	-	-	-	-	-	-
Enterococcus faecalis MTCC3159	-	-	204	-	-	-
Lactococcus lactis subsp.*lactis* MTCC440	-	-	-	-	-	-

*Zone of growth inhibition of indicator organisms in diameter (mm) was used to calculate the antimicrobial activity

% (w/v) agar containing the indicator organisms. Then the plate was incubated at 37 °C for 24 h and the antimicrobial activity of bacteriocin was screened by clear zone of inhibitions.

RESULTS AND DISCUSSION

Isolating industrially important microbial strains from natural sources have always proved to be successful and found to possess applications in both food and health care industries (Young *et al.,* 2012). In this study, well developed individual colonies (6 CFU) were observed in MRS agar plate (10^{-7}) from appam batter sample and they were labeled as AB1, AB2, AB3, AB4, AB5 and AB6 and stored at 4 °C on MRS agar slants for further studies. The cell free culture filtrates obtained from the isolated colonies were tested against the indicator organisms like *Listeria monocytogenes* (food-borne pathogen)*, Acinetobacter baumannii* (human pathogen) and Methicillin-resistant *Staphylococcus aureus (*MRSA (a multidrug resistant)*)* on MHA plates, it was found that isolate AB3 exhibited highest (Table 2) antagonistic activity especially against *A. baummannii* and *L. monocytogenes*. Upon analyzing the morphological characteristics of AB3, it was observed to be Gram (+) coccus, catalase (-) ve, non-sporulating and non motile. With respect to carbohydrate fermentation pattern, it was noted that sugars were fermented without gas production. Upon examining the characteristics of AB3 isolate, it was confirmed to be lactic acid bacterium *Pediococcus pentosaceus* and it was characterized based on its morphological, biochemical and molecular characteristics (Bajpai *et al.*, 2016b; Casaburi *et al.*, 2016). Results obtained by 16S rRNA sequencing revealed 100 % homology to *P. pentosaceus* and

Table 3. Partial purification of bacteriocin from *Pediococcus pentosaceus* isolated from fermented appam batter by ammonium sulphate precipitation method

Table 3a. Purification table of bacteriocin

Indicator strains	Bactericidal activity (AU/ml)	
	Cell-free Culture Filtrate	Partially purified (pellet obtained at 60-80 % saturation of ammonium sulphate)
Listeria monocytogenes MTCC657	3,974	8482
Acinetobacter baumannii MTCC 1425	2,969	7037

Table 3b. Activity against *Listeria monocytogenes* MTCC657

Sample	Total volume (ml)	Protein conc.) (mg/ml	Total proteins (mg)	Total activity (AU)	Specific activity (AU/mg)	Fold of purity	Act. Yield
Cell-free culture filtrate (CCF)	98.3	16.2	1592	390644	245.4	1	100
Reconstituted pellet after dialysis	12.0	0.84	10.08	101784	10098	41.2	39.7

Table 3c. Activity against *Acinetobacter baumannii* MTCC 1425

Sample	Total volume (ml)	Protein conc. (mg/ml)	Total proteins (mg)	Total activity (AU)	Specific activity (AU/mg)	Fold of purity	Act. Yield
Cell-free culture filtrate (CCF)	98.3	16.2	1592	291853	183.35	1	100
Reconstituted pellet after dialysis	12.0	0.84	10.08	84444	8377.4	45.69	28.9

the strain was designated *P. pentosaceus* NKSM 1 (Fig. 1).

According to Devirgiliis *et al*., (2013) and Yousif (2003), the most important genera of LAB associated with fermented food includes *Lactococcus, Pediococcus, Enterococcus, Oenococcus, Leuconostoc, Lactobacillus, Carnobacterium* and *Weissella*. Even in the past, researchers have demonstrated the predominance

Table 4. Effect of temperature on antimicrobial activity of partially purified bacteriocin

Temperature (in °C)	Zone of inhibition (mm)	
	L. monocytogenes MTCC657	*A. baumannii* MTCC 1425
20	2,969	2,089
35	7,037	5,718
50	5,718	5,105
65	3,456	2,513
80	440	204
95	0	0
110	0	0

Table 5. Effect of surfactants on the antimicrobial activities of partially purified bacteriocin

Surfactant	Zone of growth inhibition (mm)	
	L. monocytogenes MTCC657	*A. baumannii* MTCC 1425
Tween 20 (0.5 % v/v)	-	-
Tween 80 (0.5 % v/v)	1,005	1,335
SDS (0.1 % w/v)	2,089	2,969
EDTA Na_2 (0.1 % w/v)	-	-
CTAB (0.1 % w/v)	-	-

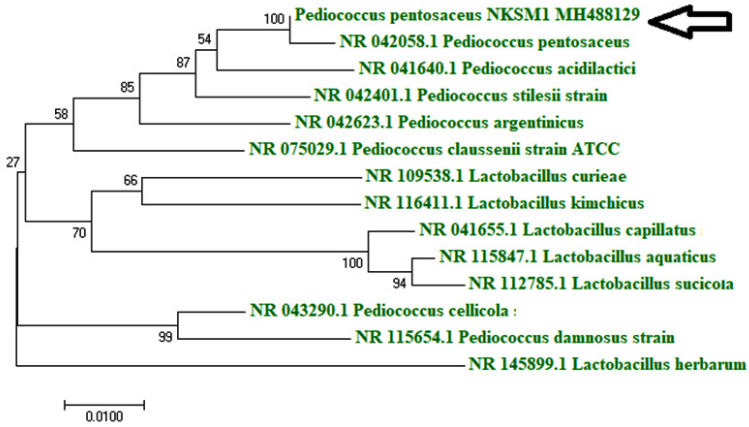

Fig. 1. Phylogenetic tree showing the taxonomic position of *P. pentosaceus* NKSM 1

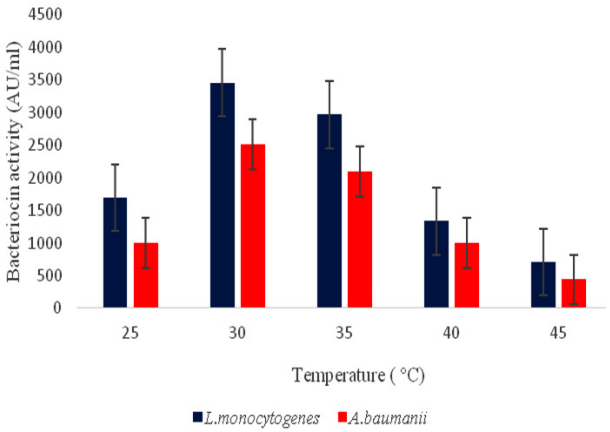

Fig. 2. Effect of temperature on bacteriocin production against *L. monocytogenes* MTCC657 and *A. baumannii* MTCC 1425

of LAB on fermented such as meat, fish, dairy products, fruits, vegetables, cereals (Abdel gadir *et al*., 2001; Grosu-Tudor *et al*., 2014; Hwanhlem *et al*., 2011; Pringsulaka *et al*., 2012; Sulieman *et al*., 2006; Yang *et al*., 2012). Generally lactobacilli represent a major LAB member within the microorganisms isolated from fermented cereal, meat, dairy and vegetable sources (Corsetti and Settanni, 2007 ; Devirgillis *et al.,* 2013). Moreover, they play a pivotal role in fermented food as a starter culture (Ratanaburee *et al.,* 2013). Therefore, *P. pentosaceus* was selected as a good bacteriocin producer and used as a starter culture for the rest of the experiments.

The antibacterial activity of CCF's of *P. pentosaceus* against the tested food borne and human pathogenic bacteria were confirmed by the presence or absence of inhibition zones on the agar well plates. Amenu, (2013) reported that other LAB have shown potential antibacterial effects against a number of foodborne pathogens. Also, a variety of pathogenic and foodborne pathogenic bacteria exhibited susceptibility to LAB (Tadesse *et al.,* 2005; Carina Audisio *et al.,* 2011; Darsanaki *et al.,* 2012; Yah *et al.,* 2014).

In the current study, we report the efficacy of bacteriocin produced by *P. pentosaceus* against

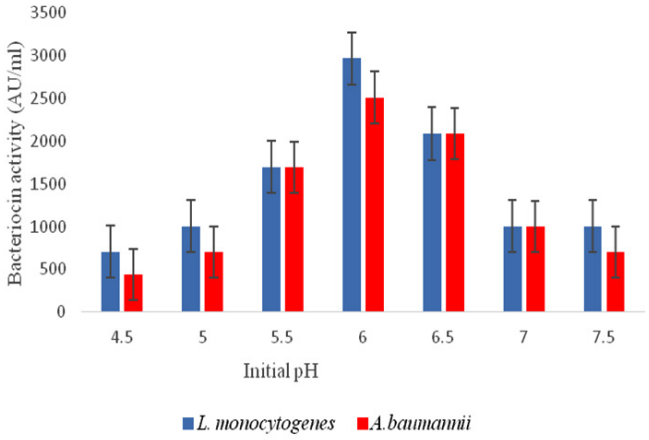

Fig. 3. Effect of different initial pH on bacteriocin production against *Listeria monocytogenes* MTCC65 and *Acinetobacter baumannii* MTCC 1425

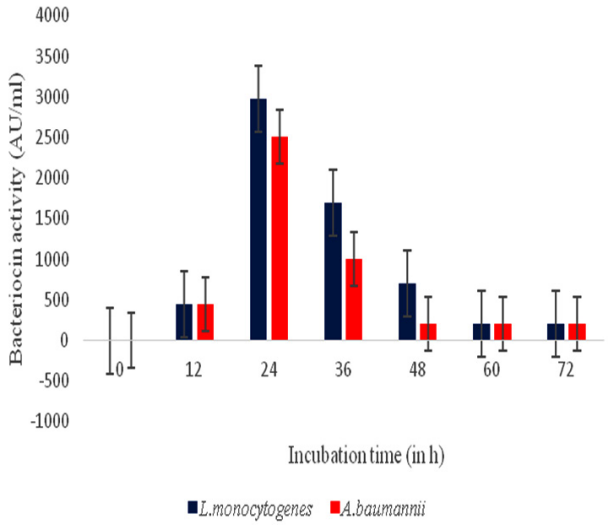

Fig. 4. Production of bacteriocin by *Pediococcus pentosaceus* at various fermentation time intervals

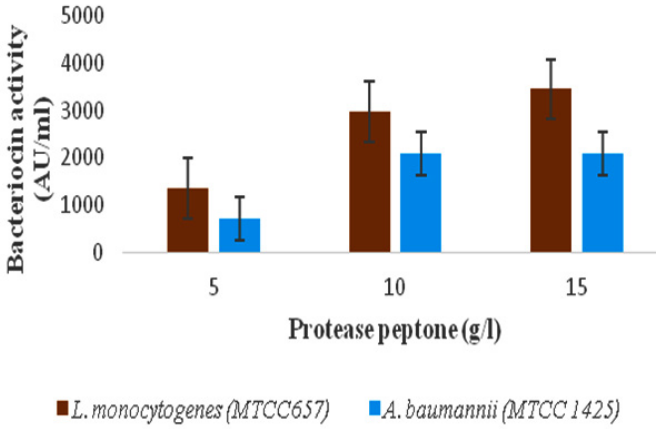

Fig. 5. Effect of protease peptone on bacteriocin production

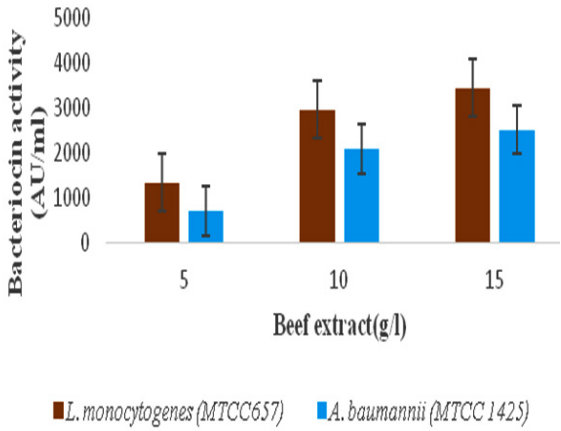

Fig. 6. Effect of beef extract on bacteriocin production

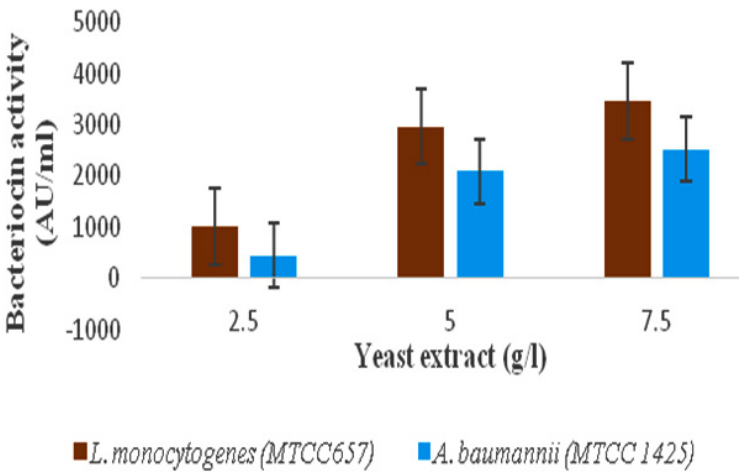

Fig. 7. Effect of yeast extract on bacteriocin production

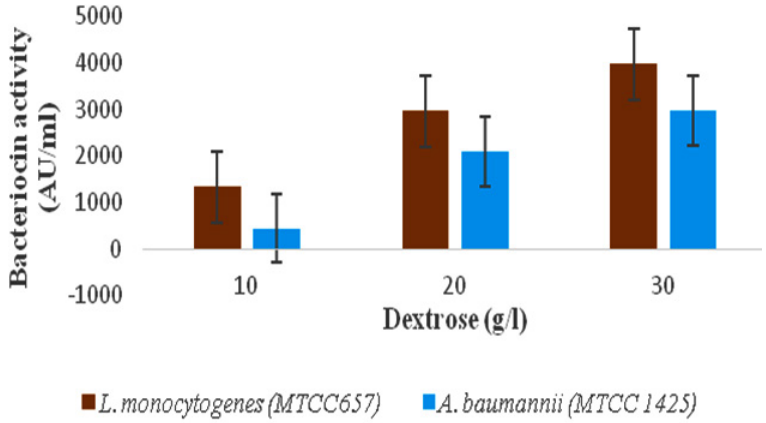

Fig. 8. Effect of dextrone on bacteriocin production

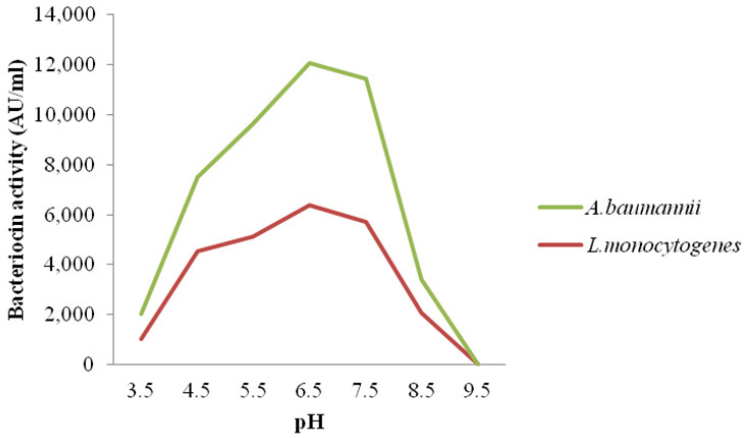

Fig. 9. Effect of pH on partially purified bacteriocin activity

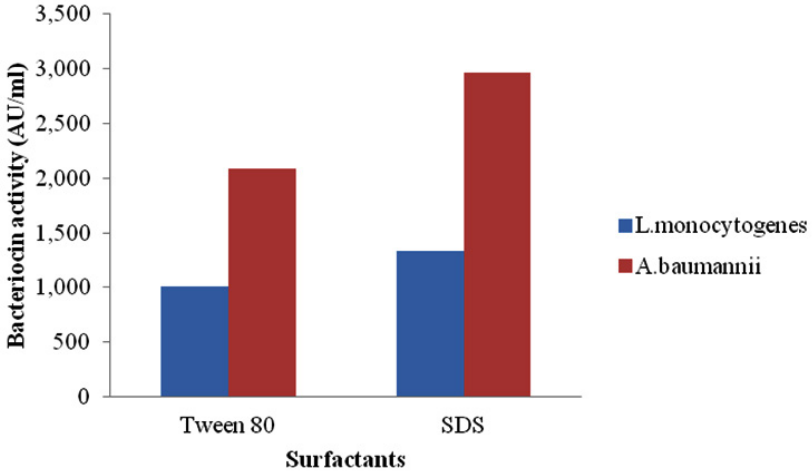

Fig. 10. Effect of surfactants on partially purified bacteriocin activity

the selected the gram negative indicator bacteria *A. baumannii*. Antimicrobial activity of LAB isolates against Gram-negative bacteria was not previously demonstrated by any of the investigators. The reasons may be attributed to the presence of lipopolysaccharide layer of the cell wall protecting the cell membrane, the site of action of bacteriocins (Stevens *et al.,* 1991). Activity against Gram negative bacteria is an unusual phenomenon which has been rarely documented (Cheikhyoussef *et al.,* 2009; De Kwaadsteniet, *et al.,* 2005; Gong *et al.,* 2010; Todorov and Dicks, 2005). Few other authors like Skytta *et al.,* (1993) reported the inhibitory activity of *P. damnosus* and *P.pentosaceus* against a range of gram negative bacteria. The inhibition activity of both gram positive and gram negative bacteria may be resulted due to competition, lactic acid, bacteriocin and hydrogen peroxide production.

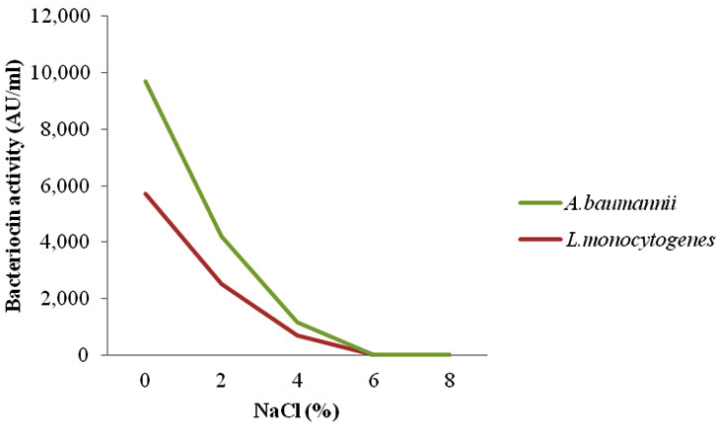

Fig. 11. Effect of salt (NaCl) concentration on partially purified bacteriocin activity

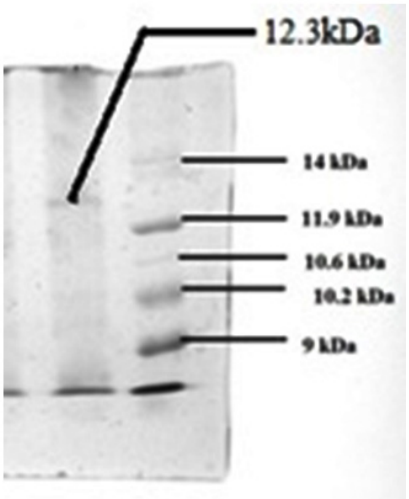

Fig. 12. Separation of bacteriocin by tricine-SDS-PAGE and stained with Coomassie brilliant blue R 250. Lane -1: The protein band with antimicrobial activity against *L. monocytogenes* and *A. baumannii*.. Lane-2: Protein molecular weight markers in low range (9-14 kDa)

Optimization of process parameters for enhanced production of bacteriocin by conventional method

The CCFs of *Pediococcus pentosaceus* NKSM1 showed maximum bacteriocin production at 30 °C for both *Listeria monocytogenes* MTCC 657 and *Acinetobacter baumannii* MTCC 1425 (Fig. 2). Here when the temperature was increased, we could notice that there was a decline in bacteriocin production. Likewise, enhanced bacteriocin production was observed at pH 6.0 (Fig. 3) for both *L. monocytogenes* MTCC 657 and *A. baumannii* MTCC 1425. Similarly with respect to incubation time period, among the tested hours, improved production of bacteriocin was noted at 24 h for both *L. monocytogenes* MTCC 657 and *A. baumannii* MTCC 1425 (Fig. 4), as the incubation period was increased we observed a decrease in bacteriocin production.

Optimization of carbon and nitrogen source medium constituents

The effect of major carbon and nitrogen

sources for the bacteriocin production using *P. pentosaceus* was evaluated (Hoda *et al.,* 2013) and an experiment was prepared by altering the carbon and nitrogen sources (protease peptone, beef extract, yeast extract, dextrose) in the MRS medium as mentioned in the table 1, presented as Figs. 5-8.

Partial Purification of bacteriocin

The partial purification results showed 70-80% saturation rate of ammonium sulphate increased the possible interactions with proteins which lead to faster elution with higher activity of the bacteriocin. The partially purified bacteriocin from *P. pentosaceus* exhibited the maximum activity of 8482 AU/mL against *L. monocytogenes* followed by 7037 AU/mL against *A. baumannii*. The amount of protein was found to be in the range of 0.84 mg/mL. Similar partial purification procedures were previously employed for bacteriocin produced by *L. plantarum* and *E. mundtii* (Todorov *et al.,* 2004; Granger *et al.,* 2005; Todorov *et al.,* 2005).

The partially purified bacteriocin activity was increased by 41.2 % and 45.69 % for *Listeria monocytogenes* MTCC657 and *Acinetobacter baumannii* MTCC 1425 respectively. The fold of purification and the overall yield and specific activity were summarized in tables-3, 3a, 3b, 3c.

Characterization of partially purified bacteriocin

The influence of pH, temperature, presence of metal ions, surfactants on the antimicrobial activity of partially purified bacteriocin by *P. pentosaceus* was examined against the indicator organisms used this study. The bacteriocin activity was assayed by subjecting the partially purified bacteriocin from *P. pentosaceus* to different temperatures were shown in table 4

The bacteriocin was incubated for 2 h at different conditions and the residual activity was measured. The partially purified bacteriocin was found to be stable at 65 °C, which enables them to be an efficient candidate for industrial applications. In the present study, bacteriocin from *P. pentosaceus* was observed to be highly stable at 65 °C with activity of over 3,456 and 2513 AU/mL for *L. monocytogenes* and *A. baumannii* respectively. On the other hand, at 80 °C, a negligible amount of bacteriocin activity was observed whereas at higher temperatures of 95 and 110 °C, bacteriocin activity was completed absent. The results clearly showed that bacteriocin from *P. pentosaceus* can withstand up to a maximum of 65°C. The bacteriocin is able to withstand pasteurisation which is related to its molecular structure composed of small peptides with no tertiary structure (Parda *et al.*, 2007) which was an important characteristic of a bio-preservative. The stability at broad temperature ranges has been attributed to the unusual amino acids in antimicrobial substances which provide strength to tolerate variations [Bhonsle *et al.,* 2013]

Effect of pH sensitivity on bactericidal activity

The partially purified bacteriocin retained its antimicrobial activity even after incubation for 2 h at pH range of 4.5 to 7.5 and the activity was found to be reduced when the pH was increased to 8.5 and 9.5. However, minimal activity was observed at pH 3.5. This shows that the bacteriocin activities are stable at acidic to neutral pH. The results are shown in figure-9. Several bacteriocins produced by *Pediococcus spp* remained stable at a pH range between 2 to 10 but when these bacteriosins were exposed to high pH (12), there was a loss in activity (Albano *et al.,* 2007). Reduction in activity is ascribed to proteolytic degradation and aggregation of protein (Aasen *et al.,* 2000). Likewise, *P. pentosaceus* MTCC 5151 showed optimum activity at pH 5.5 (Agarwal and Dharmesh 2012). *P. pentosaceus* NRC AM1 and *P. pentosaceus* NRC AM4 grew well at pH 4.0 to 8.0 (Mabrouk *et al.,* 2014).

Effect of surfactants on bacteriocin activity

The influence of both non-ionic and ionic surfactants on antimicrobial activity of bacteriocin are depicted in table 5

The anionic surfactant SDS was found to enhance the antimicrobial activity of the partially purified bacteriocin, comparing with the non-ionic surfactant Tween 80 (Fig. 9), whereas other surfactants like tween-20 and CTAB completely suppressed the bacteriocin activity when tested against *L. monocytogenes* and *A. baumannii*. The activity of bacteriocin did not decrease when treated with 0.1 % SDS. These results are similar to the findings of Todorov and Dicks, (2005) and Ivanova *et al.,* (2000).

Effect of enzymes o partially purified bacteriocin

The partially purified bacteriocin from *P. pentosaceus* lost its antimicrobial activity to the enzymes like trypsin, papain and but not for α-amylase. Hence it can be concluded that

the bacteriocin produced by *P. pentosaceus* was portentous nature.

Effect of metal ions on bacteriocin activity

The impact of metal ions on bacteriocin activity was analysed with $CuSO_4$ and $MgSO_4$ at 0.5 % (w/v) concentrations and observed that both the metals interfered with the antagonistic activity. Similar observations were reported by Graciela *et al.* (1995) in *L. casei*. In an another study, Kabore *et al.,* (2013), has reported that the activity of bacteriocin produced from *Bacillus subtilis* was completely lost when metal ions such as Fe^{2+}, Mg^{2+} or Mn^{2+} was added to growth media. But opposite findings were reported by Von Mollendorff *et al.,* (2006) in which addition of $MgSO_4$ increased bacteriocin activity in case of *L.fermentum* JW11BZ

Effect of salt (NaCl) concentration on bacteriocin activity

The effect of salt concentration on the activity of bacteriocin was screened and found that the antimicrobial activity of bacteriocin was reduced even at 4 % of sodium chloride. The growth of LAB is sometimes better in the presence of low salt concentration, usually 1 to 2 % and is inhibited above 3% NaCl, while few LAB are more resistant to NaCl (Altuntas *et al.,* 2010). On the other hand, Sodium chloride at 6 and 8 % completely inhibited the antimicrobial activity. Growth of *P. pentosaceus* decreased with increase in NaCl concentrations. The current results are in agreement with the (Mabrouk *et al.,* 2014). Whereas, Altuntas *et al.,* (2010) have reported that *P. acidilactici* grew up to 10% NaCl, but grew optimally in the absence of NaCl. According to Delgado *et al.* (2007) NaCl was required to maintain osmotic pressure in the cells, but not required for the production of bacteriocin.

Molecular weight of bacteriocin

Tricine SDS-PAGE (Fig 1) revealed the zone-producing band to a MW of 12.3 kDa Figure 12). Similarly, production of bacteriocin having a higher molecular wt of 17.5 kDa by *P. pentosaceus* was previously reported Wu *et al,* (2004). However, production of bacteriocin with a Mw of ~ 4.8 kDa by *P. pentosaceus* CFR B19 (Venkateshwari *et al.* 2010) and pediocin PA-1 having a Mw of 2.6 kDa by *P. acidilactici* PAC 1.0 (Bauer *et al.* 2005)

CONCLUSION

The results of these finds demonstrate the distribution of LAB in Indian fermented food is mainly depend on the type of raw materials from which food is prepared as well as the fermentation condition and procedure. The culture conditions and composition of the growth medium are very important for the production of individual bacteriocins. MRS is found to be the best medium for bacteriocin production by *Pediococcus pentosaceus*. The antimicrobial peptides, bacteriocins, produced by LAB represent unique antimicrobials with high diversity in their structure and physico-chemical properties. In the present study, a bacteriocinogenic LAB isolate, producing novel bacteriocin having broad spectrum of activity against food borne pathogenic and human pathogenic bacteria, isolated from appam batter. Further characterization of the identified bacteriocin and technological evaluation of the isolate for preparation of fermented food products are needed to be carried out. The current study indicates saprophytic LAB can be an ideal source for the study of new bacteriocins.

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